DOMAIN ANALYSIS FOR ESTROGEN RECEPTOR/Sp1-MEDIATED TRANSACTIVATION AND DETECTION OF ESTROGEN RECEPTOR/Sp1 PROTEIN INTERACTIONS IN LIVING CELLS

A Dissertation

By

KYOUNGHYUN KIM

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 2004

Major Subject: Toxicology

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ABSTRACT

Domain Analysis for Estrogen Receptor/Sp1-mediated
Transactivation and Detection of Estrogen Receptor/Sp1 Protein Interactions
in Living Cells. (August 2004)

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Estrogen Receptor α (ERα)/Sp1 activation of GC-rich gene promoters in breast cancer cells is dependent, in part, on the activation function 1 (AF1) of ERα. This study investigates contributions of the DNA binding domain (C) and AF2 (DEF) regions of ERα on activation of ERα/Sp1. 17Beta-estradiol (E2) and the antiestrogens 4-hydroxytamoxifen and ICI 182,780 induced reporter gene activity in MCF-7 and MDA-MB-231 cells cotransfected with human or mouse ERα (hERα or MOR), but not ERβ and GC-rich constructs containing three tandem Sp1 binding sites ($pSp1_3$) or other E2-responsive GC-rich promoters. Estrogen and antiestrogen activation of hERα/Sp1 was dependent on overlapping and different regions of the C, D, E, and F domains of ERα. Antiestrogen-induced activation of hERα/Sp1 was lost using hERα mutants deleted in zinc finger 1 (amino acids (aa) 185-205), zinc finger 2 (aa 218-245), and the hinge/helix 1 (aa 265-330) domains. In contrast with antiestrogens, E2-

dependent activation of hERα/Sp1 required the C-terminal F domain (aa 579-595), which contains a β-strand structural motif. Moreover, in peptide competition experiments overexpression of NR-box peptides inhibits E2-induced luciferase activity of $pERE_3$, which contains three tandem repeats of consensus ERE sites, whereas E2-induced hERα/Sp1 action was not inhibited by NR-box peptide expression. In contrast, overexpression of a C-terminal (aa 575-595) F domain peptide specifically blocked E2-dependent activation of hERα/Sp1, but not on activation of $pERE_3$, suggesting that F domain interactions with nuclear cofactors are specifically required for ERα/Sp1 action.

Furthermore, direct physical interactions between hERα and Sp1 protein in vivo have been investigated by using Fluorescence Resonance Energy Transfer (FRET) microscopy and image analysis. Consistent with results from transient transfection assay, E2, 4OHT, and ICI enhanced hERα/Sp1 interactions in living cells and these interactions were also confirmed by coimmunoprecipitation. In addition, endogenous hERα/Sp1 action was evaluated by using si RNA for Sp1 and a significant decrease in ligand-induced hERα/Sp1 action was observed after decreased Sp1 expression.

DEDICATION

To my wife, So-Young Kwon

My parents, Deok-Joong Kim and Deok-Ja Ko

My father and mother in Law, Taek-Joon Kwon and Ok-Gi Kim

For their love, support, patience, and friendship

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CHAPTER I

INTRODUCTION

1.1 Cancer

1.1.1 What is cancer?

Cancer, also called neoplasia or malignant tumor, 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" (Hahn and Weinberg, 2002). 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 crab". Even though cancer is often regarded as a single condition, it consists of more than 100 different diseases depending on its tissue origin. Compared to 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 2003 and approximately 0.55 million people will die from this disease. Approximately, 1 out of 4 deaths are due to cancer. The 5-year relative survival rate of all cancer combined after first diagnosis is approximately 62%, whether in remission, under treatment, or disease-free (Jernal et al., 2003).

This dissertation follows the style of Gene.

A single and ultimate cause of cancer has not been identified. However, it is certain that multiple factors including genetics, lifestyle, working environment, or combinations of these factors are linked to the development of cancer (Table 1) (Peto, 2001).

An inherited genetic alteration is one of the major risk factors for development of cancer. Polymorphisms in genes involved in hormone production or in metabolism of exo- or endogenous mutagens can also increase the risk of cancer. For example, the N-acetyltransferase (NAT2) slow acetylator phenotype, which is involved in the metabolism of certain carcinogenic aromatic amines, is associated with an increased risk of bladder cancer (Hein, 2002; Cartwright et al., 1982). Mutations in proto-oncogenes or tumor suppressor genes also confer increased cancer risk. A single base mutation of the APC gene (I1307K) among Ashkenazi Jews almost doubles the risk of colon cancer (Woodage et al., 1998).

Although there has been a marked reduction in cigarette smoking, it is estimated that at least one third of all cancers are related to smoking. Lung cancer incidence increases dramatically among heavy smokers especially those people who begin smoking at an early age (Doll, 1978). Smoking also elevates the risk of other cancers including pancreas, bladder, kidney, larynx, and esophagus (Doll and Peto, 1981). Recent reports indicated that cigarette smoking also increases the incidence of tumors in the stomach, liver, and cervix (Doll, 1996; Liu et al., 1998).

Diet has recently received considerable attention as an important lifestyle factor that influences development of cancer since many dietary nutrients contain pro-, anti-carcinogens, or both. It has been suggested that about one third of all cancers may be related to dietary factors and some of these can be avoided by dietary changes (Josefson, 2001).

Table 1

Risk factors for cancer (Peto, 2001)

Internal Factors	External Factors
Gene-Polymorphisms Gene-Mutations Hormones immune states Aging Diet and exercise (lifestyle)	Tobacco Chemicals Occupational or environmental conditions Radiation Infectious organisms (Virus, bacteria, etc)

A variety of bioactive compounds in the diet influence either genetic or epigenetic changes, and metabolism relevant to the initiation and progression of cancer (Hong and Sporn, 1997). Vitamin D, calcium, folate, the isoflavone, genistein are currently being evaluated as chemopreventive agents that inhibit carcinogenesis at various stages (Kelloff et al., 2000).

Infectious pathogens such as bacteria, viruses, or parasites can also cause cancer by diverse mechanisms of actions. A chronic bacterial infection, helicobacter pylori, causes gastric ulcers, which leads to development of stomach cancer (Miehlke et al., 1997). Many types of human papilloma viruses are detectable in all cervical cancers (Walboomers et al., 1999). The relationship

between liver cancer and hepatitis-B virus infections has been established and its synergistic interaction of this virus with smoking has been reported (Liu et al., 1998).

Asbestos exposure can result in a high incidence of lung cancer and asbestos is the leading cause of occupational- and environmental- related cancer deaths. Its carcinogenic properties are related to asbestos fibers, which cause asbestosis and mesothelioma (Wikeley, 1992)

1.1.3 Basic characteristic of cancer

Since the National Cancer Act of 1971, signed by President Nixon, remarkable progress has been made in our understanding of the cellular, biochemical, and genetic changes that occur during cancer development. The current paradigm is that cancer development is a multistep process reflecting the progressive acquisition of mutations in growth enhancing genes (oncogenes) and recessive mutations in growth inhibitory genes (tumor suppressor genes) (Land et al., 1983 and Marshall, 1991). Transformation of primary rodent cells into tumorigenic cells only occurs by coexpression of two different oncogenes (Hahn and Weinberg, 2002). The tumorigenic conversion of human epithelial cells has been observed only when multiple oncogenes like SV-40 Large T antigen, H-ras, and telomerase are ectopically expressed together. Furthermore, various transgenic animal models of tumorigenesis support a multistep carcinogenesis model with many rate-limiting steps (Bergers et al., 1998).

Carcinogenesis has three basic steps of initiation, promotion, and progression. The initiation stage of cancer development is associated with Irreversible DNA damage in normal cells by chemicals, radiation, or viruses and this is coupled with inappropriate DNA repair, leading to formation of neoplastic cells.

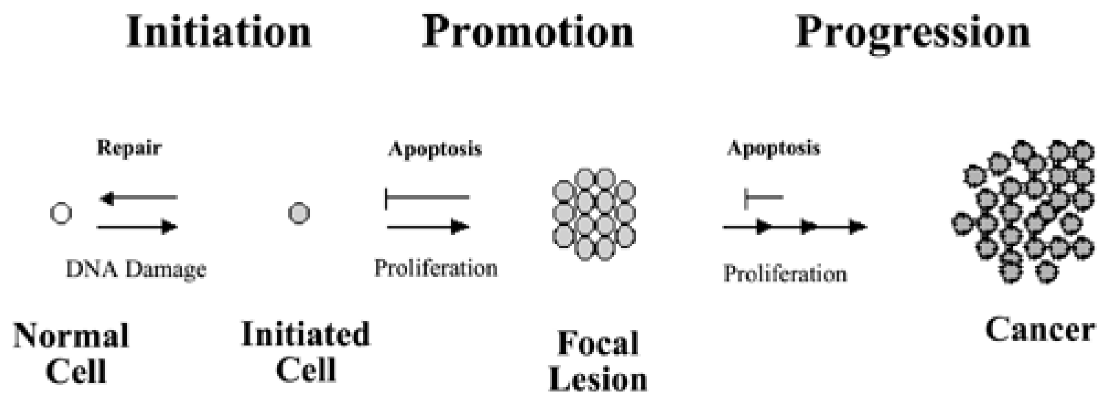

Fig. 1. Multistep carcinogenesis (Klaunig et al., 2000).

Secondly, the perturbation of growth regulatory circuits in the damaged cells are enhanced, resulting in increased cell proliferation and transformation, leading to a premalignant lesion through the process of a clonal expansion. Tumors in the final stage of progression exhibit karyotypic instability; chromosome changes including insertions, deletions, breaks, and a metastatic capacity, result in the invasion of invading neighboring tissues (Fig. 1).

Cancer cells generate their own mitogenic growth signals and become independent of exogenous growth factors. In contrast, normal cells do not grow in the absence of the exogenous mitogens. The autonomy of growth factor

signaling in cancer cells is achieved by alterations of these pathways. For example, the epidermal growth factor receptor (EGF-R/erbB) is upregulated in stomach and breast cancers (Slamon, 1987) and constitutively active form of the Ras protein is also upregulated in some cancers, resulting in increased mitogenic signals without activation by upstream growth stimulatory signals (Medema et al., 1993).

Cellular quiescence or differentiation in normal cells requires growth inhibitory signals that inhibit cell cycle progression, however, cancer cells evade those antiproliferative signals and grow exponentially. A pRB family of proteins are tumor suppressor genes and code phosphoproteins with molecular weights ranging from 104 kD to 115 kD. One copy of the wild-type RB gene is necessary for normal retinal development and loss or inactivation of both alleles at this locus results in retinoblastoma. Disruption of the pRB pathway renders cells to be insensitive to antigrowth signals (Fynan and Reiss, 1993; Kinzler and Vogelstein, 1996).

Cancer cells also acquire resistance to apoptosis or programmed cell death. p53 protein is inactivated in more than 50% of human cancers. Thus, the functionally altered p53 protein that normally triggers apoptosis to eliminate damaged cells has been inhibited in cancer cells, which thereby evade the death signal (Benard et al., 2003).

Acquisition of limitless replicative potential is essential for cancer development. For example, maintenance of teleomers at the ends of

chromosomes is observed in virtually all types of maliganant cancer cells (Shay, 1997). Ectopic expression of telomerase, an enzyme that adds hexanucleotide repeats to the ends of chromosome, resulting in the immortalization of cells with unlimited replicative potential (Bodnar et al., 1998).

Rapidly growing cancer cells need oxygen and nutrients from blood to be more proliferative and subsequently, acquire the capacity to generate signals for new vessel formation or angiogenesis (Folkman, 1997). Vascular endothelial growth factor (VEGF) is one of well-known angiogenic signals that cancer cells frequently produce.

Approximately 90% of human cancer deaths are due to the acquisition of invasive and metastatic potential of primary tumor cells, which move to distant sites and colonize other tissues. E-cadherin expression, a homotypic cell to cell interacting protein that suppresses invasion and metastasis is deregulated observed in a majority of epithelial cancers (Christofori and Semb, 1999). Some of the important acquired characteristics of cancer cells during are summarized in Table 2.

Table 2

Acquired characteristics of cancer cells (Hanahan and Weinberg, 2000)

Acquired capability	Example of mechanism
Self-sufficiency in growth signals	Activated H-ras oncogene
Insensitivity to anti-growth signals	Loss of retinoblastoma protein growth suppression
Evading apoptosis	Inactivation of p53 protein function
Limitless replicative potential	Activation of telomerase
Sustained angiogenesis	Activation of VEGF
Tissue invasion&metastasis	Inactivation of E-cadherin

1.2 Breast cancer

Breast cancer is by far the most common type of cancer and second leading cause of cancer deaths among women in the western world. Statistics indicates that one out of nine women will develop breast cancer during her lifetime. Breast cancer treatments that have decreased mortality from this disease include early detection, surgical removal combined with chemotherapy, hormone therapy, or radiation therapy (Hortobagyi and Buzdar, 2000). However, there is no effective treatment for recurrent-, endocrine-resistant, metastatic tumors.

1.2.1 Parallel between mammary gland development and breast cancer

The mammary gland is a highly unique organ in mammals responsible for providing nutrition to the young. Development of the mammary gland can be divided into 5 distinct stages from embryonic and prepubertal stage, puberty, pregnancy, lactation, to involution. Interestingly, the cycle of mammary gland development displays many characteristics associated with breast cancer.

Moreover, many of the vital factors required for mammary development are also involved in breast cancer.

In the mouse embryo, mammary gland development begins with formation of ectodermal placodes running ventrally just inside the limbs. These placodes appear around day 10 or 11 of gestation, form an epithelial bud, and increase in size up to day 15. During this period, intensive cell proliferation occurs at the tip of the epithelial bud. This epithelial bud grows out of the surrounding mammary mesenchyme toward the mammary fat pad, ultimately forming a primary sprout that bifurcates and grows into a small duct system at the time of birth. In contrast, the mammary buds in male embryos undergo a programmed cell death; the connection between the epithelial stalk and the epidermis is cut off so that nipple formation does not occur. Mammary anlage formation is arrested in mice that lack the transcription factor LEF1, suggesting the involvement of the wnt signal in this development (van Genderen et al., 1994). Mice deficient in Max1 and Max2 transcription factors exhibit a similar developmental arrest (Satokata et al., 2000). Tissue recombination experiments have demonstrated that these primary mesenchyme markers induced by the mammary epithelium are temporal and are downregulated at a later stage of development (Heuberger et al., 1982).

Parathyroid hormone related peptide (PTHrP) is an autocrine/paracrine factor involved in a variety of cellular activities. It is often overexpressed in human tumors, can lead hypercalcemia of malignancy (HHM) and is supposed

to play role in tumorigenesis. PTHrP is expressed in mammary epithelium and its signal is received by the surrounding mesenchyme expressing its receptor. The disruption of PTHrP signaling in the knock out mouse model results in mammary gland developmental arrest before bud elongation is initiated and the primary mesenchyme markers are not expressed in the absence of this signal (Wysolmerski et al., 1998). The ectopic expression of PTHrP in the epidermis causes the differentiation of the adjacent dermal cells into the primary mesenchyme and nipple cells (Foley et al., 2001). Therefore, PTHrP has been recognized as the first signaling molecule to determine cell fate in the surrounding mesenchyme. In this early stage, the crosstalk between epithelial and stromal or surrounding mesenchyme is important for the mammary development.

The secretion of ovarian hormones such as estrogen and progesterone during puberty stimulates ductal development. Large-club like structures composed of cuboidal epithelial cells, called terminal end buds (TEBs), develop at the distal end of the mammary ducts. These terminal end buds actively proliferate, elongate, and develop secondary and tertiary ducts until the ducts penetrate the entire fat pad. In the terminal end buds, there are two different cell types: body cells that give rise to mammary epithelial cells and cap cells that are precursors of myoepithelial cells. Side branching also occurs from the mature duct (Fig. 2). During each estrous cycle, cyclic changes in the level of estrogen and progesterone induce active proliferation in late proestrous, the formation of

small side branches in estrous phase, followed by the regression with increased apoptosis in diestrus.

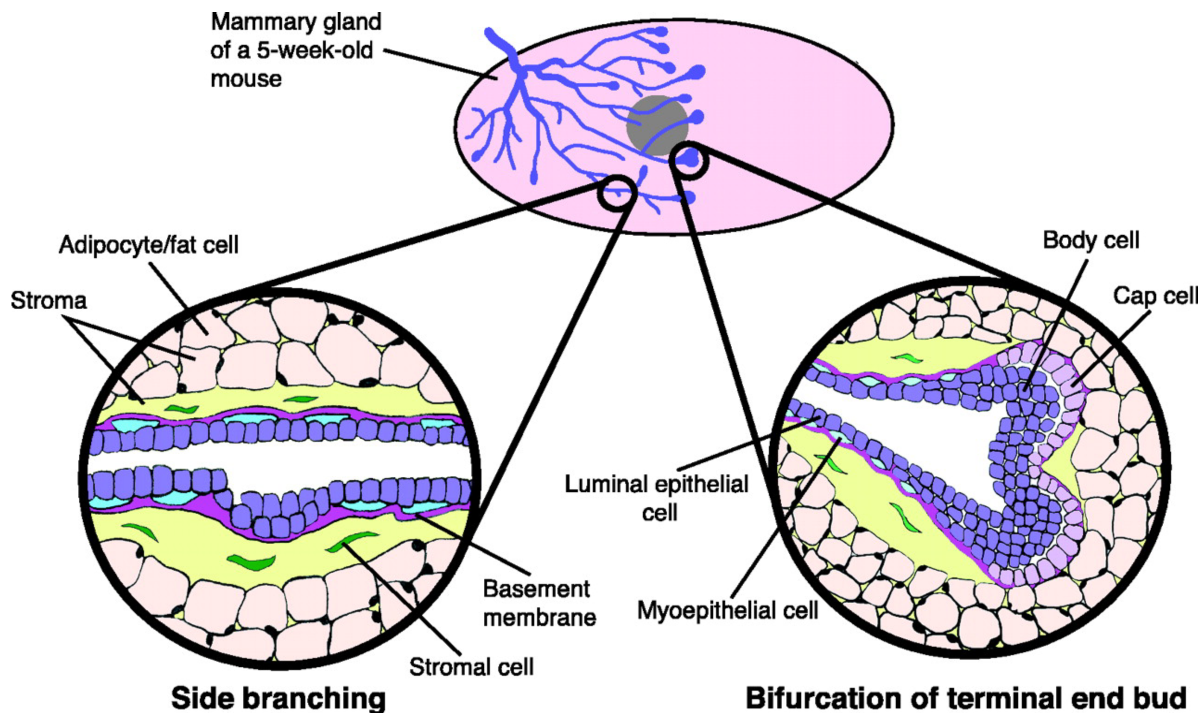

Fig. 2. The two distinct mechanisms of branching morphogenesis in the pubertal mouse mammary gland (Wiseman and Werb, 2002).

The role of both estrogen and progesterone in puberty for ductal growth has been demonstrated by hormone ablation and reconstitution experiments (Imagawa et al., 2002). Most steroid hormone action is mediated through ligand binding to nuclear receptors such as the estrogen receptor (ER) or progesterone receptor (PR). Both ER and PR are highly expressed in ovary, uterus, mammary and pituitary glands. There are two ER isoforms, denoted as ERα and ERβ,

which exhibit different tissue distribution and functionality. Differences between two ER will be discussed in section 1.5.

Transplantation studies have demonstrated that ERα expression is required in both epithelial and stromal compartments for normal duct growth. High dose estrogen and progesterone treatment induces mammary duct growth in ERα-deficient epithelium but not in ERα-deficient stroma, suggesting a rudimentary role of ERα in the epithelium in mammary duct growth (Mueller et al., 2002). Gene targeting approaches have characterized infertility and impaired mammary duct growth during puberty in female ERα-null mice. It is also reported that levels of prolactin inhibit mammary duct development (Bocchinfuso et al., 1999).

Progesterone receptor A (PR-A) and Progesterone receptor B (PR-B) are transcribed from two distinct promoter start sites on the same gene and PR-A contains 165 additional N-terminal amino acids. Mice lacking both isoforms display limited ductal side branching and deregulated ovulation (Lydon et al., 1995). By using Cre/loxP recombination strategy, mice only expressing PR-B form have been generated. Unlike the mice deficient both isoforms, the PR-A null mice develop normal ductal branching and alveolar budding, suggesting that PR-B not PR-A mediates the progesterone signaling in mammary gland development (Mulac-Jericevic et al., 2002). The epithelial glucocorticoid receptor (GR) is required for normal duct development but the function of the GR in alveolar development can be rescued by upregulation of the mineralcorticoid

receptor during pregnancy (Kinsley-Kallesen et al., 2002). Interestingly, transplantation studies have shown the direct involvement of the IGF signaling pathway in ductal outgrowth. IGF-R1 deficient epithelium displayed reduced cell proliferation in terminal end buds (Bonnette and Hadsell, 2001).

Mammary gland development is completed only when pregnancy and lactation occur. During pregnancy, reproductive hormones stimulate active cell proliferation in ducts and alveoli, resulting in the expansion of lobular compartment of the gland and induction of terminal differentiation of mammary epithelium into lobular alveoli that secrete and produce milk. Prolactin and placental lactogens are the major hormones in alveolar development and in differentiation of secretary cells. It is therefore obvious that disruption of prolactin receptor (PrlR) signaling pathway inhibits alveolar development. Deletion of the prolactin gene resulted in reduced ductal growth in adult animal virgins (Horseman et al., 1997). Heterozygous mice containing only one intact PrlR allele fail to lactate after their first pregnancy. However, older female mice or mice after second pregnancy successfully lactate, indicating that continuous hormone stimuli will lead to fully functional mammary gland development (Ormandy et al., 1997). Even though Stat5a and 5b exhibit 96% homology and similar biochemical features in tissue culture, Stat5a-deficient mice are incapable of lactation due to the failure of mammary gland differentiation during pregnancy whereas Stat5b-deficient mice maintain their pregnancy, deliver litters and lactate normally (Liu et al., 1997). Inactivation of transcription factor

C/EBPβ unexpectedly causes the phenotype similar to that of PrlR knock out mice and exhibit reduced ductal growth, abrogation of alveolar differentiation and high levels of PR positive cells in the mammary gland. It was suggested that cell-to-cell communication in paracrine manner is affected in C/EBPβ-null mice (Robinson et al., 1998, Seagroves et al., 1998 and 2000).

Cyclin D1-null mice also fail to expand and differentiate alveoli during pregnancy (Sicinski et al., 1995;Fantl et al., 1995). Interestingly, transgenic mice expressing the neu and ras oncogenes develop mammary tumors within a few months in mice overexpressing cyclin D1 whereas tumors are not induced in the absence of cyclin D1 (Yu et al., 2001). In contrast, mice overexpressing c-myc and wnt1 develop mammary tumors independent of cyclin D1 expression, indicating that at least two distinct pathways leads to mammary tumor development. Unexpectedly, normal mammary development is restored in the double knockout mice carrying null mutations on both cyclin D1 and p27, a cyclin-dependent kinase inhibitor, whereas the impaired mammary development is observed in either p27- deficient or cyclinD1-deficient mice, respectively (Geng et al., 2001). The Id2 gene encodes a basic helix-loop-helix transcription factor that inhibits cell proliferation and differentiation in many developmental processes. Moreover, silencing of Id2 expression in mammary epithelium decreased proliferation and differentiation (Mori et al., 2000). After lactation is terminated, there is decreased prolactin release from pituitary when the pups no longer suckle on the mammary gland. The secretory mammary gland epithelium

induces proapoptotic genes and subsequent apoptosis occurs in alveoli cells, leading to massive remodeling of the alveolar compartment with high tissue protease activity, resulting in a process called involution. In stat3-deficient alveolar epithelial cells, the involution is delayed at an early stage, suggesting that Stat3 may trigger apoptosis at this early stage (Humphreys and Hennighausen, 1999). Mice lacking plasminogen, a protease that degrades extracellualr matrix, also display reduced apoptosis and remodeling of the mammary gland whereas mice deficient in TIMP3, an inhibitor of metalloprotease, exhibit accelerated involution (Lund et al., 2000; Fata et al., 2001). Thus, remodeling of the mammary gland requires different proteases and protease inhibitors at the different stages of mammary development.

As described above, the developing mammary gland displays many of the same properties associated with the stepwise development of cancer. Namely; invasion of the terminal end bud into stromal tissue or fat pad, much like a solid tumor, maintenance of epithelial cell proliferation potential throughout its lifetime, resistance of the lactating mammary gland to premature involution, inhibitors of apoptotic signals and angiogenic-dependent remodeling of the blood supply required for mammary gland development. It is therefore not surprising that the factors essential for mammary gland development are also associated with breast cancer development.

1.2.2 Classification, grade, and stage of breast cancer

Breast cancers are histologically divided into three major categories: Noninvasive carcinoma (in situ carcinoma), invasive (nonfiltrating) carcinoma and Paget's disease. Noninvasive carcinoma consists of two subtypes of intraductal carcinoma (Ductal Carcinoma In Situ: DICS) and lobular carcinoma in situ (LICS). DICS is defined as a malignant population of epithelial cells within ducts originating from the end of the terminal lobular duct, which lacks the capacity to invade through the basement membrane. It is frequently diagnosed by mammography in approximately 20-30% of all breast cancer patients. However, these cells are still capable of spreading out throughout the ducts to the entire breast. There are 4 microscopic variants of DICS: comedo, solid, papillary, and cribiform carcinomas. Lobular carcinoma in situ (LCIS) is confined to the lobules and is generally considered as a marker for a precancerous stage of breast cancer. The cells in the lesion are loosely cohesive and are larger than normal cells.

Invasive or infiltrating carcinoma has two subtypes: invasive ductal carcinoma (IDC) and invasive lobular carcinoma (ILC). Invasive ductal carcinoma is the most common type, accounting for 65-80% of all breast cancers. The tumor cells invade the connective stromal tissue and display malignant cells lining the ducts, solid cell nests, tubules and glands. Medullary carcinoma is one of variants of IDC and accounts for only 1-5% of all mammary carcinomas. These tumors are usually 2-3 centimeters in size but can exceed 5

centimeters. Desmolplasia, a formation of fibrous tissue, which is commonly observed in many cancerous lesions, does not appear in medullary carcinoma, which exhibits more a soft and fleshy consistency. Colloid or mucinious carcinoma, other variants of IDC, tend to occur in older women and grow slowly in the course of cancer development. Extreme softness and appearance of pale gray-blue gelatin features characterize this type of tumor. Invasive lobular carcinoma is usually bilateral and multicentric with a distinct morphology probably arising from the terminal ductules of breast lobule.

Paget's disease is characterized by the presence of large cells with abundant clear or light staining cytoplasm and atypical nucleoli in the surface epithelium of the nipple. Skin lesions are associated with an underlying DICS, which are frequently fissured and ulcerated.

All types of breast cancers are rated depending on the tumor cell growth rate and pattern ranging from 1 to 3. Higher ratings are given to tumors with more disorganized and irregular patterns of the cell growth. Breast cancers are also staged from 0 to IV by their size, invasiveness and pattern of spreading. Stage 0 describes non-invasive breast cancer. In stage I, the tumor cells invade neighboring normal tissue but not lymph nodes and their size is less than two centimeters. Invasion of the tumor cells into the lymph nodes under the arm is observed in stage II tumors and the tumor size varies from two centimeters to five centimeters. Tumors larger than five centimeters that have spread to the breast skin and chest walls are classified as stage III. The breast skin looks like

the peel of naval orange and is a sign of inflammatory breast cancer. Finally, stage IV tumors display massive invasion of the tumor cell beyond the breast, under the arms and into internal mammary lymph nodes.

1.2.3 Genetic and epigenetic alterations of breast cancer

The accumulation of molecular alterations during tumor progression results from interactions between genetic and environmental factors, leading to deregulated cell proliferation, apoptosis, and loss of genetic stability. It has been demonstrated that aberrant activities of genes involved in these processes also predispose women to breast caner.

In hereditary breast cancer, germline mutations in BRCA1 have been identified and account for 15-20% women with a family history of both breast and ovarian cancer (Couch et al., 1997; Peto et al., 1999). BRCA1 is a 220 kD nuclear protein, containing a zinc-binding ring finger domain at the amino terminal and a conserved transcriptional coactivation domain at the carboxy terminal. This protein functions in DNA repair and cell cycle checkpoint control. Several studies have identified a new role of the ring finger domain as an essential structural subunit of E3 ubiquitin ligases. Moreover, BRCA1 exhibits ubiquitin ligase activity in vitro (Venkitaraman, 2002; Lee and Boyer, 2001). The BRCT domain is found predominantly in proteins involved in cell cycle checkpoint functions responsive to DNA damage. The C-terminal domain of BRCA1 is characterized by tandem a BRCT domain that has been found in a diverse group of proteins but has no known specific cellular function. However,

this BRCT domain mediates its interaction with many proteins such as RNA helicase A, CtIP, and histone deacetylase. Several studies indicated that BRCA1 is involved in DNA repair and cell cycle progression. Moreover, Ataxia telangiectasia mutated (ATM) and check point kinase 2 (CHK2) catalyze phosphorylation of BRCA1 in response to DNA damage and the arrest of the cell cycle in G2 after DNA damage in cells lacking functional BRCA1. However, tissue specificity of the BRCA1 mutation and its precise roles in development of breast and ovarian cancer are not fully understood (Venkitaraman, 2002). BRCA2, a second breast caner susceptibility gene, also functions in DNA damage pathways. Men with germline mutations in BRCA2 have 100-fold increase over the normal male population in their risk for the development of breast cancer (Phelan et al., 1996). This mutation may be also associated with an increased risk for colon, prostate, and stomach cancers. Phosphatase and tensin homologue on chromosome 10 (PTEN), a lipid phosphatase, is germline-mutated in Cowden syndrome and PTEN mutations are risk factors for breast cancer. Loss of heterozygocity at the PTEN locus occurs 30-40% of human breast cancers (Perren et al., 1999). p53 is a well-characterized tumor suppressor gene that functions as a transcription factor for regulation of cell cycle progression. The germline mutation of p53 cause Li-Fraumeni syndrome which is associated with childhood leukemias, brain tumors, breast carcinomas, soft tissue sarcomas, and osteosarcomas (Vogelstein, 2000). ATM is a serine/threonine kinase that functions as a signal transducer of DNA damage

responses such as DNA double strand breaks. Recent data indicate that the ATM missense mutation may exert a dominant negative effect on wild type ATM (Scott et al., 2002) and account for the occurrence of breast cancer in about 3% of families (Finkel, 2002). However all of these germline mutations found in breast cancer including BRCA1, 2, and other low penetrance variants only account for 5-10% of breast cancer overall and 15-20% of hereditary breast cancer.

For sporadic breast cancer, Somatic mutations, amplification, deletion or truncation of tumor suppressor genes and proto-oncogenes have been identified. The epidermal growth factor (EGF) ligand/receptor family has been strongly implicated in breast cancer. EGFs as potent mitogens, binds the EGF receptor tyrosine kinases that activate downstream target such as c-myc and cyclin D1 (Hynes, 2000). There are four closely related EGF receptor genes: EGFR/HER/erbB1, HER2/erbB2/Neu, erbB3/HER3, and erbB4/HER4. ErbB2 is the most frequently upregulated gene among members of ErbB family and is amplified or overexpressed in 15-30% of breast cancers (Slamon, 1987). Increased ErbB2 level may occur in an early stage of tumorigenesis and is detected in up to 60% of DCIS, particularly in the comedo type (Revillion et al., 1998). HER/ErbB1 overexpression is also observed in 20-40% of breast tumors and is associated with poor prognosis (Toi et al., 1991). The upregulation of several EGF ligands such as epidermal growth factors (EGFs) or transforming

growth factor α (TGFα) is also observed in primary and metastatic breast cancers (Salomon et al., 1995).

Insulin-like growth factor-II (IGFII) is highly expressed in the stromal compartment of breast tumors and is also correlated with poor prognosis. Among breast cancer patients, elevated serum IGF-1 levels was associated with increasing risks of developing breast cancer. IRS-1, a downstream signaling molecule in the IGF pathway and the main docking protein for binding and activation of insulin-stimulated PI 3-kinase, is correlated with ER status, and associated with poor prognosis in breast cancers (Zhang and Yee, 2000). Wnts are a family of secreted signaling proteins that exert critical roles in development. Overexpression of specific subset of these proteins such as Wnt2, Wnt4, and Wnt5A has been identified in some breast cancers. In the Wnt pathway, β catenin act as a downstream signaling mediator for regulating cyclin D expression and overexpression of β catenin in some type of breast cancer has been observed (Lin et al., 2000).

c-Src is an intracellular non-receptor tyrosine kinase that acts as proto-oncogene by augmenting signals from extracellular growth factors and by morphogenetic remodeling of cells to promote tumorigenesis. Overexpression of c-Src is observed in up to 70% of breast cancer specimens and overexpression of both c-Src and HER1 has also been identified in a subset of breast cancers (Biscardi et al., 2000). In approximately 20% of mammary carcinomas, cyclin D1 is amplified and especially overexpressed in 50% of primary ductal carcinomas.

Interestingly, cyclin D1-deficient mice develop normal mammary glands, which fail to proliferate in pregnancy, indicating the unique role of cyclin D1 in mammary development (Sutherland and Musgrove, 2002). Recent data show that truncation of CHK2, a serine/threonine kinase and a downstreram mediator of ATM, is 4-5 times more frequently observed in individuals with breast cancer. This kinase–inactive variant is considered as one of the low penetrance breast cancer susceptibility genes (Meijers-Heijboer et al., 2002). Compared to normal breast epithelium, chemokine receptors CCR7 and CXCR4 are significantly upregulated in primary breast cancers.

Chemokines are small-secreted proteins that bind G-protein coupled cell surface receptors to direct the migration and invasion of specific tumor sets to their preferred metastatic sites (Muller et al., 2001). E-cadherin is a large glycoprotein that is involved in cell-to-cell and cell to extracellular matrix adhesion. Up to 85% of lobular breast cancers do not express any E-cadherin due to LOH at 16q22.1, suggesting that E-cadherin functions as a tumor suppressor gene to repress breast cancer invasion (Berx and Van Roy, 2001).

1.2.4 Estrogen and breast cancer treatment

Estrogens are ovarian steroid hormones required for establishment and maintenance of the female reproductive tract. They also play important roles in development of the male reproductive tract, in bone formation, lipid metabolism and maintenance of the cardiovascular and nervous systems (McDonnell et al., 2001; Nilsson et al., 2001). Because of their homeostatic functions, estrogens

have been widely used for treatment of menopausal symptoms such as hot flash, urogenital atrophy, and osteoporosis. These benefits of hormone replacement therapy (HRT) for treatment of symptoms in postmenopausal women have been documented (Ettinger, 1998). However, it has been repeatedly shown that prolonged exposure to cycling estrogen levels contribute to the development of breast cancer. Over 100 years ago, it was first reported that the removal of endogenous estrogen via oophorectomy resulted in remission of breast cancer, suggesting a role for estrogens in breast cancer development (Leake, 1996).

Indeed, clinical and experimental data also indicate that breast cancer is dependent upon estrogen exposure (Clemons and Goss, 2001). Late menopause and early age at menache are associated with an increased risk for developing breast cancer (Trichopoulos et al., 1972; Kampert et at al., 1988) The use of estrogen and oral contraception increase the risk of breast cancer (Ursin et al., 1998). In postmenopausal women, a major source of estrogen is adipose tissue in which androgenic precursors can be converted to estrogen by the enzyme aromatase. Not surprisingly, obese postmenopausal women with obesity have increased estrogen levels and are more likely to develop breast cancer (Cauley et al., 1999; Maehle and Tretli, 1996) Therefore, those factors that increase estrogen exposure such as early menarche, late menopause, and nulliparity are associated with an increased risk

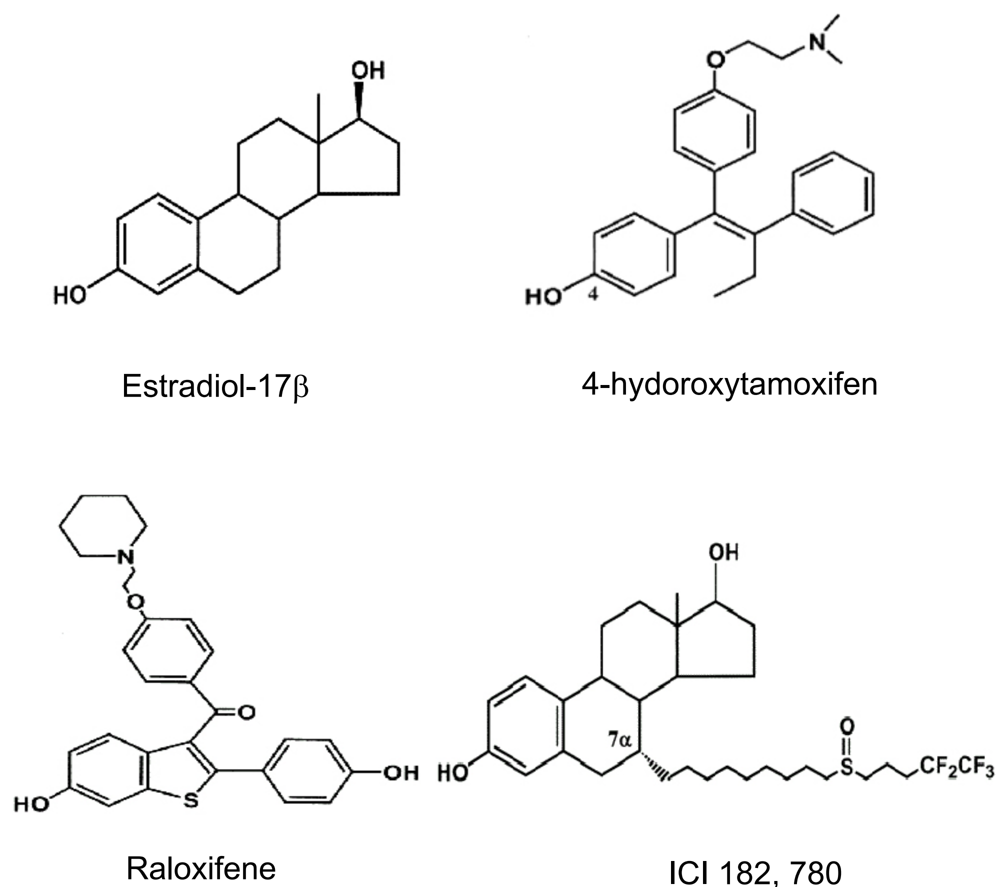

Fig. 3. Chemical structures of estrogen and antiestrogens.

of developing breast cancer. In addition, a number of studies report that long-term administration of estrogen also can increase the risk for breast, ovarian, and endometrial cancers (Persson, 2000) whereas decreased estrogen exposure is regarded as protective. Early ovarian failure substantially decreases the incidence of breast cancer. However, this is accompanied by unfavorable long-term problems such as osteoporosis (Pike at al., 1983). Similarly, a longer lactation time and moderate exercise that could decrease the total number of ovulatory cycles can be protective (Bernstein et al 1994; Yuan et al 1988). The

role of estrogen exposure in development of breast cancer has restricted the pharmacological use of estrogens and this has been replaced by selective estrogen receptor modulators (SERMs) which exhibits tissue-specific ER agonistic and antagonistic activity: SERMs that exhibit ER antagonistic activity in the mammary gland are extensively used for treatment of breast cancer (Fig. 3). Tamoxifen is a SERM that was originally developed as an oral contraceptive, but animal studies indicated that this compound was a potential antiestrogen (Harper and Walpole, 1967). Tamoxifen is beneficial for treatment of both pre- and postmenopausal women with ER-positive breast tumors. The optimal treatment period of 5 years reduces the risk of death by 28% and the incidence of contralateral breast cancer is decreased by 47% (Anonymous, 1998). For treatment of metastatic breast cancer, tamoxifen causes regression in approximately 30% of these cancers. More recent data shows that application of tamoxifen after surgery for primary breast cancer decreases micrometastasis, undetectable secondary tumors and ultimate causes of the cancer deaths among these patients (Osborne, 1998). Tamoxifen is now the first-line endocrine agent for treatment of breast cancer in pre-and post-menopausal women (Jordan, 2000). However, mixed ER agonistic and antagonistic activities of tamoxifen have been observed in animal studies. For example, tamoxifen maintains bone density in post-menopausal women and lowers the circulating cholesterol levels, but also increases the risk of endometrial and uterine cancer (Neven et al., 1997; Fisher et al., 1998; Fornander et al., 1993). Some ER-

dependent growths of breast tumors are probably related to tamoxifen resistance (Plotkin et al., 1978). To avoid adverse of effects of tamoxifen, improved SERMs such as raloxifene or ICI 182,780 (Faslodex) have been developed and these drugs have minimal effects on endometrial cancer. Raloxifene is a benzothiophene derivative that also exerts mixed ER agonist and antagonist activities. It was initially developed to prevent fractures in osteoporetic women (Delmas et al., 1997). The More trial of raloxifene was started in 1994 to evaluate its effects as another potential SERM. There was a 76% decrease in the incidence of breast cancer for women on raloxifene; significant decreases in the incidence of osteoporosis and serum cholesterol levels were observed (Cummings et al., 1999; Ettinger et al., 1999). However, unlike tamoxifen, an increased risk of heart disease was observed whereas endometrial cancer incidence was not increased after clinical studies (Barrett-Connor et al, 2002; Goldstein et al., 2000). These studies clearly indicated tissue-specific estrogenic or antiestrogenic effects of raloxifene that differed from those of tamoxifen.

Development of improved SERMs with minimal ER agonism for treatment of breast cancer risks or thromboembolism would improve clinical efficacy. ICI 182,780 is a pure antiestrogen that does not exhibit partial ER agonist activities and these antiestrogenic activities are advantageous for treatment of estrogen-dependent disease. ICI 182,780 has an ER binding affinity approximately 100 times greater than that of tamoxifen and exerts no agonistic activity on estrogen-

responsive tissues such as the uterus (Wakeling and Bowler, 1992; Parisot et al., 1995). In nude-mice xenograft studies, ICI 182,780 treatment suppressed the growth of established tumors for almost twice as long as tamoxifen treatment (Plotkin et al., 1978). Uterine stimulatory effects were not detected in animals treated with ICI 182,780 (Dukes et al., 1992). In a proportion of patients with metastatic tumors that relapsed from tamoxifen resistance, ICI182,780 still exerted inhibitory activities as an ER antagonist (Hu et al., 1993). Phase III studies of ICI 182, 780 are now underway to further examine its efficacy in comparison with other SERMs (Howell et al., 1996).

1.3 Transcription

1.3.1 Overview

Appropriate expression of genes in eukaryotes is required for the development, growth, and survival of the whole organism. Expression of genes encoding protein is regulated in a highly orchestrated and elaborate fashion to guarantee the expression of specific subsets of genes in a temporally and spatially appropriate manner. Genetic imprinting, cell growth and death signals, environmental stimulus, and tissue-specific restriction are critical factors that control gene expression in eukaryotes. These precisely controlled patterns of gene expression ultimately contribute to the overall function of the organism.

Although regulation of gene expression takes place at multiple levels from transcription, mRNA processing, translation to post-translational modifications, a major regulatory step for gene expression is transcription that is defined as “the

copying of any DNA strand nucleotide by nucleotide following the base-pairing rules by an RNA polymerase to produce a complementary RNA copy in eukaryotes in the nucleus" (Orphanides and Reinberg, 2002). RNA production in eukaryotes is regulated by three different types of RNA polymerases; RNA polymerase I transcribes ribosomal RNA (rRNA), RNA polymerase III transfer RNA (tRNA) and other small RNAs, and RNA polymerase II nuclear strucutural genes encoding mRNA for protein synthesis. The following sections will focus on RNA polymerase II-mediated transcriptional process for mRNA production in detail.

1.3.2 Chromatin structure and gene expression in eukaryotic transcription

In eukaryotic cells, genetic information is stored as the sequence of deoxyribonucleic acid (DNA) that is highly organized and densely packed in a structure known as chromatin. It is estimated that the human genome contains 30,000-75,000 genes that encode functional gene products including proteins, rRNAs, and tRNAs, and this represents only 5% of the entire genome. The remaining 95% consists of non-coding intron sequences and other repetitive DNA sequences (Venter et al., 2001). Genes are distributed among 3.2 billion base pairs of DNA that are tightly compacted into a high ordered chromatin structure via association with histones and other non-histone proteins, finally packaged into 23 pairs of chromosomes. Approximately, 146 base pair of DNA are wrapped slightly less than two turns around an octameric protein core, consisting of two copies of each histone including H2A, H2B, H3, and H4, which

result in “bead-on-a-string appearance”. The “bead” is nucleosome of around 10nM diameter and the “string” is connecting DNA. Nucleosomes are further coiled into 30nm fibers of solenoids that contain six nucleosomes per turn. A fifth histone, H1, is associated with each nucleosome on the inside of the solenoid structure. Finally, these chromatin fibers are packaged into chromosomes (Fig. 4).

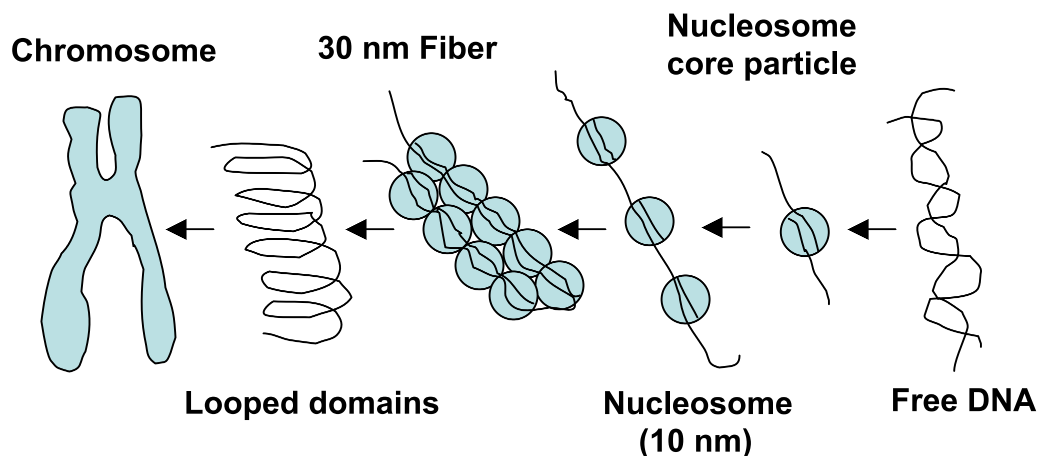

Fig. 4. Chromatin structure (Johnstone, 2002)

However, not all the chromatin is the same and chromatin structure depends on its state of packaging. In terms of DNA accessibility, there are two major types of chromatins: heterochromatin and euchromatin. The portion of genome that remains condensed during the transition from metaphase to interphase is initially described as heterochomatin (Henikoff, 2000). The richness in repetitive sequences, low gene density, and regularly spaced nucleosomes suggest that heterochromatin may function as a gene-silencing

module. In contrast, euchromatin displays irregularity in nucleosomal arrays with a punctuated pattern of nucleosome-free hypersensitive sites associated with the active transcribed region for gene expression. Packaging in a heterochromatic form typically silences genes normally active in a euchromatin region and this effect is known as a position effect varigation (PEV). This reflects heterochromatic assembly of previous euchromatic regions at the boundary between euchromatin and heterochromatin in a stochastic mechanism (Grewal and Elgin, 2002). In reality, there are a number of intermediate states of chromatin from constitutively silenced state of X chromosome inactivation to fully active states that regulate gene expression. In fact, this highly ordered hierarchy of chromatin structure in eukaryotes creates barriers for gene transcription at various levels. Most importantly, transcription requires the alteration of DNA packaging and the enhancement of DNA accessibility at the transcriptional initiation step.

The fundamental units required for gene regulation consist of three types of specific DNA sequences that determine levels of expression under specific physiological conditions. Firstly, the coding sequences that contain the information that encodes a functional gene product such as protein, rRNA, or tRNA. Secondly, the core promoter sequences that recognize RNA polymerase and include TATA box (TATA) and Initiator (Inr) sequences usually located on 5' upstream of the coding sequence. Thirdly, regulatory sequences can negatively or positively affect gene transcription. Operator or repressor sequences act as

negative control elements to inhibit unnecessary transcription. In contrast, enhancer sequences that are recognized by activators stimulate the transcription from the promoter either proximally or distally from the initiation start site (Struhl, 1999).

The complexities of gene regulation in eukaryotes arise from the fact that eukaryotic DNA is packaged into chromatin templates. In prokaryotes, RNA polymerase can access to the DNA template without any inherent restriction and initiate transcription both in vivo and in vitro without any specific activator protein. In contrast, even a strong core promoter sequence can be inactive in eukaryotes, depending on chromatin structure (Fig. 5). The ground state for transcriptional activation is inherently restrictive and chromatin acts as a general inhibitor of protein access to DNA (Workman and Kingston, 1998). Therefore, eukaryotic genes essentially require activator proteins that enhance transcription by interaction with enhancer sequences located at proximal or distal sites on the gene promoter. There are primarily two ways in which activators could enhance gene transcription in eukaryotes. Firstly, activators bound to the enhancer sequence increase gene transcription either through enhancing direct association with the basic transcriptional machinery and secondly, through increasing the recruitment of the transcriptional machinery to the promoter by altering chromatin structure (Ptashne and Gann, 1997).

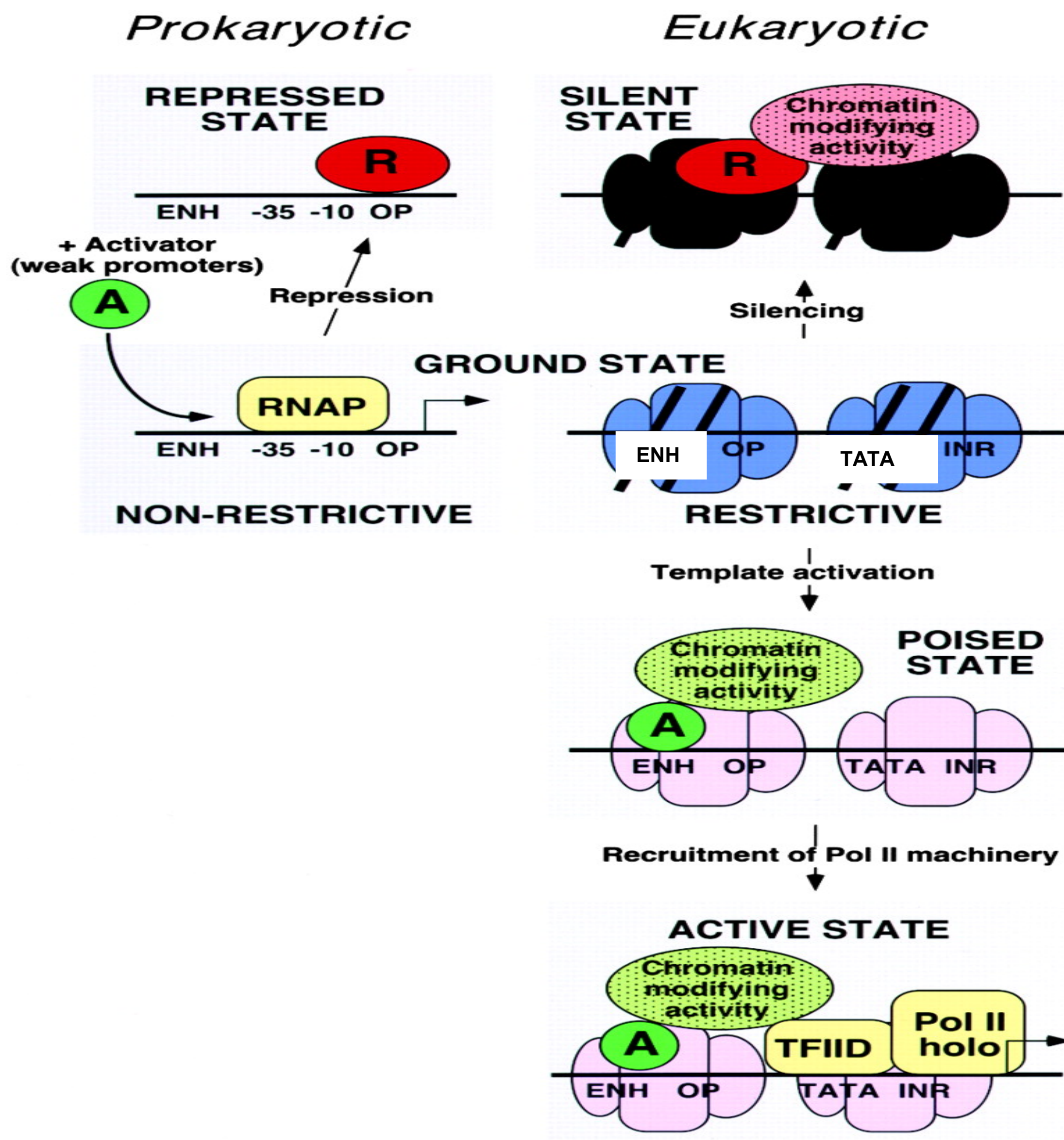

Fig. 5. Transcriptional states in prokaryotes and eukaryotes (modified from Struhl, 1999).

1.3.3 General transcription factors (GTFs) in basal transcription

The essential components of the eukaryotic transcription apparatus are General Transcription Factors (GTFs) and the Core Promoter. GTFs are

required for accurate initiation by RNA polymerase II in vitro and the core promoter is minimal DNA sequence for initiation of transcription by RNA polymerase II in a reconstituted cell-free system. RNA polymerase II is a multienzyme complex, consisting of 12 evolutionary conserved subunits, which catalyze the synthesis of mRNA from the DNA template (Roeder, 1991 and 1996). However, along with the RNA polymerase II, accurate and efficient transcription even from the strong core promoter requires other essential auxiliary factors, termed as “basal” or “general transcription factors”. There are six evolutionary well-conserved and -characterized general transcription factors that include TFIIA, TFIIB, TFIID, TFIIE, TFIIF, and TFIIH (Table 3) (Roeder, 1996). It has been demonstrated that the purified factors along with the core promoters can assemble into a transcriptional preinitiation complex (PIC) with the following sequential order: TFIID, TFIIB, RNA polymeraseII-TFIIH complex, TFIIE, and then TFIIH (Weil et al., 1979; Orphanides et al., 1996). TFIID is a multisubunit protein that consists of TBP (TATA-box binding protein) and 13 TBP associated factors (TAFs). The TFIID complex containing TATA-binding protein (TBP) and at least 12 other TBP-associated factors initially recognize and bind to the TATA box. TFIIA, composing of 3 smaller subunits, then binds the TFIID complex and stabilizes the complex. TFIIB immediately forms a TFIID-A-B complex that recruits the RNA polymerase II and TFIIF. Finally, TFIIE and TFIIH are added to the complex, which constitutes PIC that is now ready for DNA

melting and gene transcription (Roeder, 1996; Albright and Tjian 2000; Reinberg et al., 1998).

Table 3
General transcription factors associated with RNA pol II in human cells (Roeder, 1996)

Factor	Number of subunits	Mw.(kD)	Function
TFIID-TBP	1	38	Recognize promoter; Recruit TFIIB
TFIID-TAFs	12	15-250	Assist transcription activation; Assist promoter recognition
TFIIA	3	12,19,35	Stablize TFIID and promoter binding
TFIIB	1	35	Recruit RNA Pol II and TFIIF
TFIIF	2	30,74	Assist RNA Pol II to reach promoter
TFIIE	2	34,57	Recruit TFIIH;Modulate TFIIH helicase, ATPase and kinase activities
TFIIH	9	89,80,62,52, 44,34,32,38, 40	Promoter melting using helicase activity, DNA repair

1.3.4 Core promoter motifs in basal transcription

Typically, the core promoter contains the transcriptional initiation site and extends either upstream or downstream for additional –35 nucleotides (nt). There are several cis-acting DNA elements that are commonly found in core promoters such as TATA box, intiator (Inr), TFIIB recognition element (BRE), and down stream core promoter element (DPE) (Fig. 6). These motifs recognize different general transcription factors and exert specific functions in PIC formation. The TATA box was the first eukaryotic core promoter motif identified

and this motif is typically located about 25-30nt upstream of the transcription start site (Breathnach and Chambon 1981). Although the consensus sequence for the TATA box is TATAAA, a wide range of sequences still can function as a TATA box (Singer et al. 1990). The predominant TATA-box binding protein is TBP.

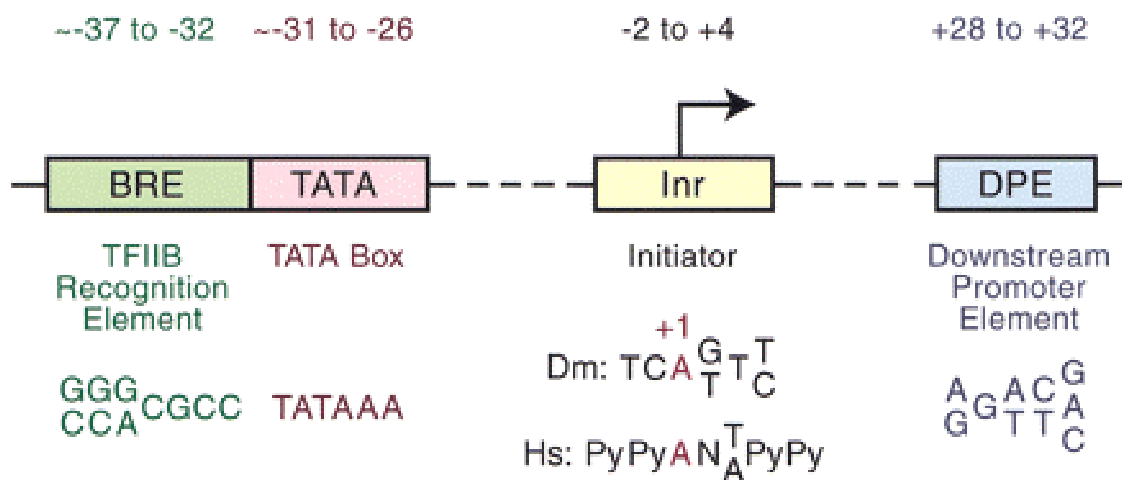

Fig. 6. Core promoter elements. Each of these elements is found in only a subset of core promoters. Any specific core promoter may contain some, all, or none of these motifs. The BRE is an upstream extension of a subset of TATA boxes. The DPE requires an Inr, and is located precisely at +28 to +32 relative to the A+1 nucleotide in the Inr. The DPE consensus was determined withDrosophila transcription factors and core promoters. The Inr consensus sequence is shown for both Drosophila (Dm) and humans (Hs) (Butler and Kadonaga, 2002).

It is, however, important to consider that there are TBP-related factors, termed TRFs, with different biochemical properties (Berk, 2000). For an example, TRF2, a TBP-related factor that does not bind to the TATA box, is

required for expression of a specific set of genes (Maldonado 1999; Moore et al., 1999).

The Initiator (Inr) motif is located at the transcription start site of many eukaryotic promoters and is found in both TATA-containing and TATA-less core promoters (Corden et al., 1980; Smale et al., 1998). TAFII150 and TAFII250, key subunits of TFIID, interact with the Inr in a sequence-specific manner (Verrijzer et al., 1995; Kaufmann et al., 1998). It also has been observed that purified RNA pol II can also bind to the Inr and mediates transcription in the absence of TAFs, suggesting that TFIID and RNA pol II may interact with the Inr in different steps of the transcriptional process (Carcamo et al., 1991; Weis and Reinberg, 1997). Interactions between the Inr and sequence specific-DNA binding factors such as TFII-I and YY1 indicate that there is communication between specific promoter–binding factors and the general transcriptional machinery (Roy et al., 1997; Grueneberg et al., 1997).

DPE, a down stream core promoter binding site for TFIID but not for TBP, is frequently found in TATA-less promoters. TAFII60 and TAFII40 interact with the DPE to stimulate DPE-dependent but TATA-less transcription and repress TATA-dependent but DPE-less transcription by recruiting the inhibitory NC2/Dr1-Drap protein complex (Willy et al., 2000). Transcriptional activation in DPE-dependent promoters vs transcriptional repression in TATA-dependent promoter illustrates fundamental mechanistic differences in transcription by assembling promoter specific protein complexes.

BRE is a TFIIB recognition motif located immediately upstream from some TATA box binding sites. This motif is frequently a GC-rich sequence in eukaryotes (Lagrange et al., 1998). Furthermore, X-ray crystallography has demonstrated the formation of a TFIIB-TBP-DNA complex and confirms the interactions of these protein-DNA complexes (Tsai and Sigler, 2000).

Since methylation of cytosine at the 5-position and subsequent deamination of the 5-methylcytosine will generate a TpG dinucleotide, which does not undergo DNA repair, the CG dinucleotide is underrepresented in vertebrate genomes. However, relatively GC-rich and mostly unmethylated stretches of DNA, termed CpG islands, are frequently found upstream from the transcription initiation sites of many genes that are transcribed at low rate and encode enzymes for intermediary metabolism. CpG islands range in size from 0.5 to 2 kbp and may contain multiple weak promoters that are distributed over a region of 100 nt. Typically, CpG islands lacks TATA or DPE core promoter elements but contains multiple GC box motifs that are bound by Sp1 and other Sp family of transcription factors. Sp1 not only contributes to the maintenance of the hypomethylated state of CpG islands but also plays role in mediating transcription initiation in concert with general transcription factors (Brandeis et al., 1994; Macleod et al., 1994). It has been observed that the Sp1 binding sites in conjunction with an Inr motif can activate transcription in the absence of TATA box (Smale and Baltimore, 1989; Emami et al., 1995).

As described above, there are different combinations of the core promoter elements such as DPE-less but TATA- and Inr-dependent, TATA-less but CpG islands-and Inr-depedent, or Inr-less but BRE-TATA dependent. These exhibit another level of transcriptional regulation depending on the combination of each individual motif on the core promoter.

1.3.5 Transcription factors and mediator complexes in eukaryotic transcription

1.3.5.1 Transcription factors

In addition to GTFs required for basal transcription in reconstituted cell-free in vitro systems, there are many inducible or sequence-specific transcription factors that bind to motifs in gene promoters to either enhance or inhibit gene transcription (Morimoto, 1992). These transcription factors (TFs) typically contain two functional domains; a sequence-specific DNA binding domain and a transactivation domain that mediates downstream events. Eukaryotic transcription factors are often classified by in their respective DNA binding domains (Harrison, 1991; Mitchell and Tjian, 1989). Typically, α helices in the DNA binding domain of transcription factors are oriented to make contacts with the major groove of DNA through hydrogen bonding and Van der Waals interactions. In some cases, the interactions of atoms between the sugar-phosphate backbone and in the DNA minor groove also contribute to the protein/DNA binding. DNA binding motifs in the TFs generally contain consensus amino acid sequences that characterize the type of transcription factors. Some examples of transcription factors classified by their conserved structural motifs

are homeobox, zinc-finger, winged helix or forkhead, leucine zipper, and helix-loop-helix families of proteins (Pabo and Saucer, 1992).

How does transcription factor binding to enhancer elements activate gene transcription from motifs that are distal (up to several kbs) from the transcriptional initiation site? What are the biochemical mechanisms of these activation processes? Briefly, there are two popular models for these processes. One is a “looping” model that involves tethering of the enhancer and promoter elements, by an interval of freely mobile DNA that enhances the probability of their interaction and results in increased gene transcription (Rippe et al., 1995; Ptashne and Gann, 1997). However, as the distance between two elements is lengthened, the “looping “structure becomes unstable and formation of large loops is less likely. The other model is a “scanning or tracking” mechanism in which enhancer binding protein complexes move along the DNA until they encounter their cognate promoters (Plon and Wang, 1986).

However, this model also does not explain how enhancers on one chromosome activate transcription from an alleic promoter on another chromosome such as a transvection event in bacteria or how an enhancer activates transcription from a tail hairpin structure, protruding from a double stranded circular DNA, results in blocking the scanning process.

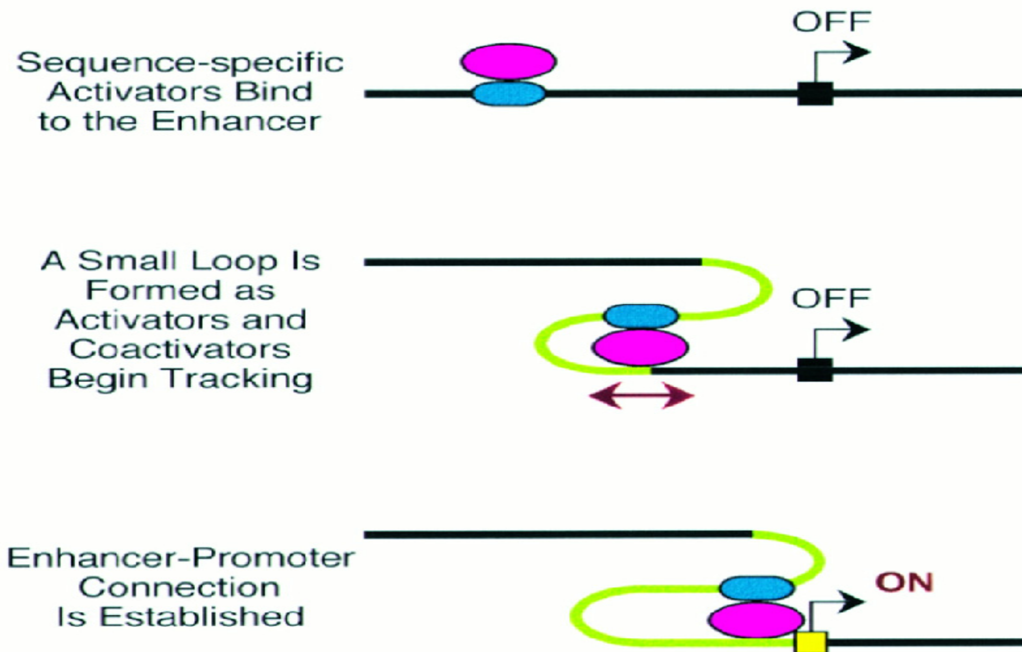

Fig. 7. A facilitated tracking model for enhancer function. (Blackwood and Kadonaga, 1998).

Recent models of a ”facilitated tracking” mechanism has shown the consistency with the broad range of phenomena associated with enhancer functions including long distance, orientation–independent transactivation, and transvection (Blackwood and Kadonaga, 1998). In this model, enhancer-bound protein complexes track along the chromatin until they encounter the cognate promoter while a stable small loop structure is maintained (Fig. 7).

In conjunction with these models, transactivation domains of transcription factors exert a variety of biochemical functions in concert with their sequence-specific DNA binding domains. These domains facilitate recruitment of chromatin

remodeling complexes and the covalent modification of chromatin structure. Enhancement of direct interaction between GTFs and their cognate promoter and the relocalization of the promoter region to active nucleolus region (Milot et al., 1996; Csink and Henikoff, 1996), and changes in the topological structure of DNA is also mediated, in part, by transactivation domains (Freeman and Garrad, 1992). These functions of transcription factors will be discussed in following sections in conjunction with other protein complexes.

1.3.5.2 Mediator complex

A minimal set of GTFs and purified RNA pol II is sufficient for accurate initiation of transcription in vitro. However, highly orchestrated transcriptional activation in response to sequence-specific transcriptional activators does not occur in these in vitro assays. This deficiency between GTFs and the transcriptional activators led to the discovery of various sets of mediator complexes (Hampsey and Reinberg, 1999). For example, the yeast mediator comprises at least 20 subunits including Srb and med proteins and this purified mediator complex binds to the Carboxy Terminal Domain (CTD) of pol II (Myers and Kornberg, 2000). In humans, several mediator complexes homologous to the yeast mediator complex have been independently identified (Rachez and Freedman, 2001). The subunit composition of these complexes ranges from 7 to at least 18 polypeptides and contains both conserved and unique components with no homology to the yeast mediator subunits. Unlike RNA pol II, GTFs, or ATP-dependent chromatin remodeling complexes, mediator itself is unable to

bind DNA but physically interacts with RNA pol II without being a component of RNA pol II.

Mediator also activates basal transcription and regulates TFIIH CTD kinase activity (Parvin and Young, 1998). The largest subunit of RNA pol II is a CTD that contains heptapeptide repeats phosphorylated by various CTD kinases. This phophorylation plays a critical role in transcriptional initiation and coordination of mRNA processing (Proudfoot et al., 2002). It has been suggested that mediator complexes are present in the nucleus free from both RNA pol II machinery and transcriptional activators. Through their ability to interact with the RNA pol II and activators, they serve as interfaces that interact with other coregulator proteins to modulate gene expression either positively or negatively, depending on subunit composition. For example, CDK8/cyclinC complex can phosphorylate the cyclin H subunit of TFIIH at two serine residues, thereby repressing both TFIIH activity and transcription. NAT mediator complex devoid of CDK8 (by immunodepletion) confers coactivation in vitro, but addition of CDK8 to this complex leads to repression. Thus, CDK8/cyclinC may act as a repressor module within the mediator complex (Akoulitchev et al., 2000). In addition, identification of the distinctive yeast Paf1 complex that transmits regulatory information from protein kinase C signaling to RNA pol II suggests that other unidentified signaling-or gene-specific mediator complexes may exist (Chang et al., 1999b). In summary, mediator is a modular complex that functions as a bridging factor between gene–specific regulatory proteins and GTFs.

However, the exact mechanism of mediator action is not yet fully understood and is currently being investigated in several laboratories.

1.3.6 Chromatin remodeling complexes in eukaryotic transcription

Before transcription is initiated, the chromatin structure of DNA/protein complexes is altered to facilitate access of transcription factors and RNA polymerase. This requires melting and reformation of the DNA duplex. Several distinct multiprotein complexes that catalyze remodeling have been identified. ATP-dependent remodeling complexes can change the position of the nucleosome, thereby exposing or occluding the specific transcription factor binding sites and RNA polymerase. Other complexes modify the nucleosome covalently either by adding or removing various chemical moieties. The N-termini of histone are extensively modified by acetylation, phosphorylation, methylation, and ubiquitination and this can impact chromatin structure.

1.3.6.1 ATP-dependent remodeling complexes

ATP-dependent remodeling complexes increase the accessibility of nucleosomal DNA by using ATP hydrolysis. There are three different classes of these complexes based on the identity of ATPase domain and the variable but distinct subdomains (SWI2/SNF2 family, ISWI family, and Mi-2 Family). The central core ATPase can alter chromatin structure in the absence of other remaining factors in the complex, However, the addition of other factors in the core ATPase complexes exhibits different biochemical activities in vivo (Kingston and Narlikor, 1999; Wang and Zhang, 2001). For an example, a Drosophila

ISWI-based complex cannot remodel nucleosomes lacking histone N-terminal tails whereas the human and yeast SWI/SNF complex can remodel these nucleosomes (Langst and Becker, 2001). The N-termini of histone 4 is critical for stimulation of ATPase activity of the ISWI complex but not the SWI/SNF complex, indicating differences in the substrate requirement by these two distinct remodeling complexes (Clapier et al., 2001). Both Mi-2 and ISWI complex can change the translational position of a nucleosome. Interestingly, the ISWI complex moves the nucleosomal histone octamer toward the end of a 248 base pair DNA fragment whereas the Mi-2 complex moves the nucleosome to the central position. Unlike ISWI, the Mi-2 complex can remodel a nucleosome in which the N-terminal tails of the histone H4, H3, and H2A have been deleted (Brehm et al., 2000). In a DNA extrusion assay that measures the capacity of the remodeling complexes to form a cruciform DNA from inverted repeats of DNA, the SWI/SNF complex can form a cruciform DNA from both naked DNA and chromatin templates. In contrast, this was not observed for the ISWI and Mi-2 complexes (Langst and Becker, 2001). This biochemical characterization of the ATP-dependent remodeling complexes provides evidence for mechanistic and functional differences between ISWI, SWI/SNF, and Mi-2 complexes.

Mechanistically, how does the exposure of a nucleosomal DNA occur? There are two models for the mechanism of ATP-dependent nucleosome remodeling. A classical “sliding” model explains that sliding of the DNA with respect to the nucleosome in the same direction can change the translational

position of the nucleosome on DNA (Fig. 8A). This leads to the exposure of the DNA that previously interacts with the histone octamer. It has been reported that all three families of ATP-dependent remodeling complexes can reposition the nucleosome on DNA. In nucleosomal arrays, certain restriction enzyme sites are blocked by SWI/SNF action, implicating that previously accessible DNA sites has been repositioned (Schnitzler et al., 2001).

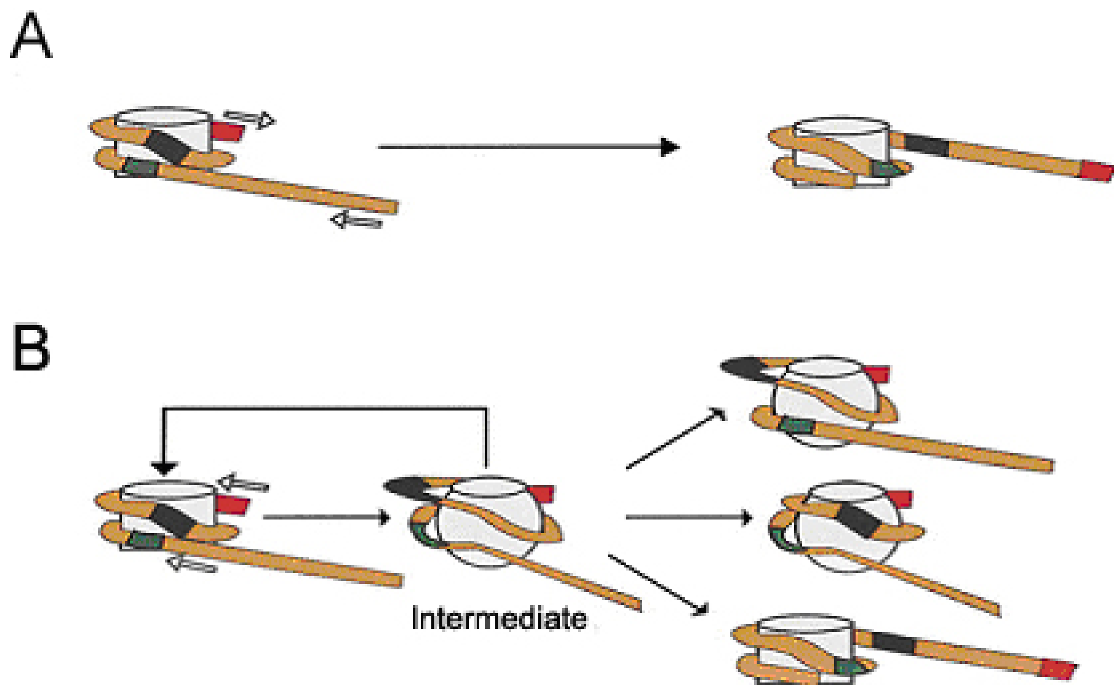

Fig. 8. Two models for the mechanism of ATP-dependent nucleosome remodeling. Sliding model (A) vs conformational change model (B) (Narlikar et al., 2002)

In addition, ISWI action can create regularly spaced nucleosomes from a randomly distributed nucleosomal array (Langst and Becker, 2001). However, this sliding mechanism cannot explain increased DNA accessibility in tightly

spaced nucleosomes since sliding will not increase the amount of exposed DNA but simply changes the location of the exposed DNA. Another unresolved problem arises from the fact that any transient changes of twist or writhe of linker DNA by repositioning the nucleosome will be transient. However, the stable topological changes introduced by SWI/SNF action can be stable and this does not fit the sliding model (Guyon et al., 2001).

Therefore, another explanation may be that the remodeling complex can induce conformational changes of nucleosomes to expose the nucleosomal DNA on the surface of the histone octamer (Fig. 8B) (Lorch et al., 1999; Studitsky et al., 1994). The SWI/SNF complex can increase DNase and restriction enzyme sensitivity on DNA sites of a mononucleosome lacking the flanking DNA space in which the histone octamer can slide (Kingstone and Narlikar, 1999). The site-specific crosslinking of the DNA to the histone octamer that prevents sliding of the DNA cannot hinder the remodeling of chromatin by SWI/SNF (Lee et al., 1999).

All of the data for ISWI-based complexes is consistent with the sliding model. Gradual movement of the histones along the DNA is characteristic of canonical nucleosomes in nucleosomal sliding, assembly, and spacing assays. ISWI-based complexes exhibit lower activity on mononucleosomes that does not contain any flanking DNA, which support the evidence of translational repositioning of histone octamers as expected for the silding model (Hamiche et al., 1999). In contrast, SWI/SNF action is more compatible with the idea that

conformational change of the histone octamer can expose nucleosomal DNA. Although the results from the SWI/SNF-and ISWI-based complex assays have suggested two different mechanisms of action, the molecular actions of ATP-dependent remodeling complexes have not been completely delineated.

1.3.6.2 Covalent modifications of chromatin

As mentioned previously, the amino termini of the core histones (H2A, H 2B, H3 and H4) are covalently modified by various chemical moieties; acetylation, phosphorylation, methylation, and ubiquitination, which can alter chromatin structure (Table 4) (Berger, 2001). The tail domains of histones, protruding from the surface of chromatin polymers, are protease sensitive and compose 25-30% mass of the individual histone (Wolffe and Hayes, 1999; Kornberg and Lorch, 1999). There are two current models for the function of histone modifications. One is that modifications directly affect the structure of chromatin by influencing either histone-DNA or histone-histone contacts. Considering the fact that maintenance of electroststic charges is required for the proper folding of chromatin structure and histone acetylation that neutralizes a positive charge and histone phosphorylation that adds a negative charge on the chromatin would cause decondensation of the chromatin fibers (Annunziato and Hansen, 2000). This would allow increased accessibility of specific DNA-binding factors to the chromatin. The second hypothesis is that various combinations of histone modifications constitute specific patterns that can recruit unique

biological complexes for mediating further downstream events. This is called "histone code" hypothesis (Strahl and Allis, 2000).

Table 4
Histone modifications (Berger, 2001)

Modification	Histones affected	Function
Acetylation	All four core histones	Transcriptional activation and repression Recombination Transcriptional silencing
Phosphorylation	H3 and H2B	Transcriptional activation Mitotic and Meiotic chromosome condensation
Methylation	H3 (arginine) H3 and H4 (lysine)	Transcriptional activation Heterochromatic silencing
Ubiquitination	H2B and Linker H1	Transcriptional activation

In yeast, the proper segregation and condensation of chromosomes during mitosis and meiosis require histone H3 phosphoryation at serine 10, possibly in conjunction with phosphorylation at serine 28 (Wei et al., 1999). The same phosphorylation at serine 10 also results in higher enzamatic acitivity of Gcn5 acetyltransferase on the neighboring lysine 14 (Cheung et al., 2000). Bromodomains are 110 amino acid long domains that are found in many chromatin-associated proteins that interact specifically with acetylated lysines. The bromodomain of PCAF, a histone acetyltransferase, promotes interactions with acetylated lysine in the context of H3 and H4 tail sequences to facilitate histone acetylation (Dhalluin et al., 1999). In contrast, the methylated lysine 9 of histone H3, targeted by the methyltransferase Su(var)39, interacts with the

chromodomain of the heterochromatic protein HP1 (Bannister et al., 2001). Increasing evidence suggests that multiple histone modifications act in a combinatorial or sequential fashion on one or multiple histone tails, resulting in recruitment of protein or protein modules to specify unique downstream functions.

Actively transcribed genes are strongly correlated with hyperacetylation state of lysines in the N-termini of the core histones whereas hypoacetylation of histones are associated with silenced genes, such as those located in heterochromatin. Not surprisingly, up to 13 of the 30-tail lysine residues are acetylated in a histone octamer (Roth et al., 2001). In vitro, histone acetylation enhances the DNA accessibility via multiple mechanisms; namely, by lowering the stability of histone-DNA interactions by introducing positive charges, by decreasing the compaction of nucleosomal arrays through disruption of internucleosomal interactions, and by recruiting additional transcription factors by forming a specific pattern of " histone code". Indeed, many transcriptional coactivators and corepressors contain subunits that possess either histone acetylase or histone deacetylase activity. Representatively, CREB-binding protein (CBP) and a related E1A-interacting protein p300 exhibit histone acetylase activity whereas Sin3 and NurD repressor complexes exhibit histone deacetyl transferase activity (Roth et al., 2001; Ahringer, 2000). Recent chromatin immunoprecipitation studies (CHIP), using specific antibodies bound to acetylated histones, have shown that there is a strong correlation between

increased acetylation in the proximal promoter region of specific genes and increased gene expression (Kuo et al., 2000). In contrast, hypoacetylation at specific promoters is clearly involved in recruiting histone deacetylase complex to the repressed genes (Khochbin et al., 2001). These histone-modifying activities exhibit substrate specificity for particular histones as well as individual lysines within the N-termini of histones. For example, yeast Gcn5 histone acetylase preferably acetylates histone H4 whereas other P/CAF and hGCN5 histone acetylase acetylate only histone 3 (Kuo et al., 1996). Steroid receptor coactivators-1 (SRC-1) acetylate all four core histones but ACTR appears to acetylate only histones 3 and 4 (Chen et al., 1997).

Coactivator-associated arginine methyltransferase 1 (CARM1) that binds to the carboxyl-terminal region of p160 coactivators, enhanced transcriptional activation by nuclear receptors, only with coexpression of GRIP1 or SRC-1 (Chen et al., 1999a). More recent studies have shown that arginine methylation in the tail of histone 3 by CARM1 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 (Xu et al., 2001; Nishioka and Reinberg, 2001). 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.

Lysine 123 within the histone H2B carboxy-terminal tail is ubiquitinated by Rad6 ubiquitin ligase and this modification is important for mitotic and meiotic growth in yeast (Robzyk et al., 2000). TAFII250 in TBP-associated TFIID complex ubiquitinates histone H1, leading to gene activation (Pham and Sauer, 2000). These data suggest that histone ubiquitination is involved in gene transcription.

1.3.7 Communications between various complexes in transcription

The identification of various types of functional coactivators or corepressor complexes including ATP-dependent remodeling complexes, mediator complexes, and histone acetylase/deacetylase complexes raise the many questions; namely, are there differences in the requirements for recruiting functionally distinct protein complexes to a specific gene promoter? Additionally, Is there any order for recruitment of different complexes temporally or spatially in expression of a specific gene?

In yeast, Gcn5p histone acetylase seems to be required for the expression of only 5% of entire yeast genes. Mutations in subunits of SWI/SNF complex reveals that this complex is not only related to the expression of only 6% of all yeast genes in genome-wide expression studies but also is required repression of some genes (Holstege et al., 1998; Sudarsanam et al., 2000). These studies suggest that a specific subset of genes requires different set of complexes in mediating their gene expression or repression.

There is evidence that ATP-dependent chromatin remodeling complexes and covalent modification complexes work to regulate gene expression either in a coordinated or sequential manner. Chromatin immunoprecipitation studies (CHIP) have shown that both BRG1 and p300/CBP are present on estrogen – responsive genes at the same time after the estrogen treatment, which supports the idea of cooperative model (Direnzo et al., 2000; Shang et al., 2000). In contrast, other studies suggest that acetylation of chromatin may stabilize the interaction between the SWI/SNF complex with specific gene promoter regions. For example, the yeast Gcn5p histone acetylase acetylates the PHO5 promoter region, and then bromodomain of Gcn5p stabilizes binding of SWI/SNF complex to the newly hyperacetylated region. It has also been shown that the SWI/SNF complex preferentially binds to an acetylated template in vitro (Hassan et al., 2001).

The temporal requirement for recruitment of different complexes to the same promoter region is also variable. In yeast, the inducible GAL1 promoter only requires Gcn5p histone acetylase during interphase. However, both the Gcn5p and SWI/SNF complexes are required for induction of this gene in late mitosis, a stage when chromatin structure is more condensed (Krebs et al., 2000).

It is assumed that there is no single dominant factor that can lead gene activation/repression in eukaryotic transcription. The combinatorial, sequential, and temporal requirement for specific gene expression is dependent on multiple

factors including DNA accessibility of the gene promoter, the context of the core promoter elements, the enhancer elements that recruit specific transcription factors, and the temporal and spatial recruitment of functionally different complexes. The covalent or non-covalent modification of the subunits of the coactivator/corepressor complexes or specific transcription factors that are recruited to the specific promoter site also contributes to the regulation of gene transcription (Freiman and Tjian, 2003).

1.4 Nuclear receptor superfamily

The nuclear receptor (NR) superfamily is the single largest family of metazoan transcription factors that plays a role in various biological and physiological processes including development, metabolism, and reproduction (Tsai and O'Malley, 1994; Aranda and Pascual, 2001). More than 50 nuclear receptors have been reported and ligands have been identified for at least half of the receptors. Evolutionary analysis subdivides these receptors into 6 subgroups (Laudet, 1997). Nuclear receptors are typically defined as ligand-inducible transcription factors that directly interact with a specific DNA element in target genes as monomers, homodimers, or heterodimers. In general, nuclear receptors consist of conserved structural modules that behave independently.

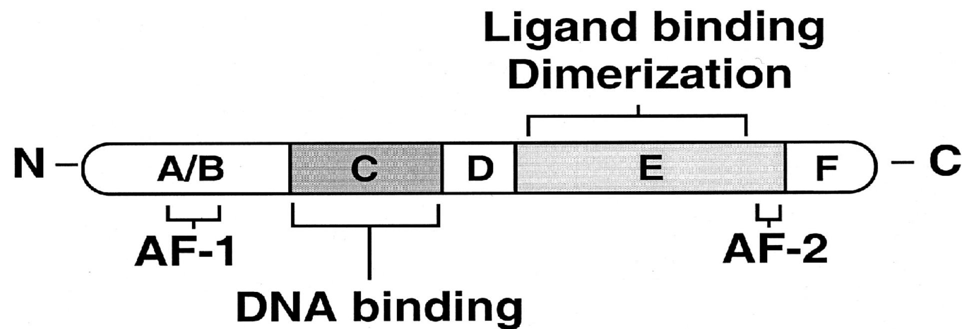

Fig. 9. Schematic representation of nuclear receptor (Aranda and Pascual, 2001).

These domains have been named A through F and include activation function 1 (AF1) in the N-terminal (A/B) region, a specific DNA binding domain (C), a variable hinge region (D), AF2 for ligand–dependent transcriptional activation (E), and another variable C-terminal region that modulates AF2 activity (F)(Fig. 9).

Different NR families interact with specific DNA sequences termed response elements (REs). The core RE sequences usually contain a single conserved hexanucleotide that exhibit variability in their spacing and orientation. These REs can be panlindromic (Pal), direct repeats (DR), inverted repeats (IR) or hemisites. Tandem repeated REs are called direct repeats (DRs) and these DRs can vary in their spacing from a one base pair insertion (DR-1) to a four base insertion (DR4). The core RE is sometimes repeated in a sense and antisense orientation to produce an IR and IR also has spacing variation. Finally, the core REs can be found as a palindrome (P), an inverted palindrome (IP) or hemisite. As described above, specific sequence, spacing, and orientation of the

core REs determine the specificity of receptor–DNA interactions (Fig. 10). For example, class III steroid hormone receptors bind palindrome sequence separated by three nucleotides. In contrast, class I receptors typically bind DRs with different spacing variations after heterodimerization with RXR (Forman and Evans, 1995).

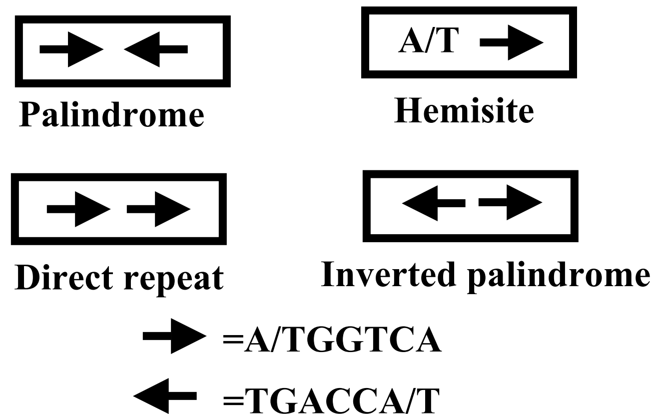

Fig. 10. Schematic representation of the core response elements (Foreman and Evans, 1995).

It is now recognized that activity of nuclear receptors can be regulated by at least 3 other different mechanisms of actions. Nuclear receptor activity can be regulated by covalent modifications including phosphorylation, acetylation, and ubiquitination. Secondly, crosstalk between nuclear receptors themselves or with other transcription factors via direct protein-protein interactions can modulate nuclear receptor functions. Finally, some nuclear receptors are also involved in nongenomic action that occurs within a few minutes after addition of ligand. The

ER can function through all of these pathways and will be described in the following section.

1.5 Mechanisms of estrogen receptor action

1.5.1 Overview

Estrogens exert most of their activity through the estrogen receptor (ER), a member of the nuclear receptor (NR) superfamily and a ligand-dependent transcription factor. There are two isoforms of ER, denoted as ERα and ERβ (Green et al., 1986a, 1986b; Kuiper at al., 1996). The discovery of ERβ from a rat prostate cDNA library has paved the way for studying the comparative roles of ERα and ERβ in normal cancerous tissues. Like other NRs, both ER consist of six defined structural domains. There is considerable variability in AF1, hinge, and F domains of ERα and ERβ. However, ligand binding and DNA binding properties associated with E and C domains of two ERs are similar (Mosselman et al., 1996; Tremblay et al., 1997). However, the two receptors have distinctly different tissue distribution and levels in normal tissues and in human breast tumors (Couse et al., 1997; Leygue et al., 1998). The following sections will primarily focus on ERα functions (Fig. 11).

In the classical model, 17β-estradiol (E2) passively diffuses across the cell membrane and binds ER associated with heat shock proteins (HSPs) in the cytoplasm. Ligand-bound ER is released from HSPs due to conformational changes and forms homo- or hetero-dimers. Transcriptionally active ER dimers interact with specific palindromic DNA sequences, called estrogen responsive

elements (EREs; GGTCANNNTGACC), on target gene promoters. This consensus sequence was first identified from the vitellogenin genes of xenopus and chicken (Burch et al., 1988; Klein-Hitpass et al., 1988).

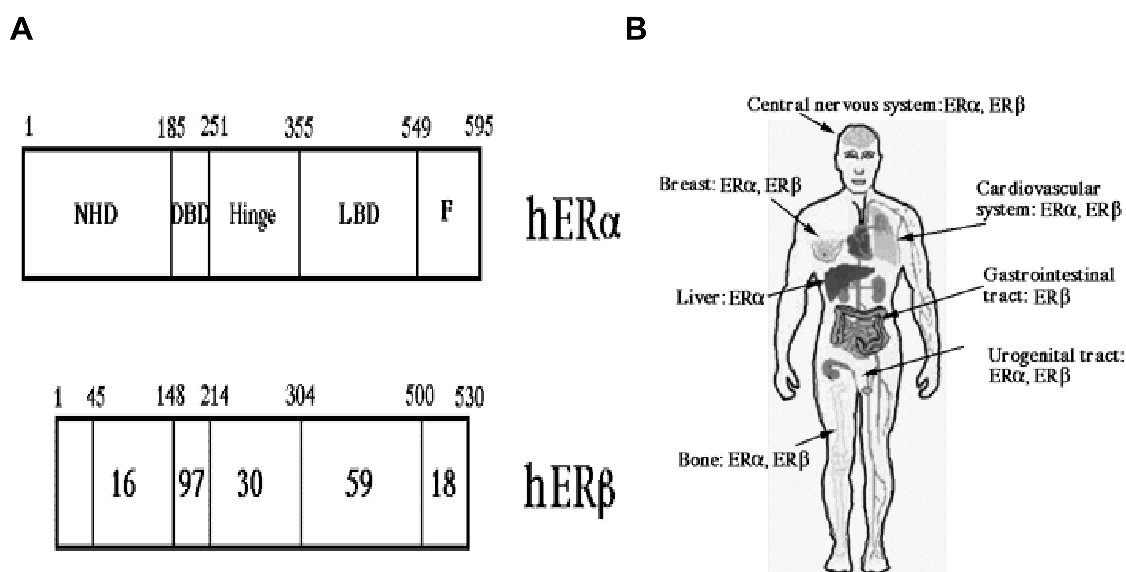

Fig. 11. The homology between ERα and ERβ. The numbers in the ER β diagram show the % sequence identity (A). The distribution of ERα and ERβ is also shown (B) (Gustafsson, 1999).

However, EREs from other E2 responsive genes exhibit considerable variability in the sequence and location compared to that of the consensus elements identified in the vitellogenin A2 gene promoter (Stancel et al., 1995). DNA-ER complexes subsequently recruit other coactivators and/or chromatin remodeling factors, and the general transcriptional machinery to the target gene promoter, resulting in gene transcription (Nilsson et al., 2001).

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. AF2 is located within the ligand binding domain (LBD)(E) and ligand binding regulates its activity. In contrast, AF1 activity is typically regulated by phophorylation in the absence of ligand. Either Independent or synergistic transcriptional activation by AF1 and/or AF2 has been observed and these activities are influenced by the ligand, the promoter context, and cell type. For example, tamoxifen inhibits AF2 activity but not AF1, and exhibits agonist and antagonist activities in a tissue-specific manner. This partial agonist activity has been observed in cells where AF1 activity is dominant (Tora et al., 1988; Berry et al., 1990; Tzukerman et al., 1994)

1.5.2 ER DNA binding domain structure and function

The DNA binding domain (DBD) of ER was first defined by deletion mutagenesis (Kumar et al., 1987). The DBD is composed of two zinc finger subdomains in which the zinc ion is tetrahedrally coordinated by four cysteine residues. Each zinc finger domain forms distinct and complementary surfaces. The first zinc finger domain adopts the S-configuration in its chirality with respect to the zinc coordination, whereas the second zinc finger domain adopts the R-configuration, suggesting these structures are not derived from a duplication event in evolution (Lee et al., 1993). The two-amphiphatic α-helices that follow the zinc finger domains are packed perpendicularly to each other. The inner side

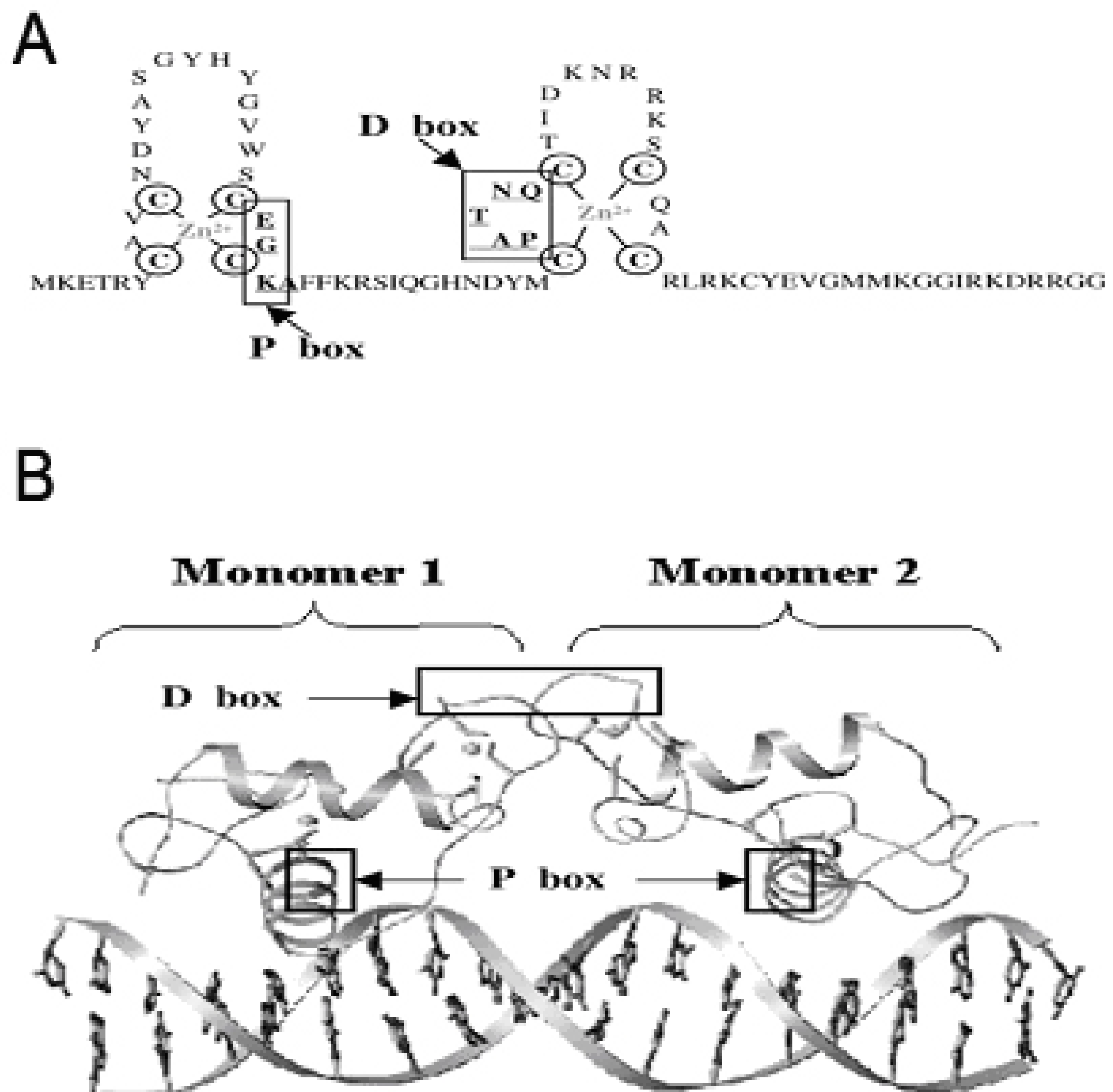

Fig. 12. Schematic representation of the DNA binding domain of estrogen receptor formed by a zinc finger Motif (Modified from Schwabe et al., 1990 and 1993).

of the first α-helix, which contains hydrophobic residues, forms the central hydrophobic core whereas the outer side, containing charged residues, provides an ideal contact surface for recognizing the major groove of the DNA-half site. Thus, this first α-helix is referred to as the DNA recognition helix that determines

DNA-half site specificity. This region in the DBD is called the P-Box (Fig. 12). In fact, mutations of three critical residues in the P-box of ER to the corresponding amino acids in the Glucocorticoid Receptor (GR) (denoted as GSV mutant) changed the specificity of DNA–half site recognition and resulted in transactivation from a glucocorticoid response element (GRE)-linked reporter gene, but not from a ERE-linked reporter gene (Mader et al., 1989).

The second α-helix in the second zinc finger domain appears to provide a number of phosphate contacts to the DNA backbone and is important for the receptor dimerization. This region in the DBD, which is required for stabilization of DNA/ER dimmer complex, is denoted as the D-Box (Schwabe et al., 1990 and 1993).

1.5.3 ER ligand binding domain structure and function

The ER ligand binding domain (LBD) is a wedge–shaped structure that consists of 12 α helices (H1-H12). These α helices are arranged into three antiparallel layers with H4, H5, H6, H8 and H9 flanked by H1 and H3 on one side and by H7, H10, and H11 on the other side (Brzozowski et al., 1997; Tanenbaum et al., 1998). The LBD forms a dimerization interface for homo-and heterodimerzation and a binding surface for coactivators and corepressors. The ER ligand-binding pocket is closed on one side by an antiparallel β-sheet structure and by H12 on the other side is also critical for regulating AF2 activity. The ER LBDs form dimers within the crystal when bound to agonists or antagonists (Fig. 13).

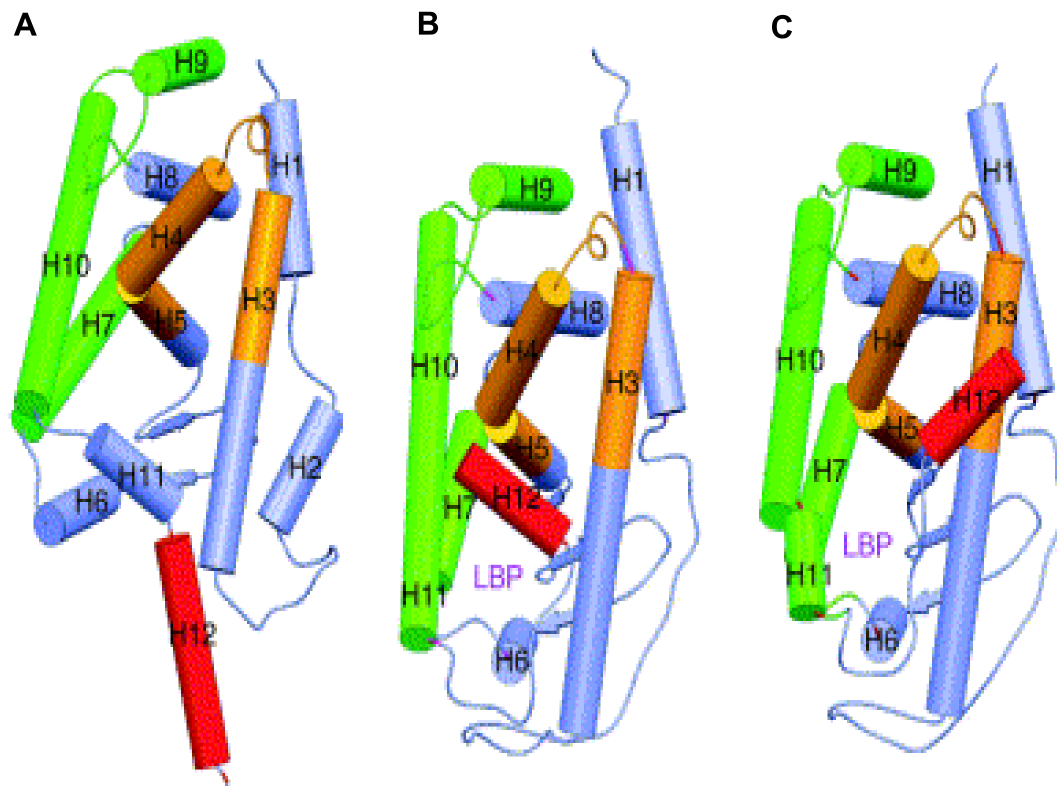

Fig. 13. Agonist- or antagonist-bound ER LBD structures. The unliganded (apo) estrogen receptor LBD. (B) The agonist-bound (holo) estrogen receptor LBD. (C) The antagonist-bound ER LBD. The α-helices (H1–H12) are depicted as rods whereas broad arrows represent the β-turn. The various regions of the LBD are coloured depending on their function: the dimerization surface is shown in green, the co-activator and co-repressor binding site, which also encompasses the nuclear receptor LBD signature motif 6, is shown in orange and the activation helix H12 that harbours the residues of the core activation function 2 (AF-2) activation domain (AD) is shown in red; other structural elements are shown in mauve. Abbreviation: LBP,ligand-binding pocket (Bourguet et al., 2000).

The residues from H8 up to H11 are mainly involved in receptor dimerization. H10 and H11 from each respective monomer contribute important contact surfaces for receptor dimerization by using a stretch of leucine-zipper like hydrophobic residues (Brzozowski et al., 1997).

The dimer interface in both ER isoforms is mainly composed of H10 and H11, which make contact with the cognate ligands and provide the link between ligand binding and dimerization (Ogawa et al., 1998). ER binds a variety of structurally diverse chemical compounds (Anstead et al., 1997). Either agonist- or antagonist-bound ER LBD structure has been determined. All of these ligands interact with the binding cavity in the LBD that is composed of residues from H3, H6, the loop region between H7, H8, H11, and H12. Ligand recognition is achieved via a combination of specific hydrogen bonds, and the complementary hydrophobic interactions between hydrophobic residues in the cavity and the non-polar moieties of ER ligands. Two polar regions located at opposite sides of the ligand-binding pocket are involved in anchoring the 3 and 17 hydroxyl moieties of E2. Glu353 from H3, Arg394 from H5, and a water molecule form a polar pocket between H3 and H6 and are hydrogen-bonded to the phenolic hydroxyl group of the A-ring (3-OH) of E2. On the other side, the hydroxyl group of the D-ring forms a single hydrogen bond to His 524 from H11 (Brzozowski et al., 1997). Antagonists such as raloxifene and tamoxifen bind across the cavity in a similar manner to agonists. However, their bulky sidechains 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 by ER antagonists disrupts interactions between the hydrophobic groove in the LBD with nuclear coactivators (Brzozowski et al., 1997; Shiau et al., 1998).

Coactivators that serve as links between liganded NRs and GTFs are recruited to the AF2 domain of ERα and contain a distinctive common signature motif termed NR-box, comprising the core consensus sequence LxxLL where L is leucine and X is any amino acid (Heery et al., 1997). A conformational rearrangement induced by agonist binding in the LBD results in formation of specific binding sites for an LxxLL motif or NR-box composed of coactivators. This binding site on ERα 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, in which is stabilized by a charge clamp. Introduction of mutations in either partner abrogates this interaction (Shiau et al., 1998). The antagonist-bound ER LBD exhibits major structural differences compared to agonist-bound LBD. The large bulky sidechain of raloxifene or tamoxifen provokes steric clashes that hinder the H12 to adopt its characteristic conformation for coactivator recruitment. Instead, H12 (which contains an NR-box like sequence of LxxML) lies tightly in the hydrophobic groove and perfectly mimics the interaction made by NR-box from coactivators (Pike et al., 1999).

The crystal structures of ERβ isoform bound to genistein, a partial agonist for ERβ, and raloxifene, a pure antagonist for ERβ, have been determined (Pike et al., 1999). Genistein, an isoflavonoid phytoestrogen, displays 7-30 fold higher affinity for ER β over ERα. The orientation of H12 in genestein–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 ERβ, 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 ERβ.

1.5.4 Classification of LXXLL motifs (NR-boxes)

Many nuclear receptor coactivators appear to bind the AF2-cleft induced by agonists and contain at least one or more copies of the LxxLL motifs (Heery et al., 1997; McInerney et al., 1998). However, not all the LxxLL motifs are the same and variations in flanking sequences determine their functional specificity (Chang et al., 1999a). In addition, over 60 different peptide sequences, which interact with agonist-bound ERα, have been identified by phage display (Paige et al., 1999). These peptide sequences are divided into three classes; SRLxxLL motifs regarded as class I, PLLxxLL motifs as class II, and SψLxxLL (ψ=L/I) motifs as class III (Table 5) (Chang et al., 1999a). With a series of ER mutants that form altered or nonfunctional AF2-clefts, the functional differences between these peptides were investigated in a mammalian two-hybrid assay. Unexpectedly, the F6 peptide of class III still interacts with an AF2-nonfunctional ER mutant containing three point mutations in the core AF2-cleft whereas all other peptide classes fail to bind this mutant ER.

Table 5

Classification of LXXLL motif (Chang et al., 1999a)

								-2	-1	1	2	3	4	5							
Class I	ER4 D2 D11 D30	S	S G V H	N S E P	H E S T	Q P G H	S K S S	S S S S	R R R R	**L** **L** **L** **L**	I L M W	E E Q E	**L** **L** **L** **L**	**L** **L** **L** **L**	S S M M	R A A E	 P N A	 V D T	 T L P	 D L T	 V T M
Class II	D14 D47 C33		Q H H	E V V	A Y E	H Q M	G H H	P P P	L L L	**L** **L** **L**	W L M	N S G	**L** **L** **L**	**L** **L** **L**	S S M	R S E	S E S	D H Q	T E W	D S G	W G A
Class III	F6 D22 D48 D43 D17 D41 D26 D40 D15 F4		G L S A G H L S P P	H P G H V H G G S V	E Y W G F N E W G G	P E E E C G R N G E	L G N S D H A E S P	T S S S S S S S S G	L L I L I I L I V L	**L** **L** **L** **L** **L** **L** **L** **L** **L** **L**	E L Y A C Y D Y E W	R K S W Q G M R Y R	**L** **L** **L** **L** **L** **L** **L** **L** **L** **L**	**L** **L** **L** **L** **L** **L** **L** **L** **L** **L**	M R S S A A R Q T S	D A D G H G Q A H A	D P R E D S E D D P	K V V Y N D N A T V	Q E S S A A P F S E	A E L S R P A D I R	V V D A L S W V L E
ERβ sp.	#293	S	S	I	K	D	F	P	N	**L**	I	S	**L**	**L**	S	R					
GRIP-1	NR1 NR2 NR3		D L K	S K K	S K K	G K E	Q H N	T K A	L L L	**L** **L** **L**	L H R	Q Q Y	**L** **L** **L**	**L** **L** **L**	T Q D	T D K	K S D	S S D	D S T	Q P K	M V D
SRC-1	NR1 NR2 NR3		Y L E	S T S	S T S	T R D	S H H	H K Q	L L L	**L** **L** **L**	V H R	K R Y	**L** **L** **L**	**L** **L** **L**	T Q D	T E K	T G D	A S E	E P K	Q S D	Q D L

∗ Sequences from the center three copies of LXXLL motifs in the SRC-1 and GRIP-1 coactivators are also included for comparison and the first conserved leucine was defined as position 1.

These data support idea that distinct conformational rearrangements in the AF2 induced by different ligands recruit different types of coactivators and this explains the distinct pharmacology of many ER ligands in vivo.

However, other distinct receptor interacting motifs has been identified. Androgen receptor (AR) and certain AR coregulators are distinguished by an FXXLF motif that specifically interacts with the AR AF2 site (He at al., 2002; He and Wilson, 2003). Moreover, some of nuclear corepressor proteins interact with

other NRs through I/LXXII motifs (Webb et al., 2000; Perissi et al.,1999). Even some of coactivators of NR-mediated transactivation do not require LxxLL motifs for their function. For example, the LxxLL motif is not required for RIP140 binding to AhR and for enhancing AhR-mediated transcription (Kumar and Perdew, 1999).

1.5.5 Estrogen receptor-dependent coactivators

Ligand-bound ER activates gene expression by stimulating recruitment of functionally different coactivator complexes and the general transcriptional machinery through activation domains.

Direct interaction of ER with components of general transcriptional machinery has been described. ER interacts with TFIIB through its AF2 domain (Ing et al., 1992). Human TAFII30, a TBP-associated factor within the TFIID complex also binds the AF2 domain of ER, but not AF1, and this binding enhances transcription (Jacq et al., 1994). A human TBP enhances both AF1 and AF2 activity of ER and TBP is associated with both domains of ER in vitro. However, over the past few years, many different types of coregulator proteins have been identified. They either act as adaptor molecules to facilitate recruitment of GTFs to the target promoters through direct interaction or they mediate chromatin remodeling to increase access of TFs and GTFs to their respective promoters.

Steroid receptor coactivators (SRC) or P160 family of proteins are ligand-dependent coactivators that enhance transcriptional activation of several nuclear

receptors such as ER, PR (McKenna and O'Malley, 2002). These proteins are divided into three classes based on their sequence homology. The N-terminal domains of SRC/p160 family of proteins exhibit the most extensive sequence similarity and contain conserved helix-loop-helix and PAS (per/ARNT/sim) motifs that mediate homo-and heterodimerization. Representatively, SRC-1/NcoA-1 belong to class I, TIF2/GRIP1/NCoA-2 belong to class II, and pCIP/ACTR/AIB1/SRC-3 are class III coactivators. One of the most distinct structural features of this family of coactivators is the presence of multiple LxxLL signature motifs. Upon ligand binding, the hydrophobic cleft is formed by repositioning of helix 12 in the ER-LBD which acts as "a charged clamp" to interact with LxxLL motifs in the coactivators. A majority of identified coactivators contain this motif. Some coactivators such as SRC-1 and ACTR exhibit weak intrinsic histone acetylase activity. More interestingly, the C-terminal domain of class II coactivators GRIP1 can recruit CARM1, a novel arginine methyltransferase, whereas the N-terminal LxxLL motif in GRIP1 interacts with several NRs. This arginine methyltransferase can methylate histone H3 in vitro. Another protein arginine methyltrasnferase, PRMT2, directly interacts with multiple regions of ERα and enhances both AF1 and AF2 activity (Qi et al., 2002), suggesting the involvement of these coactivators in chromatin remodeling.

CREB Binding Protein (CBP) and its homologue p300 are another class of coactivators that exhibit histone acetylase activity. CBP was initially identified

as transcription factor CREB associated protein and p300 was purified as a binding protein of adenovirus protein E1A. Both CBP and P300 have been associated with regulation of a large numbers of transcription factors (Goldman et al., 1997) Competition for limiting levels of these proteins within a cell can result in cross-talk between different signaling pathways, suggesting that CBP/p300 proteins are key mediators of signal integration (Janknecht and Hunter, 1996). For NRs, the interaction also occurs between the LxxLL motifs in the CBP/p300 and their LBD in ligand–dependent manner. In addition, purified p300 significantly enhances ligand-dependent ER action only on a chromatin template, suggesting a role for the histone acetyltransferase activity of p300 in chromatin remodeling (Kraus and Kadonaga, 1998). PCAF, p300/CBP associated factor, also exhibits histone acetyltransferase activity and enhances transcriptional activation of several NRs including ER, independent of p300/CBP binding but in ligand-dependent manner (Blanco et al., 1998).

Brahma-related gene 1(BRG1), a catalytic subunit in the mammalian SWI/SNF complexes that are ATP-dependent chromatin remodeling enzymes, is required for transcriptional activation of ER (Ichinose et al., 1997). BRG-1-mediated coactivation of ER involves in histone acetylation and Inhibition of histone deacetylation by trichostatin A, a reversible histone deacetylase inhibitor, significantly increases BRG-1-mediated coactivation of ER signaling. This enhancement is reversed by overexpression of histone deacetylase 1(Direnzo et al., 2000). However, the mechanisms by which the complex is recruited to

estrogen-responsive gene promoters are unknown. Recent data reveals that estrogen stimulates the interaction between ER and BAF57, a subunit present only in mammalian SWI/SNF complexes, and an additional interaction between BAF57 and p160 family of coactivators has also been reported (Belandia et al., 2002).

The multisubunit complex that interacts with vitamin D receptor (VR) and thyroid hormone receptor (TR) was identified and was called the DRIP/TRAP complex (Rachez et al., 1999; Fondell et al., 1996). At least seven subunits out of 13-15 proteins in the complex are homologous to the proteins identified in Srb mediator complex that is associated with carboxyterminal repeat domains (CTDs) of a large subunit in the RNA polymerase II complex (Hampsey and Reinberg, 1999). DRIP205/TRAP220, the largest subunit in the complex, interacts with the ER-LBD through its LXXLL motifs in ligand–dependent manner (Burakov et al., 2000). In addition, a cyclic association and dissociation of different types of coactivators with the estrogen responsive pS2 gene promoter in MCF-7 cells has been observed and recruitment of p160s and DRIPs occurs in opposite phases, suggesting coactivator exchange between these coactivator complexes at the target promoter (Burakov et al., 2002).

E6-associated protein (E6-AP/UBE3A) directly interacts with and potentiates the transcriptional activity of ER in a ligand-dependent manner. E6-AP protein can function as an ubiquitin-protein ligase (E3) in the presence of the E6 protein from human papillomavirus types 16 and 18. However, the ubiquitin-

protein ligase function of E6-AP is dispensable for its ability to coactivate ER (Nawaz et al., 1999). ER α transcriptional activity is also enhanced by T:G mismatch-specific thymine DNA glycosylase (TDG) that is required for base excision repair of deaminated methylcytosine, providing an important link between DNA repair proteins and estrogen receptor function (Chen et al., 2003).

As indicated above, full activity of ER requires a synergy between AF1 and AF2. It was reported that some SRC/p160 coactivators enhance transcriptional activation of ERα via both AF1 and AF2 (Webb et al., 1998). The p68 helicase protein was identified as an AF1 specific coactivator of ERα but not ERβ. This protein displays enhanced affinity toward phosphorylated ER-AF1 domain, providing a link between phosphorylation and transactivation (Endoh et al., 1999). In addition, an in vitro association of p68 helicase protein with p300/CBP was observed, suggesting a possible role of p68 as a bridging factor in recruitment of AF2-dependent coactivators. Another AF1-dependent coactivator, steroid RNA activator (SRA), acts as an RNA transcript rather than a translated protein and mediates transcriptional activation of ER. SRA exists as a large riboprotein complex that contains SRC-1 and may serve as a scaffold that facilitates SRC-1 recruitment to ER (Lanz et al, 2003).

1.5.6 Modulation of ER activity by posttranslational modifications

Several growth factors can also stimulate ER activity in the absence of ligand (Smith, 1998). Epidermal growth factor (EGF) can mimic estrogenic effects in ovariectomized mice, resulting in increased uterine-and vaginal cell

proliferation (Ignar-Trowbridge et al., 1992). The inhibitory effects of ICI 164,384 on EGF-stimulated cell proliferation was observed in wild type mice but not in ER-knock out mice, suggesting association of growth factor signaling with ligand-independent activation of the ER (Curtis et al., 1996). In fact, growth factor-activated ER transcriptional activity is dependent on the phosphorylation states of ER. Phosphorylation of serine 118 within AF1 of ERα is mediated by the mitogen activated protein kinases (MAPKs; ERK1/2), which are activated by treatment with EGF or IGF, leading to ligand-independent transactivation of ER (Kato et al., 1995). A 90k Da ribosomal S6 kinase (RSK) is an ERK substrate and a mediator of ERK signaling pathway (Frodin and Gammeltoft, 1999). EGF- or phorbol myristate-activated RSK specifically phophorylates serine 167 within AF1 and ectopic expression of RSK also increased serine 167 phosphorylation (Joel et al., 1998). ER phosphorylation is also ligand–dependent and serine 118 is phosphorylated by CDK7, a cyclin-dependent kinase associated with the general transcription factor TFIIH. Interestingly, CDK7 overexpression significantly enhances agonistic activity of tamoxifen (Chen et al., 2000).

AKT is a serine/threonine protein kinase that promotes cell proliferation and anti-apoptotic responses and is a downstream target of phosphatidylinositol -3-OH kinase (PI3K)(Datta et al., 1999). PI3K and AKT activate hER in the absence of estrogen. AKT increased only AF1-dependent activity through phosphorylation of serine 167 (Martin et al., 2000; Campbell et al., 2001). Frequent alterations of the PI3K-AKT pathway can occur by increased activity

and expression of all three AKT family members or by inactivation of PTEN, a negative regulator of AKT, suggesting possible role for this pathway in breast cancer. In addition, ERα directly binds p85, the regulatory subunit of PI3K, in a ligand-dependent manner, leading to AKT activation and induction of endothelial nitric oxide synthase (eNOS) (Simoncini et al., 2000). Other In vitro transcription studies showed that casein kinase II also phosphorylated human ER (hER) at serine-167. This data suggested that a conformational change of hER induced by E2 binding may expose serine-167 to casein kinase II, resulting in ER-mediated transactivation (Castano et al., 1997).

Cyclins are subunits of cyclin-dependent kinase (CDK) complexes and that regulate cell cycle progression. The CyclinA/CDK2 complex phosphorylates serine 104 and 106 of ERα and these modifications potentiate transcriptional activity of ER in ligand–independent manner (Rogatsky et al., 1999). Direct interaction between Cyclin D1 and ER also enhance ER activity without involving phosphorylation and CDK activity (Zwijsen et al., 1997; Neuman et al., 1997).

Protein kinase A (PKA) regulates ligand-independent ER activity by phosphorylating serine 236 within the DNA binding domain of ER α. This phosphorylation induced by PKA overexpression or activation inhibits dimerization and DNA binding of ER α (Chen et al., 1999b). It has been reported that PKA overexpression is associated with high proliferation in normal breast, malignant transformation in the breast, poor prognosis in established breast cancer, and resistance to antiestrogens (Miller, 2002).

Phosphorylation of ERα has not only been observed in AF1 but also in AF2 of ERα. The phosphrylation of tyrosine 537 on human ER by Src kinase regulates receptor dimerization, DNA binding and estrogen binding (Arnold and Notides, 1995). Interestingly, not only phosphorylation but also direct acetylation of ERα regulates its activity. Two conserved lysine residues (302 and 303) within hinge/LBD region of ERα are acetylated by p300, resulting in altered ligand sensitivity (Wang et al., 2001).

1.5.7 Nonclassical ER actions

ER homodimers binds to EREs in target gene promoter and recruit various coactivators to stimulate gene transcription. This classical nuclear ER-dependent ER transcriptional activation pathway has been well characterized. However, some of E2-responsive genes do not contain consensus or nonconsensus EREs in their promoters. In fact, ligand-bound ER can activate many target genes through protein-protein interactions without direct DNA binding of ER. The following section will focus on two major nonclassical actions of ER; namely, ER/AP-1 and ER/Sp1 pathways.

1.5.7.1 ER/AP-1 pathway

An E2-responsive AP-1 element was initially identified in the proximal promoter of the ovalbumin gene (Tora et al., 1988) 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 (Gaub et al., 1990; Tzukerman et al., 1991; Philips et al., 1993; Umayahara et al., 1994; Montano

and Katzenellenbogen, 1997; Geum et al., 1997; Altucci et al., 1996). 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 response to various extracellular stimuli (Shaulian and Karin, 2002).

The antiestrogen tamoxifen activates AP-1 target genes in uterine cells but not in breast cancer cells (Webb et al., 1995). This cell-type specific ER/AP-1 action is intriguingly parallel to the effect of tamoxifen on growth of these cell types where tamoxifen acts as an ER agonist in uterine cells and stimulates cell growth. In contrast, tamoxifen inhibits the growth of breast cancer cells where tamoxifen acts as antagonist.

Mechanistic studies of ER/AP-1 actions have shown that the requirement for ER structural domains is dependent on ligand structure. For example, the ER DBD is not necessary for estrogen-mediated AP-1 actions but is required for tamoxifen-activated ER/AP-1-depedent activity. Furthermore, ICI, 182,780, another SERM that inhibits ER dimerization and ER DNA (ERE) binding, activates an AP-1 reporter construct. Interestingly, the ER LBD alone can strongly activate an AP-1 reporter construct and requires an intact AF2 function. The p160 coactivator GRIP1 synergistically enhances ER/AP-1-mediated transcription in an LXXLL-dependent manner. However, full length ERα containing mutations in AF1 also compromised estrogen-mediated AP-1 activity, indicating that ER/AP-1 action requires both AF1 and AF2 (Webb et al., 1999).

ERβ also activates transcription from an AP-1 element. However, the effects of estrogen and antiestrogen on ERβ/AP-1 exhibits contrast to those observation for ERα/AP-1. E2, ICI,182,780, tamoxifen, and raloxifene all activate AP-1 reporter construct in cells cotransfected with ERα whereas, in the presence of ERβ, E2 not only acts as antagonist but also inhibits the activity of tamoxifen and raloxifene dependent induction of ERβ/AP-1. However, either tamoxifen or raloxifene alone behave as full agonists (Peach et al., 1997).

Direct protein-protein interaction between ER and c-Jun is required for ER/AP-1 action. Recent data have shown that the hinge region of ERα is associated with the C-terminal region of c-Jun in a ligand–independent manner whereas ER does not interact with c-Fos. Moreover, the coactivator GRIP1 forms a triple complex with c-Jun and ERα in both in vitro and in intact cells, suggesting that GRIP1 stabilizes the ERα/c-Jun complex (Teyssier et al., 2001).

1.5.7.2 ER/Sp1 pathway

E2-responsive GC-rich elements were initially identified in the c-myc gene promoter (Dubik and Shiu, 1992). This site contains a nonconsensus ERE-half site (ERE½) and an Sp1 binding site that was required for estrogen-mediated induction. Similar ERE½/Sp1 elements have been subsequently characterized in the cathepsin D (Krishnan et al., 1994), heat shock protein 27 (Hsp27) (Porter et al., 1996), TGF α (Vyhidal et al., 2000), prothymosin α (Martini and Katzenellenbogen, 2001), and human PR A (Petz and Nardulli, 2000), and rabbit uteroglobulin gene promoters (Scholz et al., 1998). With exception of Hsp27,

these promoters require ERα binding to ERE ½ sites for E2-mediated induction since mutations of the ERE ½ site abolish estrogen-responsiveness of these promoters. In the Hsp27 promoter, ERα/Sp1 also mediated transactivation when the ERE ½ sites was mutated (Krishnan et al., 1994; Porter et al., 1997) and ERα/Sp1 action is observed in cell transfected with wild type ER or mutant ER with a deletion of the DBD.

ERα/Sp1 protein-protein interaction was investigated in vitro using GSTpull down assays, which showed interaction between the C-terminal end of Sp1 and multiple regions of ERα. Interestingly, the antiestrogens such as tamoxifen and ICI 182,780 activated ERα/Sp1 in cells transfected with construct containing a GC-rich promoter (pSp1 or $pSp1_3$). DNA-independent ERα/Sp1 action has been observed for several genes including retinoic acid receptor α (Sun et al., 1998), c-Fos (Duan et al., 1998), insulin-like growth factor-binding protein-4 (Qin et al., 1999), bcl-2 (Dong et al., 1999), adenosine deaminase (Xie et al, 1999), thymidylate synthase (Xie et al., 2000), cyclin D1 (Castro-Rivera et al., 2001), cad (Khan et al., 2003), E2F-1 (Ngwenya and Safe, 2003).

Although both ERα and ERβ forms complexe with Sp1 protein, only ERα induces consensus Sp1 element-linked reporter gene activity whereas ERβ, exhibits minimal or decreased the basal reporter gene activity and these responses are ligand- and cell type-specific. Additionally, it has been shown using a series of ER α deletion mutants and ERα/ERβ chimeric mutants that the

AF1 domain of ERα is critical for ERα/Sp1-mediated transactivation. These data indicated that ERα/Sp1 action is dependent on cell type, ligand, and ER subtype (Saville et al., 2000). Interestingly, it was recently reported that both ERα and ERβ 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 ERβ/Sp1 action (Salvatori et al., 2003).

ERα 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 found that vascular endothelial growth factor (VEGF) gene expression is regulated by ERα/Sp1 or ERα/Sp3 either positively or negatively and Sp1/Sp3 ratios and 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 ERα and Sp1 expression plasmid whereas downregulation of the same promoter activity was observed when cells cotransfected with ERα and Sp3 expression plasmid (Stoner et al., 2000 and 2004).

1.6 Sp family of transcription actors

Sp1 was originally identified as a mammalian transcription factor that binds and activates transcription from multiple GC-rich sequences in the simian virus 40 (SV40) early promoter and the thymidine kinase promoter (Dynan and Tijan, 1983; Gidoni et al., 1984; Jones et al., 1985). The human cDNA that

partially encodes the C-terminal 696 amino acids of Sp1 was initially cloned from Hela cells and the entire Sp1 cDNA from rat and mouse have also been cloned later (Kadonaga et al., 1987; Imataka et al., 1992). Other Sp1-related transcription factors including Sp2, Sp3, and Sp4 were cloned and form a multigene family of transcription factors (Hagen et al., 1992: Kingsly and Winoto, 1992; Supp et al., 1996). All four human Sp proteins share similar domain structures (Fig.15) and contain DNA binding domains at the C-termini and glutamine rich domains adjacent to serine/threonine stretches at the N-termini. The DBD consists of 81 amino acids that contain three C2H2 type zinc finger motifs and this region is a highly conserved region among Sp proteins. Sp1, Sp3, and Sp4 recognize the classical GC-rich Sp1 element with similar affinities (Pavletich and Pabo, 1991; Hagen et al., 1992 and 1994). In contrast, Sp2 does not bind GC-rich elements but to a GT-rich motif since the conserved histidine residue in first zinc finger domain is replaced by leucine residue (Kingsly and Winoto, 1992). The following sections primarily focus on Sp1 and Sp3 proteins (Fig. 14).

1.6.1 Sp1

Sp1 protein is ubiquitously expressed and is important for regulation of TATA-less genes that encode housekeeping proteins (Pugh and Tjian, 1991). It has been shown that its activity and cellular content is regulated during development, cell proliferation and apoptosis (Armstrong et al., 1997; Jane et al., 1993; Piedrafita and Pfahl, 1997). Sp1-dependent gene regulation occurs

through direct protein-protein interactions, post-translational modifications, and alterations in Sp1 protein levels.

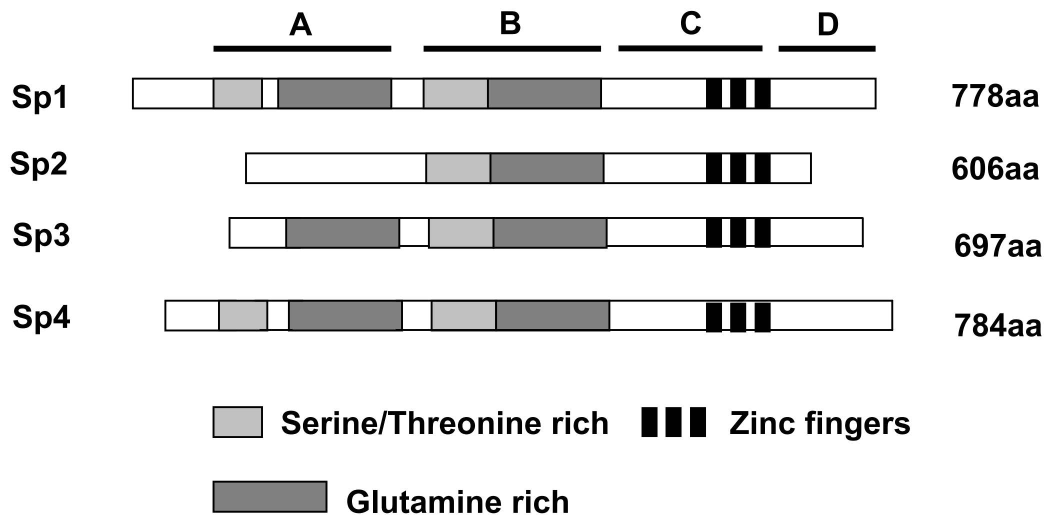

Fig. 14. Schematic representation of the functional domains of Sp family proteins (Modified from Suske et al., 1999).

The glutamamine-rich A and B regions of Sp1 act as strong transcactivation domains (Courey and Tjian, 1988) and the interspersed bulky hydrophobic amino acids within the activation domain play critical role (Gill et al., 1994). Sp1 activates transcription synergistically by forming homomultimeric complexes, which requires the activation domains A and B and carboxy-terminal domain D (Pascal and Tjian, 1991; Tanese et al., 1991). Sp1 directly interacts with subunits of general transcription machinery including TBP, TBP-asoociated factors TAFII110, and TAFII55 to stabilize the transcriptional initiation complex (Emili et al., 1994; Chiang and Roeder, 1995). Recent studies show that cofactors required for Sp1 activation (CRSP) complex is required for Sp1-

mediated transactivation and is composed of six to eight proteins that range in size from 30-200kDa (Naar et al., 1998). Sp1 also interacts with many transcription factors to modulate transcription including GATA1, YY1, E2F, c-Jun, p53, pRb (Gregory et al., 1996; Seto et al., 1993; Karlseder et al., 1996; Wang and Chang, 2003; Schavinsky-Khrapunsky et al., 2003; Datta et al., 1995).

Phosphorylation and glycosylation are posttranslational modifications that modulate Sp1-dependent activity. DNA-dependent protein kinase phosphorylates Sp1 by viral infection and is associated with DNA damage responses (Jackson et al., 1990; Anderson, 1993). Casein Kinase II (CKII) phosphorylates a threonine residue in the second zinc finger of Sp1 and this inhibits DNA binding whereas N-terminal phosphorylation by PKA increases transcriptional activity of Sp1 in part through increasing its DNA binding affinity (Armstrong et al., 1997; Rohlff et al., 1997; Zheng et al., 2000). Cyclin A/cdk2 complex directly binds to and phosphorylates Sp1 and increases its DNA binding activity (Haidweger et al., 2001). In contrast, direct cyclin D1 binding to Sp1 inhibits its transcriptional activity (Opitz and Rustgi, 2000). Glycosylation of Sp1 through O-linkage of monosaccharide, N-acetylglucosamine (O-GlcNAc) is another important posttranslational modification. Sp1 acetylation is linked to multiple functional changes including altered self association, altered interaction with GTFs and proteasome-dependent degradation (Roos et al., 1997; Han and Kudlow, 1997).

1.6.2 Sp3

There are 3 isoforms of Sp3, which were derived from three different initiation sites from the same gene (Kingsly and Winoto, 1992). Sp3 is ubiquitously expressed and acts as either a transcriptional activator or repressor dependent on promoter and cell context. Initial studies implicated Sp3 as strictly a repressor because Sp3 did not activate GC-rich elements containing promoters from HIV-1 and HTLV-1 genes (Hagen et al., 1994; Dennig et al., 1995). However, later studies show that Sp3 also has activator function in both mammalian and SL2 cells (Dennig et al., 1996; Majello et al., 1997). In fact, Sp3 stimulates expression of the HERV-H long terminal repeat in teratocarcinoma cells but acts as a repressor in both Hela and insect cells (Sjottem et al., 1996). Therefore, It has been suggested that the relative abundance of Sp1 and Sp3 in specific cells determines the activity of Sp3 as an activator or a repressor. Sp1/Sp3 cellular ratios are highly variable; endothelial cells contains high levels of both Sp1 and Sp3 whereas Sp3 level is lower in non-endothelial cells, which regulates KDR/flk-1 promoter activity (Hata et al., 1998). The repression domain of Sp3 is mapped to a small amino acid stretch (KEE) located at the 5' of the zinc finger domain (Dennig et al., 1996). Sp3 interacting protein (SIF-1) that is associated with the repressor domain has been cloned (Suske, 1999). Post-translational modifications also modulate Sp3 repressor activity in conjunction with cellular location. For example, removal of a small ubiquitin-related modifier-1 (SUMO-1) from Sp3 by mutation of the SUMO acceptor lysines or expression of

the SUMO-1 protease Senp1 converted Sp3 to a strong activator with a diffuse nuclear localization (Ross et al., 2002).

Sp1-/- embryos are retarded in development, exhibit a broad range of abnormalities and die around day 11 of gestation. Cell cycle-regulated genes are not affected in Sp1-/- embryos and CpG islands remain methylation free (Marin et al., 1997). Sp3-deficient embryos are also growth retarded and invariably die at birth due to respiratory failure. In addition, histological studies of individual organs indicate a pronounced defect in late tooth formation in Sp3(-/-) mice (Bouwman et al., 2000). Recent data shows that the absence of Sp3 also results in impaired hematopoiesis by affecting in some of the erythroid and myeloid cell lineages (Van Loo et al., 2003). These comparisons of the Sp1 and Sp3 knockout phenotype show that Sp1 and Sp3 have distinct functions in vivo.

1.7 Research objectives

1.7.1 Objective 1

The DBD of ER consists of two zinc finger motifs. Each motif establishes a distinct and complementary surface with different chirality; the first zinc finger motif exhibits an S-configuration and the second zinc finger motif exhibits an R-configuration, suggesting that these substructures were not derived from a duplication event in evolution. The first zinc finger motif (ZF1) contains a DNA recognition helix that contacts the major groove at the DNA halfsite. In contrast, zinc finger 2 (ZF2) contacts the phosphate backbone and provides the dimer interface (Schwabe et al., 1990 and 1993). The introduction of point mutations to

either ZF1 or ZF2 resulted in different patterns of agonistic or antagonistic activities on ER/AP-1 action. ERα mutated in zinc finger 1 (E207A/G208A) did not affect ICI 182,760-induced transcriptional activity on an AP-1 promoter whereas a zinc finger 2-point mutation (A227T) resulted in loss of inducibility by ICI 182,760 (Jakacka et al., 2001). Therefore, the first objective of this project is to characterize and compare the functional properties of ER mutants containing deletions of either the first zinc finger or the second zinc finger motif in cells transfected with constructs containing 3 tandem EREs ($pERE_3$) or GC-rich ($pSp1_3$) elements linked to a luciferase reporter gene and treated with estrogen, antiestrogens or their combination. Additionally, immunocytochemistry will be employed to determine if the zinc finger domain deletion mutants translocate into the nucleus after treatment with E2 or antiestrogens.

1.7.2 Objective 2

Previous studies in this laboratory showed the critical role of the AF1 domain in hERα/Sp1 action. Deletion of aa 51-117 resulted in loss of E2-dependent transcriptional activation in cells transfected with a GC-rich construct (pSp1). In contrast, ERβ or a chimeric ERβα consisting of the AF1 domain of ERβ and the DEF domain from ERα induced minimal reporter gene activity in cells transfected with pSp1 (Saville et al., 2000). However, the role of the AF2 domain including the hinge region (D) and E/F domains of hERα on hERα/Sp1 action has not previously been investigated. Deletion of either D or F domain of hERα does not results in loss of hormonal dependent transactivation in cells

transfected with pERE (Kumar et al., 1987). However, in cells transfected with hERα mutants lacking the F domain or containing point mutation in the F domain showed altered responses to antiestrogens, suggesting that a predicted helix 13 or beta strand substructure in the F domain may form distinct conformations through interaction with other domains of ERα (Nichols et al., 1998; Schwartz et al., 2002). The second objective of this research is to investigate the effects of deletions or mutations of the hinge region, the AF2 core, or the F domain of hERα on E2/antiE2-induced transactivation in breast cancer cells transfected with pSp13 or pERE3. hERαTAF1 contains three amino acid mutations in helix 12 (D538Q, E542Q and D545N) and has a nonfunctional AF2 that does not interact with SRC family coactivators through LxxLL motifs (NR box). By generating hERαTAF1 expression plasmid containing either zinc finger 1 (hERαTAF1ΔZF1) or zinc finger 2 (hERαTAF1ΔZF1), the effects of estrogen and antiestrogen-induced transcriptional activity can be determined in cells transfected with $pSp1_3$.

1.7.3 Objective 3

GAL4 fusion peptides containing LxxLL motifs derived from NR boxes of different coactivators inhibit estrogen-induced transactivation in cells transfected with $pERE_3$. Depending on flanking amino acid sequence and variation of the LxxLL motif, these peptides exhibit different affinities in the hydrophobic groove formed in the LBD (ligand binding domain) after binding to estrogen or antiestrogens. This suggests that the conformation of the LBD induced by

various ligands may recruit different coactivator complexes for gene transcription. Peptides derived from helix 10/11 or 12 specifically inhibited ERα binding to an ERE (Norris et al., 1999; Chang et al., 1999a). The effects of these different peptides on ERα/Sp1 action in breast cancer cells will be determined in this study. The identification of selective inhibitory peptides for activation of hERα/Sp1 will provide fundamental information on the contribution of this pathway to gene expression of breast cancer cells. Disruption of transcriptional activation by different classes of peptides containing LxxLL motifs derived from coactivators will be investigated in transient transfection assay in breast cancer cells transfected with $pERE_3$ or $pSp1_3$.

1.7.4 Objective 4

Direct interactions between ERs and Sp proteins have been detected using coimmunoprecipitation and GST pull down assay in vitro; Both ERs bind to the zinc finger domain of either Sp1 or Sp3 protein (Porter et al., 1997; Saville et al., 2000; Stoner et al., 2000). However, direct physical interactions between ERα and Sp1 protein have not been investigated in vivo.

Fluorescence resonance energy transfer (FRET) can be used to quantify the distance between two different fluorophores by measuring the transfer of energy from a fluorescent donor in its excited state to another excitable fluorescent moiety, the acceptor (Clegg, 2002). With the development of spectral variants of the green fluorescent protein (Heim, 1999), FRET has been extensively used as a method to image molecular events in living cells such as

protein-protein interactions (Mahajan et al., 1998) or protease and kinase activities (Heim and Tsien, 1996; Sato et al., 2002). Bascically, FRET requires the overlap between the donor molecule's fluorescence emission spectrum and the acceptor molecule's excitation spectrum, a limited distance between the donor and the acceptor molecules (1-10nm), and the appropriate orientation between the two fluorophores reside in both the donor and the acceptor molecules (Gordon et al., 1998; Elangovan et al., 2003). Recently, ligand-dependent ERα-LxxLL peptide interactions, NRs-Steroid Receptor Coactivators (SRCs) interactions have been detected in living cells using FRET (Liopis et al., 2000; Weatherman et al., 2002; Bai and Giguere, 2003). Here, ligand-dependent hERα-Sp1 protein interactions will be investigated in living cells by using FRET microscopy and image analysis.

CHAPTER II

MATERIALS AND METHODS

2.1 Chemical and biochemicals

DMEM nutrient mixture F-12 (DME/F12) without phenol red, PBS, E2, 4-OHT, BSA (Fraction V), and 100x antibiotic/antimycotic solution were purchased from Sigma (St Louis, MO). Fetal bovine serum (FBS) was obtained from JRH Biosciences (Lenexa, KS). ICI 182,780 was kindly provided by Dr. Alan Wakeling (Astra USA, Inc.-Zeneca Pharmaceuticals, Macclesfield, UK). [γ-32P]ATP (3000 Ci/mmol) was purchased from NEN Life Science Products (Boston, MA). Polydeoxy-(inosinic-cytidylic)acid, and T4-polynucleotide kinase were purchased from Roche Molecular Biochemicals (Indianapolis, IN). All the restriction enzymes and modifying enzymes (T4 DNA ligase, calf intestinal alkaline phosphatase) used in this study were purchased from Promega Corp. (Madison, WI) or Roche Molecular Biochemicals. Plasmid preparation kits were purchased from QIAGEN (Valencia, CA), and 40% polyacrylamide was obtained from National Diagnostics (Atlanta, GA). All other chemicals were obtained from commercial sources at the highest quality available.

2.2 Cell maintenance and transient transfection assay

MCF-7, Chinese Hamster ovary (CHO), and MDA-MB-231 cells were

obtained from the American Type Culture Collection (ATCC, Manassas, VA). MCF-7, MDA-MB-231 and CHO cells were grown in DME/F12 (Sigma) supplemented with 2.2 g/L sodium bicarbonate, 5% fetal bovine serum (FBS), bovine serum albumin (Sigma), and 10ml/L antibiotic/antimycotic solution (Sigma). Cells were cultured and maintained in 150 cm2 tissue culture dishes in a 37°C in 5% CO2:95% air. For transient transfection assays, cells were seeded onto 6-well tissue culture plates in DME/F12 without phenol red supplemented with 2.2 g/L sodium bicarbonate, 5% dextran-coated charcoal-stripped FBS, bovine serum albumin, and 10 ml/L antibiotic/antimycotic solution (Sigma). After 24 h, cells were transfected with the calcium phosphate method with 2 μg of luciferase reporter construct ($pSp1_3$, $pERE_3$, pADA, and pRARα1), 250 ng pcDNA3/His/lacZ (Invitrogen, Carlsbad, CA) as a standard reference for transfection efficiency, and 1 μg or 100 ng (for cotransfection with pERE3) of the appropriate ER expression plasmid. In studies where variable amounts of coactivators were also used, the amount of DNA transfected was kept at a constant value by adding sufficient amount of empty vector. After 5 to 6 h, the media was removed and the cells were shocked with 20% glycerol in phosphate buffered saline (PBS) (pH 7.4) for 1 min. Cells were rinsed twice with 1 ml of PBS and treated with 5% charcoal-stripped DME/F12 either containing Me2SO, E2 (10 nM), HOTAM (1 μM), or ICI 182, 780 (1 μM) for 36 to 40 h. After harvesting cells by scraping in 1X reporter lysis buffer (Promega, Madison, WI), luciferase activity of aliquots of this extract was determined using the luciferase

assay system (Promega). β-Galactosidase activity was performed using Tropix Galacto-Light Plus assay system (Tropix, Bedford, MA). Light emission was detected on a lumicount micro-well plate reader (Packard, Meriden, CT) and luciferase reporter gene activity was corrected by normalizing against β-galactosidase activity, obtained from the same sample. Results are expressed as means ± SD with at least three determinations for each treatment group.

2.3 Oligonucleotides and plasmids

hERα expression plasmid was kindly provided by Dr. Ming-jer Tsai (Baylor College of Medicine, Houston, TX); ER-null and hERαTAF1 were obtained from Dr. D. McDonnell (Duke University, Durham, NC). The human ER deletion construct hER11C was originally obtained from Dr. Pierre Chambon (Institut de Genetique et de Biologie Moleculaire et Cellulaire, Illkirch, France). Mouse estrogen receptor (MOR) was generously provided by Dr. Malcom G. Parker (Imperial Cancer Research Fund, London, United Kingdom) and hERβ was supplied by Dr. J. A. Gustafsson (Karolinska Institute, Huddinge, Sweden). ERα DBD point mutants (K210A, A227T, 207AA, and 207GS) were obtained from Dr. Larry Jameson (Lurie Comprehensive Cancer Center, Northwestern University, Chicago, USA). Our experiments were carried out using a shorter variant form of ERβ; however, in preliminary experiments using a longer form of ERβ (provided by Dr. S. Mosselman, N.V. Organon, Oss, Netherlands), minimal induction of ERβ/Sp1 was also observed. SRC-1, SRC-2 (GRIP1), SRC-3 (A1B1) and p68 RNA helicase were graciously provided by Drs. B. O'Malley

(Baylor College of Medicine, Houston, TX), M. R. Stallcup (University of Southern California, Los Angeles, CA), P. Meltzer (National Cancer Institute, Bethesda, MD) and S. Kato (University of Tokyo, Tokyo, Japan), respectively. The hER cDNAs and the MOR cDNA were inserted into vectors pcDNA3 or pcDNA3.1/His C (Invitrogen, Carlsbad, CA) in this laboratory for in vitro translation and for expression in mammalian cells in transient transfection assays. For gel mobility shift assays, a consensus estrogen response element (ERE); 5' GTCCAAAGTCAGGTCACAG TGACCTGATCAAAGTT 3' (SENSE) was used and obtained from the Gene Technologies Laboratories, Texas A&M University (College Station, TX). The DNA oligonucleotides used for construction of plasmids were synthesized by the Gene Technologies Laboratories (College Station, TX). pXP1 luciferase reporter construct was obtained from ATCC and the minimal TATA sequence were inserted into pXP1 in this laboratory. The following promoter sequences were cloned into HindIII and BamHI sites of pXP1 TATA-luciferase reporter construct; three consensus GC-rich Sp1 binding sites for $pSp1_3$; 5' GCTTATTCGATCGGGGCGGGGCGAGCATTCGATCGGGG CGGGGCGAGCATTGATCGGGGCGGGGGCGAGCG 3' (sense); three consensus EREs for $pERE_3$; 5' AGCTTTCCGGATCTAGGTCACTGTGA CCCGGGATCCTAGGTCACTGTGACCCGGGATCCTAGGTCACTG TGACCTGATCAAAGTG 3' (sense); consensus AP1 binding site for pAP1; 5'GATTCGAGGTGTCTGACTCATGACT 3' (sense). The GC-rich genomic promoter sequence from RARα1 gene (pRARα1; -79 to -49); 5' AGCTTGA

TTGGTCGGTGGGCGGGCAGGGGCGG GCCT 3' (sense) and the GC-rich genomic promoter sequence from the adenosine deaminase gene (pADA; -86 to -65); 5' AGCTTGGCGAGAGGGCGG GCCCCGGGA 3' (sense) were also cloned into HindIII and BamHI sites of pXP1 luciferase reporter construct. The GC-rich and ERE motif are underlined.

2.4 Generation of ER deletion mutant constructs

ER DBD deletion constructs were prepared by site-directed mutagenesis by overlap extension using the polymerase chain reaction as previously described (Ho et al., 1989). For example, hERαΔZF1 in pcDNA3 was constructed by carrying out the following procedures. One set of primers (A1/B1) from the Hind III site (A1) in the multiple cloning site in pcDNA3 to the site before the first amino acid in the region to be deleted was amplified by PCR; the latter primer (B1) has an overlapping region of about 15 to 20 bp that starts at the next amino acid in the deletion construct. In addition, another set of primers beginning just after the last amino acid to be deleted to the Hind III site in hERα cDNA sequence were also used and amplified by PCR. This second set of primers (A2/B2) contained a 15 to 20 bp overlapping region complementary to the last 15 to 20 bp DNA sequence in the first PCR product. Both PCR reaction products have their own regions of overlap, and these were coincubated to anneal the overlapping regions; this was followed by PCR amplification with the primer (A1) that starts at the multiple cloning site and the primer (B2) that starts at the hERα cDNA sequence.

Table 6

Summary of primers used for mutagenesis of the DNA binding domains of hERα and MOR

ER Deletion Mutant	PCR-Primer Sets (A1/B1, A2/B2) for Mutagenesis	Deletion
HERαΔZF1	A1; 5' GCAAATGGGCGGTAGGCGTGTA 3' B1; 5' CTTGAAGAAGGCCTTGTAGCGAGTCTCCTTGG 3' A2; 5' AAGGAGACTCGCTACAAGGCCTTCTTCAAGAG 3' B2; 5' GAGACCAATCATCAGGA	21 amino acid deletion (185 aa - 205 aa)
HERαΔZF2	A1; 5' GCAAATGGGCGGTAGGCGTGTA 3' B1; 5' CATTCCCACTTCGTAGTTATGTCCTTGAATACT 3' A2; 5' GTATTCAAGGACATAACTACGAAGTGGGAATGAT 3' B2; 5' GAGACCAATCATCAGGA 3'	28 amino acid deletion (218 aa - 245 aa)
HERβΔZF1	A1; 5' GACGTCAATGGGAGT 3' B1; 5'CTTTTAAAAAAGGCCTTGAAGTGAGCATCCCTCTTC 3' A2; 5' GGATGCTCACTTCAAGGCCTTTTTTAAAAG 3' B2; 5' GAGACCAATCATCAGGA	21 amino acid deletion (96 aa - 111 aa)
HERβΔZF2	A1; 5' GTGTACGGTGGGAG 3' B1; 5' CATTCCCACTTCGTAATTATGTCCTTGAATGCTTC 3' A2; 5' CAAGGACATAATTACGAAGTGGGAATGG 3' B2; 5' AACTCTCGAAACCTTGAA 3'	28 amino acid deletion (124 aa - 131 aa)
MORβΔZF1	A1; 5' GCATCGCCTACGG 3' B1; 5' CTTAAAGAAAGCCTTGTAGCGAGTCTCCTTGGC 3' A2; 5' GGAGACTCGCTACAAGGCTTTCTTTAAGAGAAGC 3' B2; 5' GGTCAATAAGCCCATCA 3'	21 amino acid deletion (189 aa - 209 aa)
MORΔZF2	A1; 5' GCATCGCCTACGG 3' B1; 5' CATGCCCACTTCGTAATTGTGTCCTTGAATGCT 3' A2; 5' TCAAGGACACAATTACGAAGTGGGCATGATG3' B2; 5' GGTCAATAAGCCCATCA 3'	28 amino acid deletion (222 aa - 249 aa)

* Overlapping regions are noted in bold type.

The resulting PCR product containing the desired deletion and a unique restriction site at both ends (Hind III) was purified, digested with HindIII, and finally cloned into pcDNA3 construct to give the appropriate expression plasmid for electrophoretic mobility shift and transient transfection assays.

The primers used for the mutagenesis assays are summarized in the previous Table 6). hERαΔZF2 in pcDNA3 was also generated by using a unique Hind III restriction site for cloning into this vector.

Generation of hERβΔZF1 and hERβΔZF2 used the unique Nhe I and EcoR I sites of previously modified hERβ in pcDNA3.1 (Saville et al., 2000). MOR cDNA was inserted into EcoR I site of pMT2 mammalian expression vector that contains unique Not I and Xba I sites in MOR cDNA sequence suitable for cloning the PCR-amplified insert containing deletion of one of zinc finger domain, into pMT2. The EcoR I fragment containing the desired deletion from pMT2 MOR vector was cloned into EcoR I site of pcDNA3.1for in vitro translation. The hERαTAF1 construct (in pcDNA3) has three point mutations (D538N, E542Q, and D545N) in AF2 (Tzukerman et al., 1994). hERαTAF1ΔZF1 and hERαTAF1ΔZF2 constructs were created by cloning the Xba I fragment (≅ 0.7 Kb) from hERαTAF1 (in pcDNA3) into pcDNA3.1 and zinc finger mutants were prepared as described above. All constructs were mapped by restriction enzymes and sequenced to confirm that proper deletion or insertions were introduced into the target cDNA.

2.5 In vitro translation and detection of the translated proteins

Wild-type ER and ER deletion mutants were synthesized in vitro using TNT T7 quick coupled transcription/translation System (Promega) in the presence or absence of [^{35}S] methionine for electrophoretic mobility shift assays and separation by 10% SDS polyacrylamide gel electrophoresis.

2.6 Electrophoretic mobility shift assays

The consensus [32P]ERE oligonucleotide was annealed and end-labeled using T4-polynucleotide kinase and [γ-32P]ATP. To characterize DNA binding of wild-type ER and corresponding zinc finger deletion mutants, 0.5 μl of in vitro translated protein or 0.5 μl of unprogrammed lysate, was incubated in 1X binding buffer (25% glycerol, 0.5 mM EDTA, 0.5 mM dithiothretiol, 50 mM potassium chloride, 10 mM HEPES at pH 8.0) for 5 min at 4°C. Radiolabeled consensus ERE oligonucleotide (60,000 cpm) was added to the reaction and the reaction mixture incubated at 25°C for 15 min. Samples were then applied to the gel and separated by polyacrylamide gel electrophoresis at 120 V in 0.9 mM Tris, 0.9 M borate, 2 mM EDTA (pH 8.0) for 2 to 3 h. Protein-DNA complexes were visualized by autoradiography using X-OmatTM film (Eastman Kodak, Co., Rochester, NY).

2.7 Fluorescence immunocytochemistry

MDA-MB-231 cells were subcultured in four-well Lab-Tek chambered slides (Nunc Inc., Naperville, IL) in DME/F12 medium without phenol red 5% FBS stripped with dextran-coated charcoal. After 24 h, cells were transiently

transfected with 500 ng of hERα or hERα mutant expression plasmids. For transient transfection studies, cells were incubated with FuGENE Transfection Reagent (Roche) at 37 C for 5 h, followed by 24 h of recovery in DME/F12. Before fixation, slides were washed three times in Dulbecco's PBS (DPBS) and then fixed for 10 min at -20 C at 100% methanol. Slides were subsequently washed three times in DPBS followed by a 1-h blocking step in 3% normal goat serum (G-9023; Sigma). For nuclear localization of ER, the rat monoclonal antibody raised against the N-terminal domain of the hERα (H184; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was diluted to a final concentration of 3 μg/ml in DPBS containing 0.5% BSA, 0.1% goat serum, and 0.3% Tween 20. Rat IgG at the same concentration was used as a control. Tween 20 (0.3%) was included in all antibody, blocking steps, and washes for nuclear localization of ER. Following by incubation with H184 antibody for 16 h, cells were washed with DPBS (three times) and then incubated for 1 h in a 1:200 dilution of fluorescein isothiocyanate-conjugated goat-antirat IgG (62–9511; Zymed Laboratories, Inc., South San Francisco, CA) in DPBS containing 0.1% goat serum. Cells were then washed (four times) over a period of 2 h and transferred to DPBS before coverslip mounting with ProLong Antifade mounting reagent (Molecular Probes, Inc., Eugene, OR). For each treatment, representative fluorescence images were recorded using an Axioplan microscope (Carl Zeiss, Thornwood, NY) equipped with a Hamamatsu chilled three charge-coupled device color camera (Hamamatsu, Japan) using Adobe Photoshop 5.0 (Adobe Systems, Seattle, WA)

image capture software. Images from all treatment groups were captured at the same time using identical image capture parameters.

2.8 Statistics

For transient transfection studies result are expressed as means ± SD for at least three separate experiments for each treatment group. Statistical differences ($p < 0.05$) between control (Me_2SO) and treatment groups or between E2-induced responses and treatment groups (coactivator experiments) were determine by ANOVA and Scheffe's post hoc test.

2.9 Coimmunoprecipitation and Western blot analysis

Cells were seeded into 35-mm six-well tissue culture plates in phenol red-free medium (Dulbecco's modified Eagle's medium [DMEM] Ham F-12) containing 2.5% charcoal-stripped fetal bovine serum (FBS) and when cells were 60-80% confluent, YFP-hERα and Sp1 expression plasmids were transfected using LipofectAMINE Plus Reagent (Invitrogen). After 24 hr, transfected cells were treated with DMSO, E2 10 nM, 4OHT 1 μM, and ICI 1 μM for 30min, 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) was added and cells were disrupted by repeated aspiration through a 21-gauge needle. Cellular debris was removed by centrifugation at 10,000xg for 10 min at 4º C and the supernatant was transferred to a fresh microcentrifuge tube on ice. Lysate was precleared by adding 1.0 μg of the appropriate control normal rabbit IgG together with 20 μl of

appropriate suspended (25% v/v) protein A/G-Agarose agarose conjugate and incubated at 4º C for 30 min. After centrifugation for 30sec, the supernatant (800 µg total cellular proteins) was transferred to a new microcentrifuge tube, 5 µl of rabbit polyclonal anti-GFP antibody (1 µg) (Santa Cruz) was added and incubate for 2–3 hours at 4º C. 20 µl of protein A/G-agarose (Santa Cruz) was added and incubated at 4º C for 1 hr. The immunoprecipitate was collected by centrifugation and the pellet was gently washed 2 times with 1.0 ml RIPA buffer and then with PBS. The agarose pellet was then resuspended in 50 µl of 1x Laemmli buffer (50 mM Tris-HCl, 2% sodium dodecyl sulfate [SDS], 0.1% bromphenol blue, 175 mM ß-mercaptoethenol), boiled, and centrifuged. The suspended sample was separated by SDS-10% PAGE, electrophoresed to a PVDF membrane. The membrane was blocked in 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 for Sp1 PEP2 (1:500) for 3 hr at room temperature. Following incubation with peroxidase-conjugated secondary antibody, immunoglobulins were visualized using the ECL detection system (NEN, Boston).

2.10 siRNA transfection

siRNA for Sp1 were prepared by IDT (Coralville, IA) and targeted the coding region 1811-1833 relative to the start codon of Sp1 gene (Abdelrahim, et al., 2002). Scrambled siRNA as negative control was purchased from Ambion (Austin, TX). Cells were cultured in 12-well plates in 1 ml of DME/F12 medium

supplemented with 5% fetal bovine serum. After 16-20 hr when cells were 60-75% confluent, iRNA duplexes and reporter gene constructs were transfected using LipofectAMINE Plus Reagent (Invitrogen); 0.375 μg of iRNA duplex to give a final concentration of 50 nM, $pSp1_3$ (500 ng), and β-galactosidase control plasmid were transfected in each well. The effects of small interfering Sp1 RNA (iSp1) transfection on hormone-induced transactivation were investigated in ZR-75 cells treated with 50 nM E2, 1μM 4OHT, 1μM ICI 182, 780. Cells were harvested 36-44 h after transfection by manual scraping in 1x lysis buffer (Promega) and luciferase activity was measured by the same method described in section 2.2.

2.11 FRET microscopy and analysis

To perform fluorescence resonance energy transfer (FRET), cells were washed with DME/F12 medium containing 5% serum and then put on the stage of the BioRad 2000MP system equipped with a Nikon TE300 inverted microscope with a 60x (NA 1.2) water immersion objective lens and a Titanium: Saphire laser tuned to 820 nm wavelength. Control images were acquired before treatment of cells with DMSO, E2, 4OHT or ICI. Additional images were acquired between 8 and 18 min after addition of each ligand at a speed of 25lps. FRET data in MCF-7 cells transfected with CFP and YFP fusion constructs alone or in combination, were collected using 2 photon-820 nm excitation wavelength. Emission of CFP (donor signal) was collected using a 500DCLP dichroic and 450 nm /80 nm filter while emission of YFP (acceptor signal or FRET signal) was

collected using a 528 nm/50 nm filter. Donor bleed through signal to the FRET channel was calculated by measuring the FRET channel signal resulting from MCF-7 cells transfected only with the CFP fusion construct. Acceptor bleed through to the FRET channel was calculated by measuring the FRET channel signal resulting from MCF-7 cells transfected with YFP fusion construct alone. To correct for variations in fluorophore expression resulting from different transfection efficiencies, minimum levels of YFP expression and maximum levels of CFP were selected based on data collected from each experiment. Cells that did not match the selection criteria were eliminated from the FRET analysis. Negative (CFP empty and YFP empty) and positive (CFP-YFP chimera) controls were used to calculate the approximate FRET efficiency in cells treated with different ligands; it was assumed that the signal from cells transfected with the positive CFP-YFP chimera construct will exhibit 50% FRET efficiency when compared to signals from cells transfected with CFP/YFP empty constructs.

For identification of Region Of Interest (ROI) and FRET analysis, MetaMorph software version 6.0 was used (Universal Imaging Corp. Downingtown, PA). Acceptor signal acquired with the FRET channel was corrected by subtracting the background signal as well as the donor bleed through signal. Ten to fifteen images were collected from each sample and 1-5 cells per image captured were analyzed. Three to five experiments per each combination of transfected fusion constructs were conducted on different days. Student’s t test was used to analyze the statistical significance between control

and ligand-treated cells at $p<0.05$ and this analysis was performed using Prism software version 4.0 (GraphPad Software Inc. San Diego, CA).

2.12 Plasmid construction for FRET studies

CFP-C1 and YFP-C1 mammalian expression vectors were obtained from BD Biosciences CLONTECH Laboratories, Inc. (Palo Alto, CA). The CFP-YFP chimera was generated by PCR using the following primer set: 5' TCCCCGCGGTAGCCGCCATGGTGAGCAAGGGC-GAGGAGCTG 3' (sense) and 5-CGGGATCCCTTGTACAGCTCGTCCATGCCGAG 3' (antisense). The PCR product was digested with SacII and BamHI and cloned into the CFP-C1 vector (Bai and Giguere, 2003). CFP-hERα and YFP-hERα were made by PCR using following primer set: 5' TTCGAATTCTATGACCATGACCCTCC ACACCAAAGCA 3' (sense) and 5' TAGTCGACTCAGACTGTGGCAGGGA AACCCTC 3' (antisense) and the primer set for CFP-Sp1 is 5' TTCGAATTCTACAGGTGAGCTTGACCTCACAGCC 3' (sense) and 5' TAGTCGACTCAGAAGCCATTGCCACTGATATT 3' (antisense). The PCR product was digested with EcoRI and Sal I and cloned into either the CFP or YFP construct. Dominant negative Sp1 plasmid (Sp1DN) was provided by Drs.Yoshihiro Sowa and Toshiyuki Sakai (Kyoto Prefectural University of Medicine, Kyoto, Japan).

CHAPTER III

RESULTS*

3.1 Role of zinc fingers 1 and 2 of ERα and ERβ in hormonal activation of GC-rich promoters

Previous studies in this laboratory showed that hormone-induced activation of hERα/Sp1 in breast cancer cells required the AF1 domain of hERα (Saville et al., 2000), and activation by E2 was also observed in cells cotransfected with the DBD deletion mutant (aa 185–251) hER11 (Porter et al., 1997; Wang et al., 1999; Xie etal., 1999; Sun et al., 1998; Qin et al., 1999; Duan et al., 1998; Dong et al., 1999; Samudio et al., 2001). The role of other domains of hERα on estrogen and antiestrogen activation of hERα/Sp1 has not been defined and has been investigated in this study. Although hERα11/Sp1 is activated by E2 in transactivation assays, deletion of the entire DBD resulted in loss of antiestrogen-induced transactivation (Xie et al., 2000; Saville et al., 2000), and therefore initial studies determined the role of zinc fingers 1 and 2 deletion mutants on estrogen/antiestrogen activation of ERα/Sp1.

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Wild-type and zinc finger deletion mutants for hER (α and β) and mouse ERα (MOR) were cloned into pcDNA3 and translated in vitro using [^{35}S]methionine and the radiolabeled proteins were separated by SDS-PAGE (Fig. 15). The results show that in vitro translated proteins gave distinct bandswith comparable intensities and the expected molecular weights, indicating that the ΔZF1 or ΔZF2 deletions did not cause unexpected frame shifts.

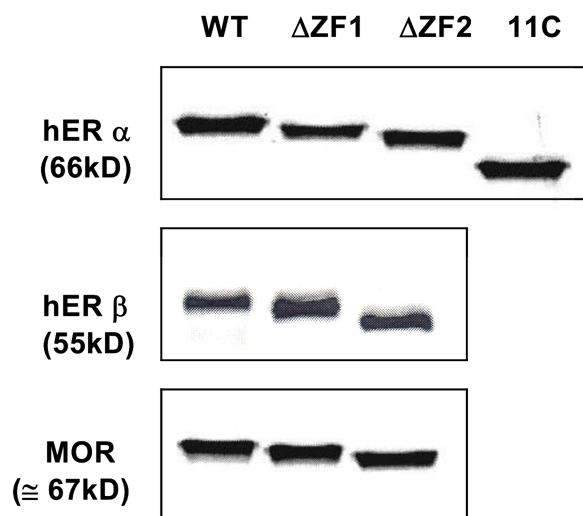


Fig. 15. SDS-PAGE separation of in vitro translated ^{35}S-labeled proteins. Wild-type hERα, MOR, and hERβ and their corresponding zinc finger deletion mutants were in vitro translated using [^{35}S]methionine and separated by SDS-PAGE as described in Materials and Methods. 11C refers to a DBD deletion mutant (aa 185–281) of hERα. Intensities of the radiolabeled proteins were similar and electrophoretic mobilities were consistent with their expected molecular masses.

The effects of ΔZF1 and ΔZF2 mutations on DNA binding were determined by EMSAs of the in vitro expressed proteins. The results showed

that only wild-type hERs and MOR formed retarded bands after incubation with [^{32}P]ERE (lanes 3, 7, and 10), whereas DNA-bound complexes were not observed with the zinc finger deletion mutants (Fig. 16). Transcriptional activation assays in ER-negative MDA-MB-231 cells cotransfected with an ERE-dependent promoter (pERE$_3$) and wild-type ER or ER deletion mutants showed that E2 induced activity only in cells transfected with wild-type hER (α or β) or MOR (Fig. 16).

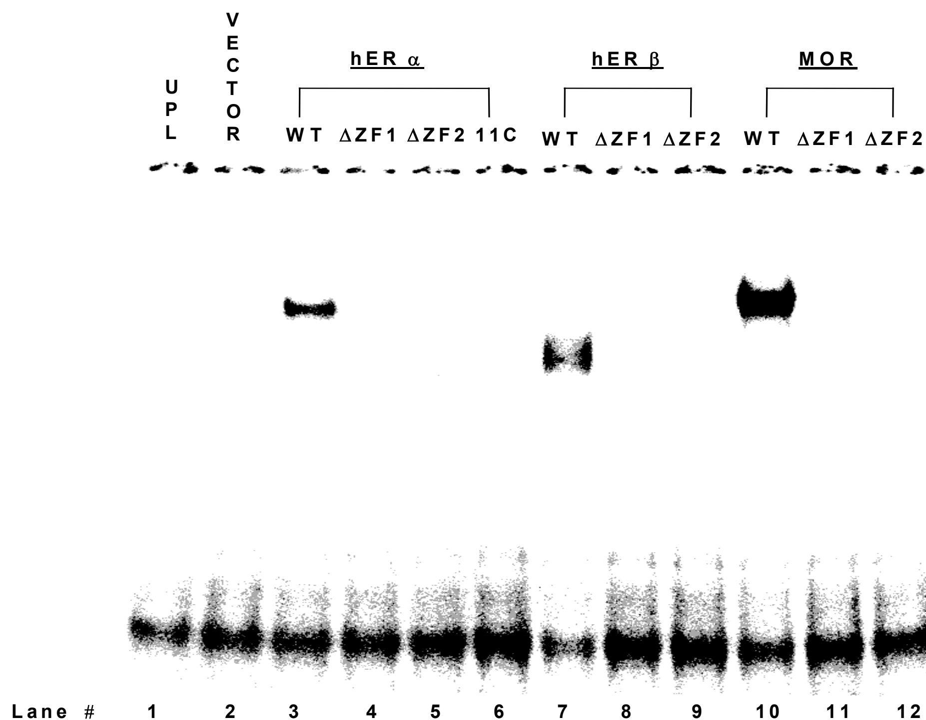


Fig. 16. Gel mobility shift assays. Unlabeled wild-type hERα, MOR, and hERβ and their corresponding zinc finger deletion mutants were in vitro translated, incubated with [^{32}P]ERE, and analyzed by gel mobility shift assays as described in Materials and Methods. UPL refers to unprogrammed lysate. Only wild-type hERα (lane 3), hERβ (lane 7), and MOR (lane 10) formed retarded bands. Competition with excess unlabeled ERE decreased intensities of these bands (data not shown).

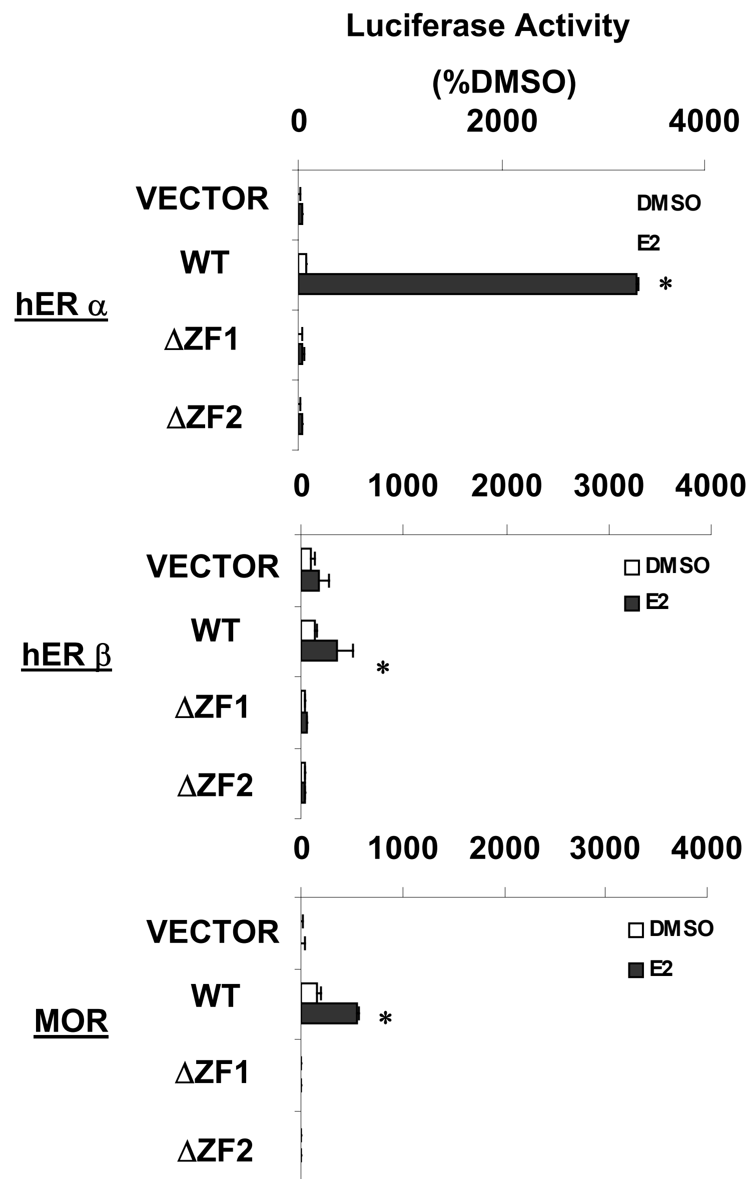


Fig. 17. Transactivation in MDA-MB-231 cells transfected with $pERE_3$. MDA-MB-231 cells were treated with 10 nM E2, cotransfected with $pERE_3$ and wild-type hERα, MOR, and hER or their zinc finger deletion mutants, and luciferase activities were determined as described in *Materials and Methods*. Significant ($P < 0.05$) induction is indicated (*). Results are expressed as means ± SD for at least three separate determinations for each treatment group.

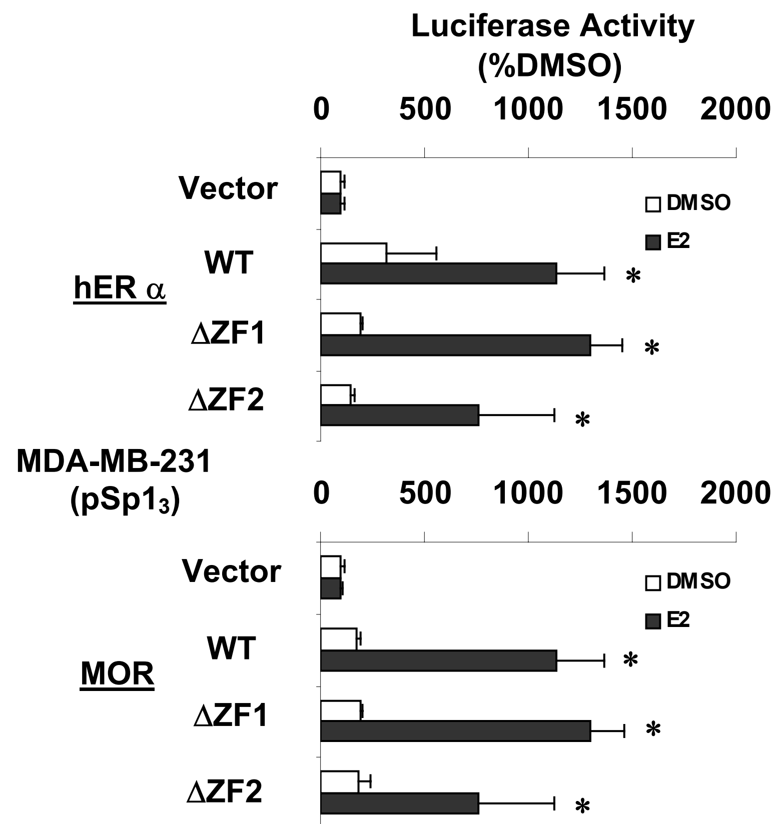


Fig. 18. Transactivation in MDA-MB-231 cells transfected with pSp1$_3$ and zinc finger deletion mutants. MDA-MB-231 cells were treated with 10 nM E2, transfected with pSp1$_3$ and wild-type hERα or MOR and their zinc finger deletion mutants. Luciferase activities were determined as described in *Materials and Methods*. Significant ($P < 0.05$) induction is indicated (*).

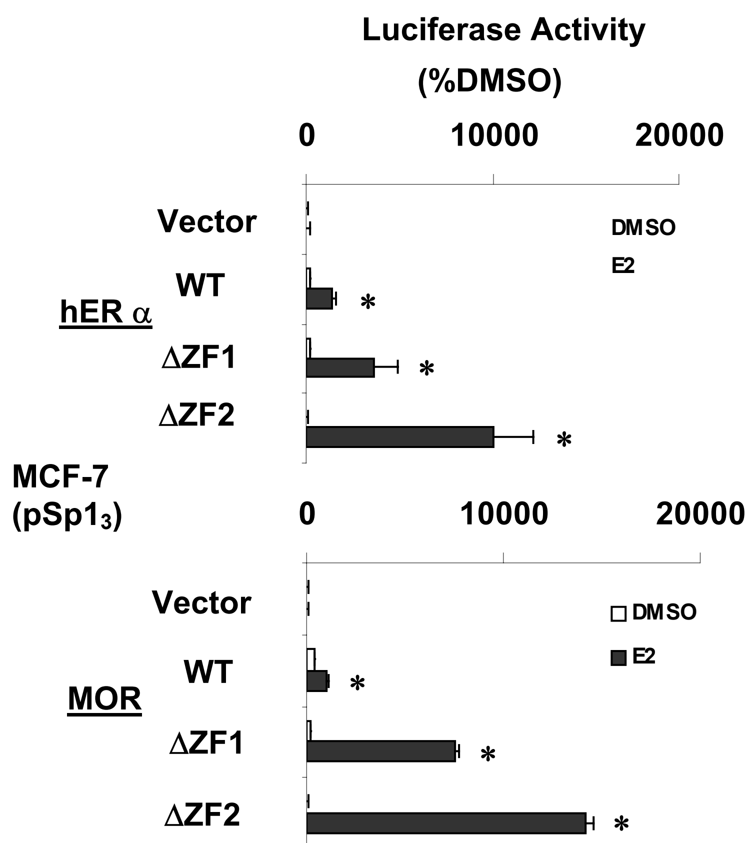


Fig. 19. Transactivation in MCF-7 cells transfected with $pSp1_3$ and zinc finger deletion mutants. Cells were treated with 10 nM E2, transfected with $pSp1_3$ and wild-type hERα or MOR and their zinc finger deletion mutants. Luciferase activities were determined as described in *Materials and Methods*. Significant ($P < 0.05$) induction is indicated (*). Only minimal responses were observed for hERß/Sp1 (data not shown) as previously reported (Saville et al., 2000), and similar results were obtained with the hERβ zinc finger deletion mutants. Results are expressed as means ± SD for three separate determinations for each treatment group.

These results were consistent with the gel mobility shift assays showing that the zinc finger mutants do not bind EREs (Fig. 17). E2 induced reporter

gene activity in MDA-MB-231 and MCF-7 cells transfected with $pSp1_3$ and expression plasmids for wild-type hERα or MOR (Figs.18 and 19).
In contrast to results obtained in cells transfected with pERE3, induction responses were observed for both zinc finger deletion mutants of hERα and MOR. hERβ and the DBD deletion mutants were only minimally active in MDA-MB-231 cells (≅2-fold induction) and inactive in MCF-7 cells (data not shown). The fold-induction using wild-type and zinc finger deletion mutants of hERα and MOR was lower in MDA-MB-231 than MCF-7 breast cancer cells due, in part, to higher basal activity in the former cell line. The highest induction responses using $pSp1_3$ and E2 were observed in MCF-7 cells transfected with ΔZF2 mutants; in MCF-7 cells, all the ER-DBD deletion mutants were more potent activators of $pSp1_3$ than wild-type hERα or MOR. The overall pattern of induction responses was similar for wild-type hERα and MOR and their zinc finger deletion mutants, and subsequent studies used only hERα.

Several genes that are induced by E2 in MCF-7 breast cancer cells through hERα/Sp1 interactions with GC-rich motifs have previously been identified (Porter et al., 1997; Wang et al., 1999; Xie et al., 1999; Sun et al., 1998; Qin et al., 1999; Duan et al., 1998; Dong et al., 1999; Samudio et al., 2001; Khan et al., 2003; Ngwenya et al., 2003), and these include retinoic acid receptor α1 and adenosine deaminase (Xie et al., 1999; Sun et al., 1998).

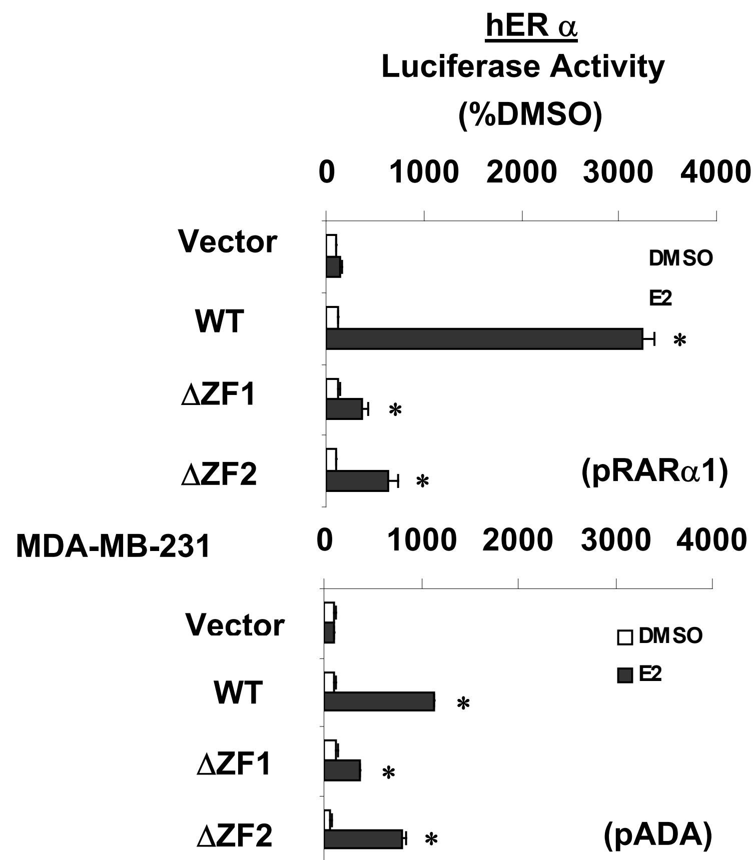


Fig. 20. MDA-MB-231 cells transfected with pRARα1 and pADA. Cells were treated with E2 or DMSO, transfected with pADA or pRARα1 constructs, wild-type hERα, or zinc finger deletion mutants of hERα, and luciferase activities were determined as described in *Materials and Methods*. Significant ($P < 0.05$) induction by E2 is indicated (*). Results are presented as means ± SD for three separate determinations for each treatment group.

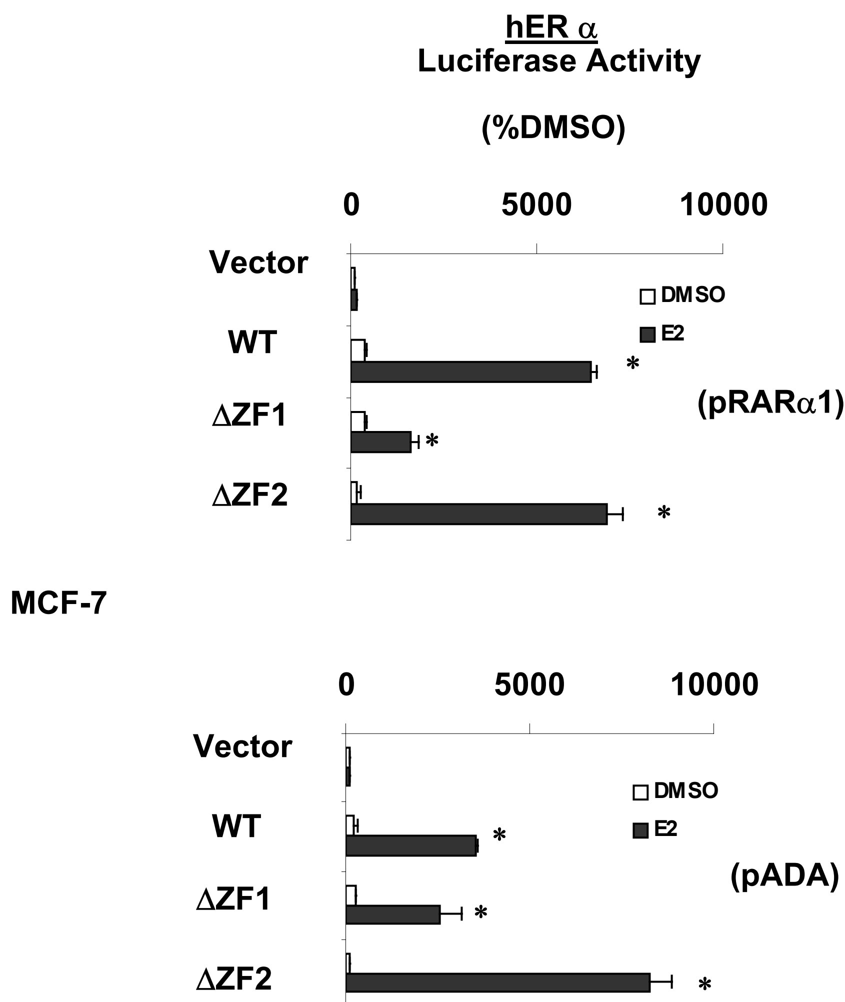


Fig. 21. MCF-7 cells transfected with pRARα1 and pADA. Cells were treated with E2 or DMSO, transfected with pADA or pRARα1 constructs, wild-type hERα, or zinc finger deletion mutants of hERα, and luciferase activities were determined as described in Materials and Methods. Significant ($P < 0.05$) induction by E2 is indicated (*). Results are presented as means ± SD for three separate determinations for each treatment group.

hERα/Sp1 activation of constructs derived from GC-rich E2-responsive regions of the adenosine deaminase (pADA; -86 to -65) and retinoic receptor α1 (pRARα1; -79 to -49) gene promoters were investigated in MCF-7 and MDA-MB-231 cells transfected with wild-type or zinc finger deletion mutants of

hERα (Figs. 20 and 21). The pattern of induction by E2 was dependent on expression of wild-type or variant hERα, promoter, and cell context. For example, hormone inducibility was higher in MDA-MB-231 cells transfected with either pADA or pRARα1 and wild-type hERα compared with responses observed for the zinc finger deletion mutants. In MCF-7 cells, hormone inducibility was highest in cells transfected with pADA or pRARα1 and the ΔZF2 deletion mutant of hERα.

3.2 Role of zinc fingers 1 and 2 in antiestrogen activation of GC-rich pSp1$_3$

The results in Fig. 22 summarize the effects of E2 and the antiestrogens ICI 182,780 and 4-OHT on induction of luciferase activity in MCF-7 cells transfected with pSp1$_3$ and wild-type or zinc finger deletion mutants of hERα. Both estrogens and antiestrogens induce transactivation in cells transfected with wild-type hERα, whereas E2, but not the antiestrogens, were active in cells transfected with ΔZF1 or ΔZF2 deletion mutants of hERα. These results suggest that antiestrogen-bound zinc finger mutants of hERα are transcriptionally inactive and their recruitment of functional coactivators requires cooperative interactions that are directly or indirectly dependent on the zinc finger domains of hERα.

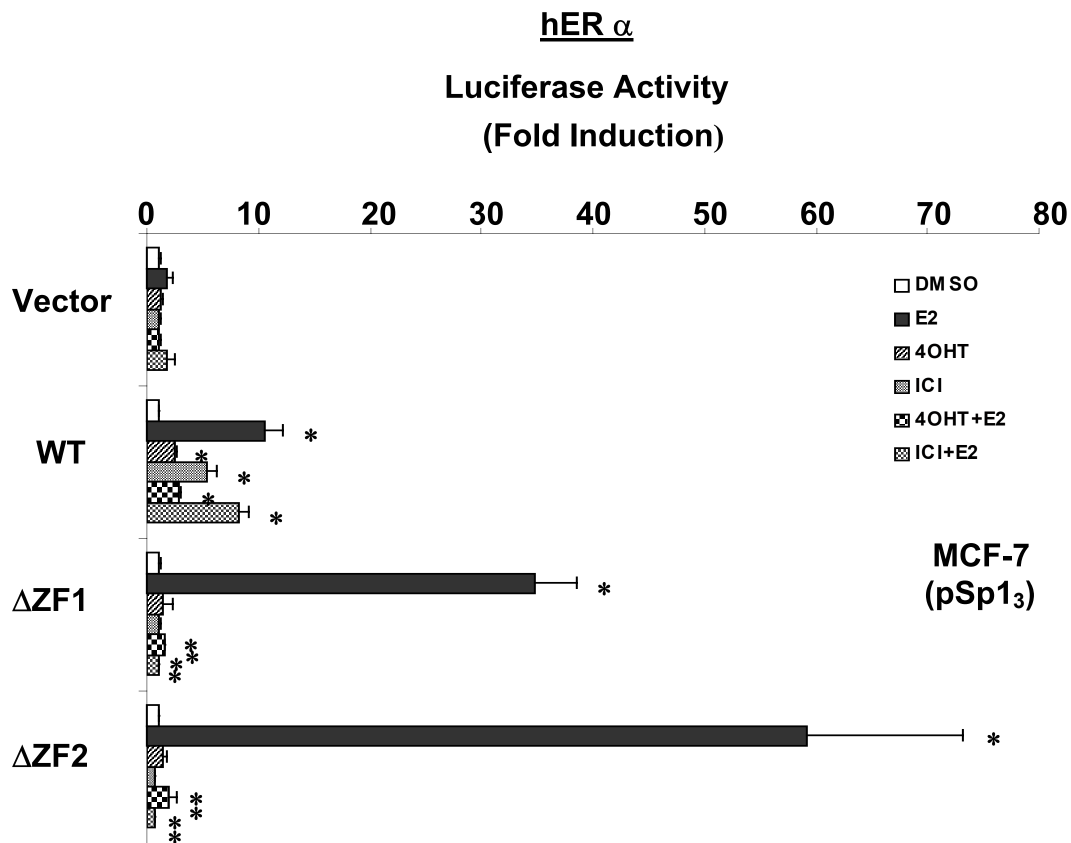


Fig. 22. Effects of zinc finger DBD mutants of hERα on activation of $pSp1_3$ by estrogens and antiestrogens in MCF-7 cells. MCF-7 cells were treated with 10 nM E2, 1 μM 4-OHT, 1 μM ICI 182,780 (ICI), or antiestrogens plus E2, transfected with pSp13 and hERα, hERαΔZF1, and hERαΔZF2, and luciferase activities were determined as described in Materials and Methods. Significant ($P < 0.05$) induction (*) and inhibition of E2-induced activity (**) are indicated.

Although deletion of one or both zinc fingers resulted in loss of antiestrogen-dependent hERα/Sp1 agonist activity, 4-OHT and ICI 182,780 inhibited E2-induced ERα/Sp1 action using these DBD deletion constructs,

suggesting that the antiestrogen-mediated responses are intact in cells expressing zinc finger mutants of hERα.

We also investigated the possibility that the failure to observe antiestrogen activation of zinc finger mutants of hERαSp1 may be due to failure of the transfected constructs to accumulate in nuclei of breast cancer cells.

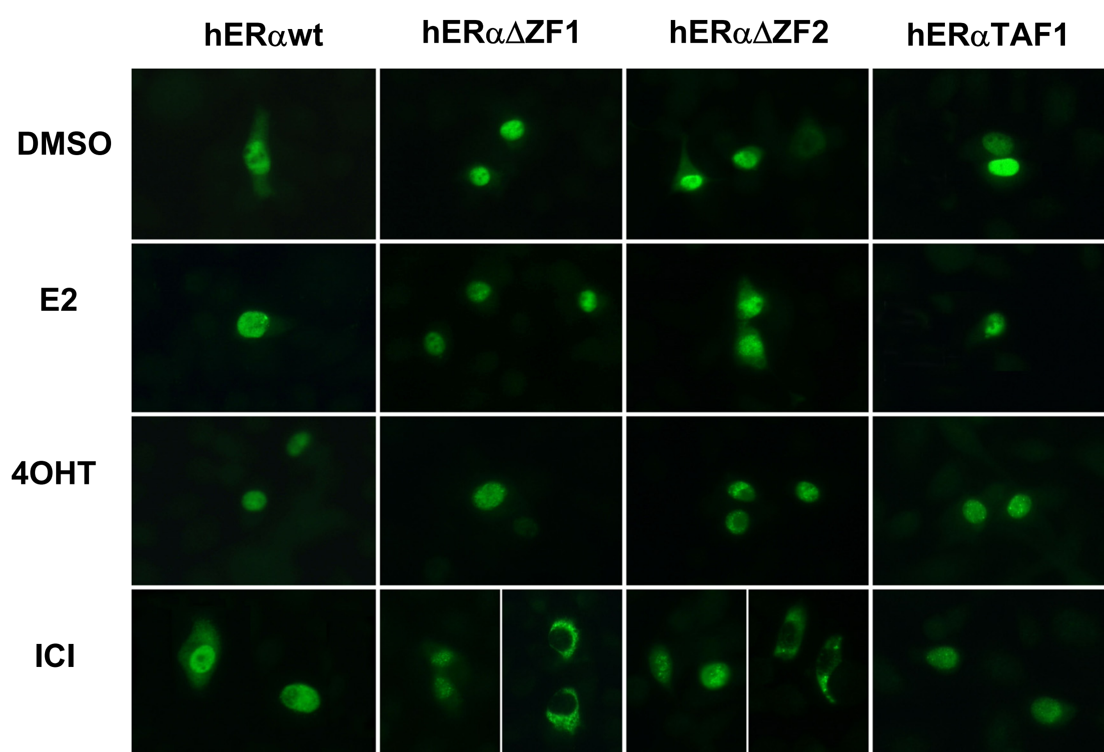


Fig. 23. Immunostaining of transfected wild-type and mutant hERα constructs in MDA-MB-231 cells. Cells were transfected with hERα, hERαΔZF1, or hERαΔZF2, treated with 10 nM E2, 1 μM 4-OHT, or 1 μM ICI 182,780 for 24 h, and immunostaining of transfected ER constructs was determined as described in Materials and Methods. Nuclear staining was observed in all groups; however, cells treated with ICI 182,780 exhibited perinuclear staining.

However, results of immunofluorescent studies in MDA-MB-231 cells transfected with hERα or the zinc finger mutants showed that wild-type and mutant constructs were primarily nuclear in cells treated with dimethylsulfoxide (DMSO) or E2/antiestrogens, although some perinuclear staining was observed with hERαΔZF1 using ICI 182,780 (Fig. 23).

3.3 Role of zinc fingers 1 and 2 or DBD in estrogen or antiestrogen activation of AP1 promoter

ERs also regulate transcription of AP1-depedent promoters not by direct DNA-protein interactions but by indirect protein-protein interactions as observed for ERα/Sp1, and this involves ER interaction with the AP1(Fos and Jun) complex (Pfahl, 1993). It has been previously reported that estrogen regulates AP1 dependent transcription either positively or negatively, depending on cell type and promoter context (Gaub et al., 1990; Philips et al., 1993; Umayahara et al., 1994; Webb et al., 1995). ERα containing a point mutation in the first zinc finger or complete deletion of DBD was shown to be efficient in regulating AP1 responses (Gaub et al., 1990; Webb et al., 1995; Philips et al., 1998). Direct physical interactions between the C-terminal part of c-Jun and amino acids 259-302 present in the ERα hinge domain are responsible for the formation of c-Jun•ERα•GRIP1 coactivator complex (Teyssier et al., 2001).

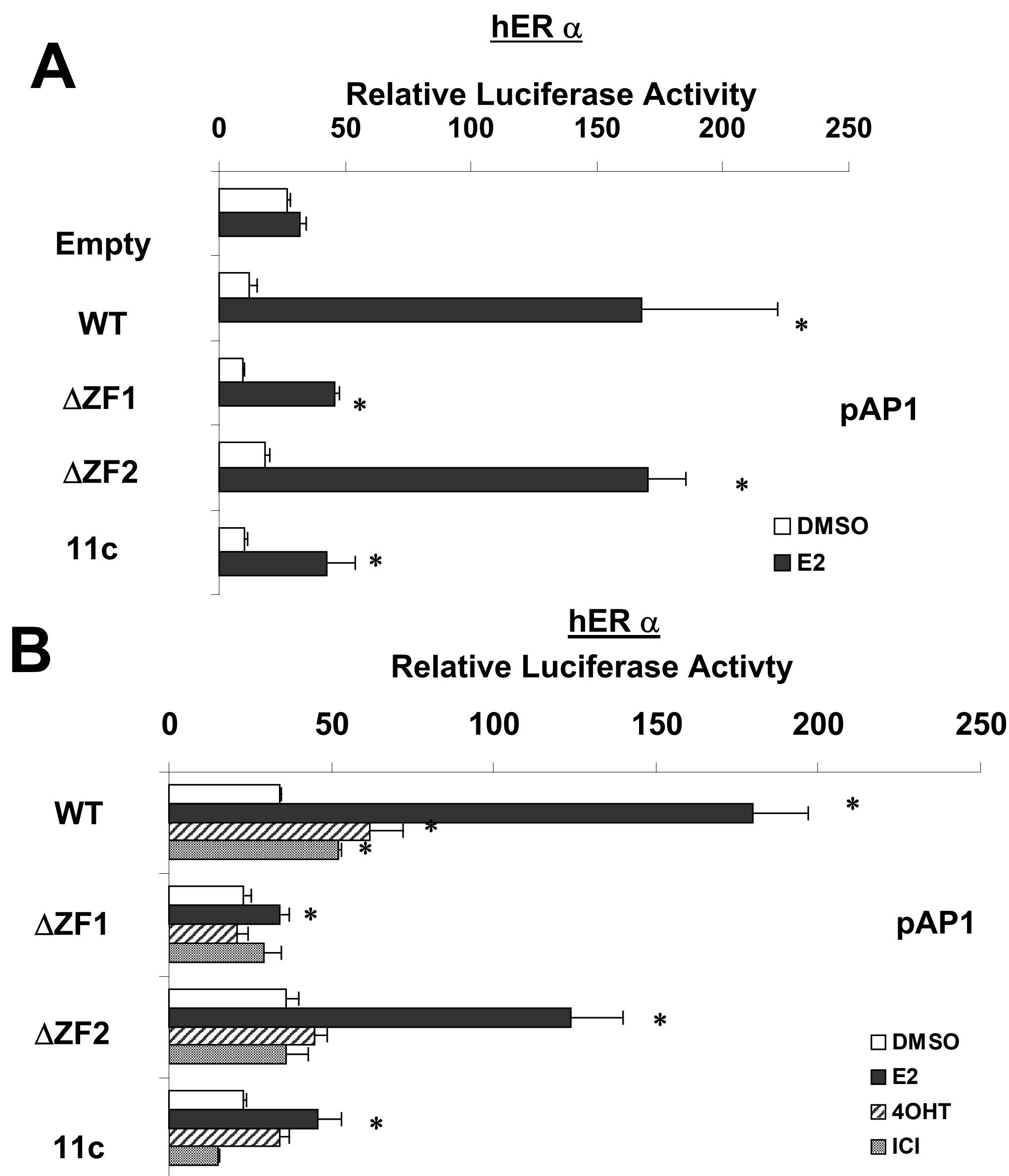


Fig. 24. Effects of zinc finger DBD mutants of hERα on activation of pAP1 by estrogens and antiestrogens in MCF-7 cells. MCF-7 cells were treated with 10nM E2, transfected with pAP1, and wild type hERα, 11c, or their zinc finger deletion mutants (A). MCF-7 cells were treated with 10 nM E2, 1 μM 4-OHT, 1 μM ICI 182,780 (ICI), transfected with pAP1 and hERα, hERαΔZF1, and hERαΔZF2 (B). Luciferase activities were determined as described in Materials and Methods. Significant ($P < 0.05$) induction (*) is indicated.

ERα-mediated regulation of AP1 activity was investigated in this study using deletion constructs of either zinc fingers or the complete DBD and these expression plasmids were transfected into MCF-7 cells along with an AP1-TATA luciferase reporter construct.

In agreement with previous results (Philips et al., 1998), either deletion of entire DBD or one of zinc fingers did not show any effect on estrogen-induced AP-1 activity (Fig. 24 A). The highest induction responses were observed in MCF-7 cells transfected with hERα wt and hERαΔZF2 mutants. The effects of E2, antiestrogen ICI 182, 780 or 4OHT were observed in MCF-7 cells transfected with hERwt or deletion mutants and pAP-1 luciferase reporter construct (Fig. 24 B). Only wild type hERα exhibited hormone inducibility after treatment of MCF-7 cells with estrogen and antiestrogen treatments. In contrast, cells transfected with DBD or zinc finger domain deletion mutants showed minimal inducibility with antiestrogen treatment and the results with the mutant constructs were similar to those coresponding estrogen/antiestrogen activation of GC-rich promoters.

3.4 ERα/Sp1-mediated transactivation in cells transfected with hERα zinc finger point mutants

The three mutants of E207G/G208S (207GS), E207A/G208A (207AA) and K210A have point mutation in the “P-box” of the first zinc finger whereas the A227T mutant contains a point mutation in the “D-box” of the second zinc finger. It was previously reported that with the exception of the K210A mutant these

mutant ERs retained their hormonal responsiveness on an AP-1 promoter but did not activate an ERE-dependent promoter (Jackacka et al., 2001).

To assess transcriptional activation of the GC-rich promoter ($pSp1_3$) by these mutants, MCF-7 cells were transfected with $pSp1_3$ and treated with estrogen and antiestrogens. Only two mutants, A227T and K210A, exhibited hormone responsiveness after treatment with estrogen whereas no transactivation was observed after treatment with antiestrogens (Fig. 25).

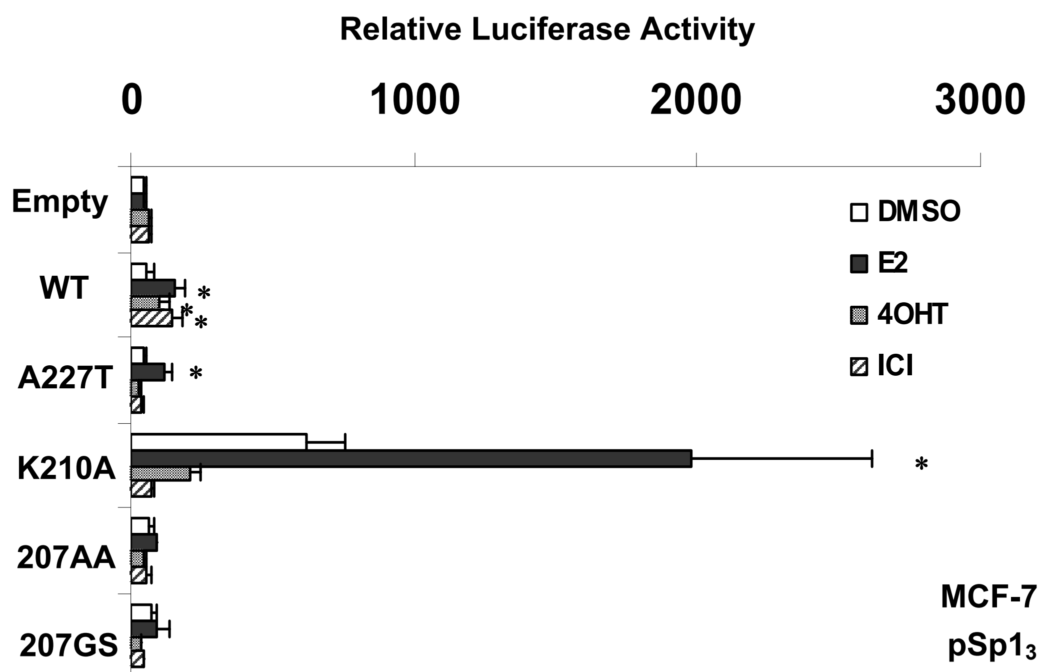


Fig. 25. Effects of hERα zinc finger point mutants on activation of of $pSp1_3$ by estrogens and antiestrogens in MCF-7 cells. MCF-7 cells were transfected with $pSp1_3$, and wild type hERα, A277T, K210A, 207AA or 207GS point mutation(s) mutants. MCF-7 cells were treated with 10 nM E2, 1 μM 4-OHT, 1 μM ICI 182,780 (ICI) and luciferase activities were determined as described in Materials and Methods. Significant ($P < 0.05$) induction (*) is indicated.

Surprisingly, unlike the hERα deletion mutants, the 207AA and 207GS mutants did not show hormone responsiveness demonstrating significant differences between the ZFdeletion and ZF point mutant ERα in activation of ERα/Sp1.

3.5 Role of histone deacetylase inhibitors in ERα/ERE- or ERα/Sp1-mediated transactivation

Acetylation and deacetylation of histones in nucleosomes are linked to formation of transcriptionally active chromatin structure (Kuo and Allis, 1998; Struhl, 1998; Workman and Kingston, 1998). Histone deacetylase inhibitors, such as trichostatin A (TSA) and sodium butyrate, increase the histone acetylation in many types of cells (Yoshida et al., 1995). In addition, it has previously been reported that TSA significantly enhanced estrogen-induced transactivation in cells stably transfected with the E2-reponsive vitellogenin-CAT construct (Mao and Shapiro, 2000).

In order to assess the role of histone acetylation/deacetylation processes in ERα/ERE- or ERα/Sp1-dependent transactivation, two reversible histone deacetylase inhibitors, TSA and sodium butyrate were added to MCF-7 cells transfected with $pERE_3$ and $pSp1_3$, respectively. Both TSA and sodium butyrate significantly increased the fold E2-induced activity in cells transfected with $pERE_3$. In contrast, the overall fold induction level was not affected in MCF-7cells transfected with $pSp1_3$ and this was due to a parallel increase in basal and induced activation (Fig. 26).

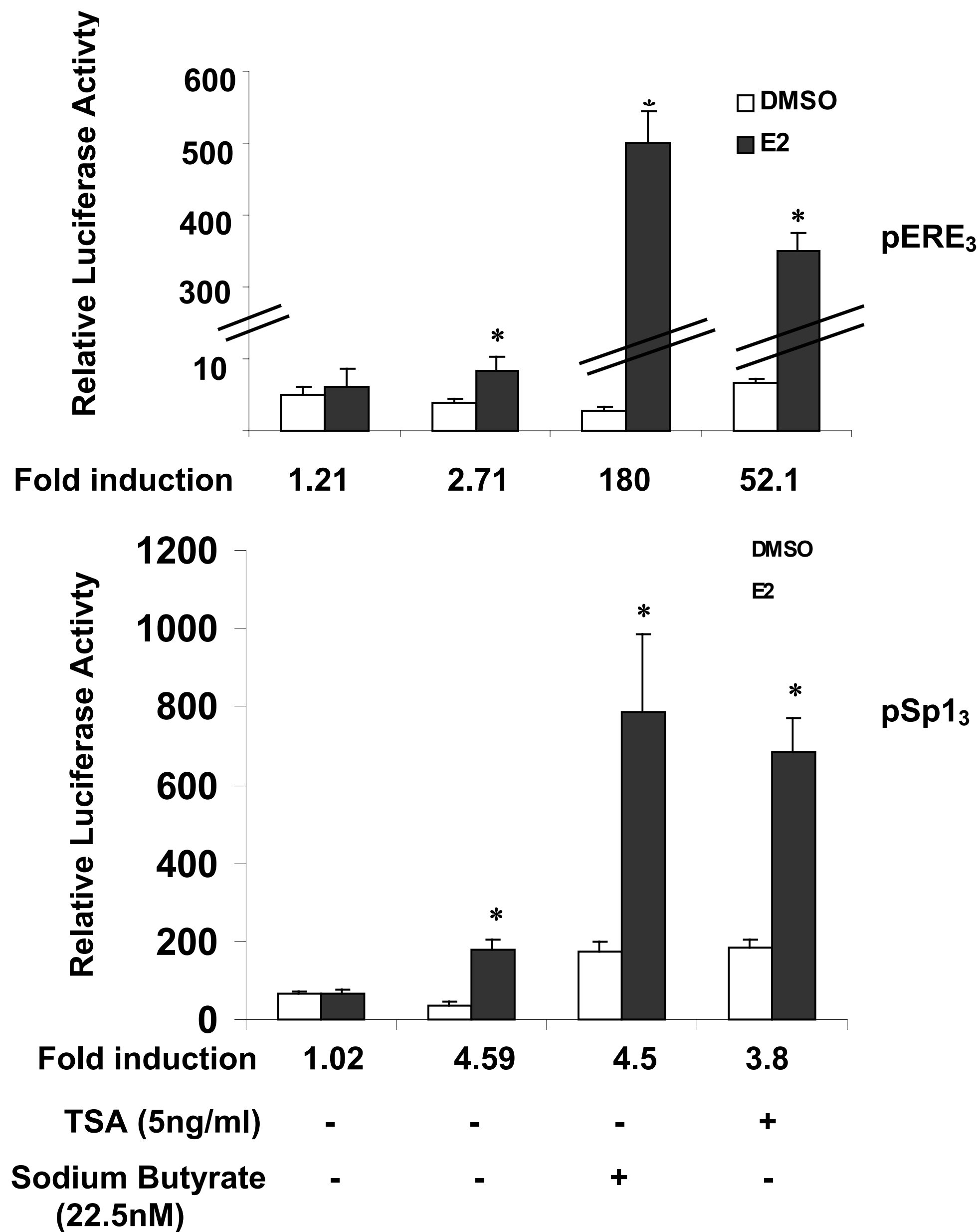


Fig. 26. Effects of histone deacetylase inhibitors on activation of $pERE_3$ and $pSp1_3$ in MCF-7 cells. Luciferase activities were determined as described in Materials and Methods. Significant ($P < 0.05$) induction (*) is indicated.

These results show that ERα/ERE-dependent transcription is more sensitive to histone deacetylase inhibitor treatments than ERα/Sp1-dependent

transcription, suggesting that differences in nucleosomal accessibility in vivo, which are dependent on promoter context.

3.6 Activation of hERα/Sp1 by E2, 4-OHT, and ICI 182,780 does not require AF2-helix 12-coactivator interactions

It was previously reported that activation of hER/Sp1 by E2 was lost after deletion of aa51–117(Saville et al., 2000); however, this did not exclude a role for AF2 alone or as a modifier of AF1-dependent hERα/Sp1 action. hERαTAF1 contains three aa mutations (D538N, E542Q, and D545N) that do not affect ligand binding but inactivate AF2 by selectively blocking interactions with AF2-dependent coactivators (Chang et al., 1999a; Schaufele et al., 2000; Tzukerman etal., 1994; McDonnell et al., 2000). In MCF-7 cells transfected with $pSp1_3$ and hERαTAF1, E2 significantly induced reporter gene activity, and ICI 182,780 and 4-OHT also slightly increased this response (Fig.27). Hormone-mediated transactivation was also observed in MCF-7 cells transfected with $pSp1_3$ and hERαTAF1 containing deletions of zinc finger 1 or zinc finger 2 in the DBD; in contrast, the antiestrogens ICI 182,780 and 4-OHT did not activate luciferase activity using the hERαTAF1 DBD mutants, and similar results were observed for hERαΔZF1 and hERαΔZF2 (Fig.22). Increased hormone-induced transactivation was observed in MCF-7 cells transfected with $pSp1_3$ and hERαΔZF1 or hERαΔZF2 compared with wild-type hERα (Figs. 19 and 22). In contrast, deletion of zinc fingers 1 or 2 in hERαTAF1 did not result in increased hormone responsiveness in MCF-7 cells transfected with the zinc finger deletion

mutants compared with hERαTAF1 (Fig. 27). This suggests that helix 12 may contribute to E2-induced hERαΔZF1/Sp1 and hERαΔZF2/Sp1 action. hERα19 and hERαnull were also inactive, and this was consistent with previous studies showing the importance of AF1 for hERα/Sp1 action (Saville et al., 2000).

The results in Figs. 28 and 29 shows that expression of the LXXLL-peptide 2XF6 and Grip (Chang et al., 1999a) significantly decreased hormone-induced transactivation in MDA-MB-231 cells and MCF-7 cells transfected with pERE3, and inhibition was not observed in cells transfected with $pSp1_3$. Furthermore, various LXXLL peptides including 2XF6, Grip, and C33 inhibited E2-induced transactivation in ZR-75 cells transfected with $pERE_3$ but not with $pSp1_3$ (Fig. 30). These data suggest that interactions of hERα with prototypical steroid receptor coactivators containing LXXLL motifs may not be critical for hERα/Sp1 action.

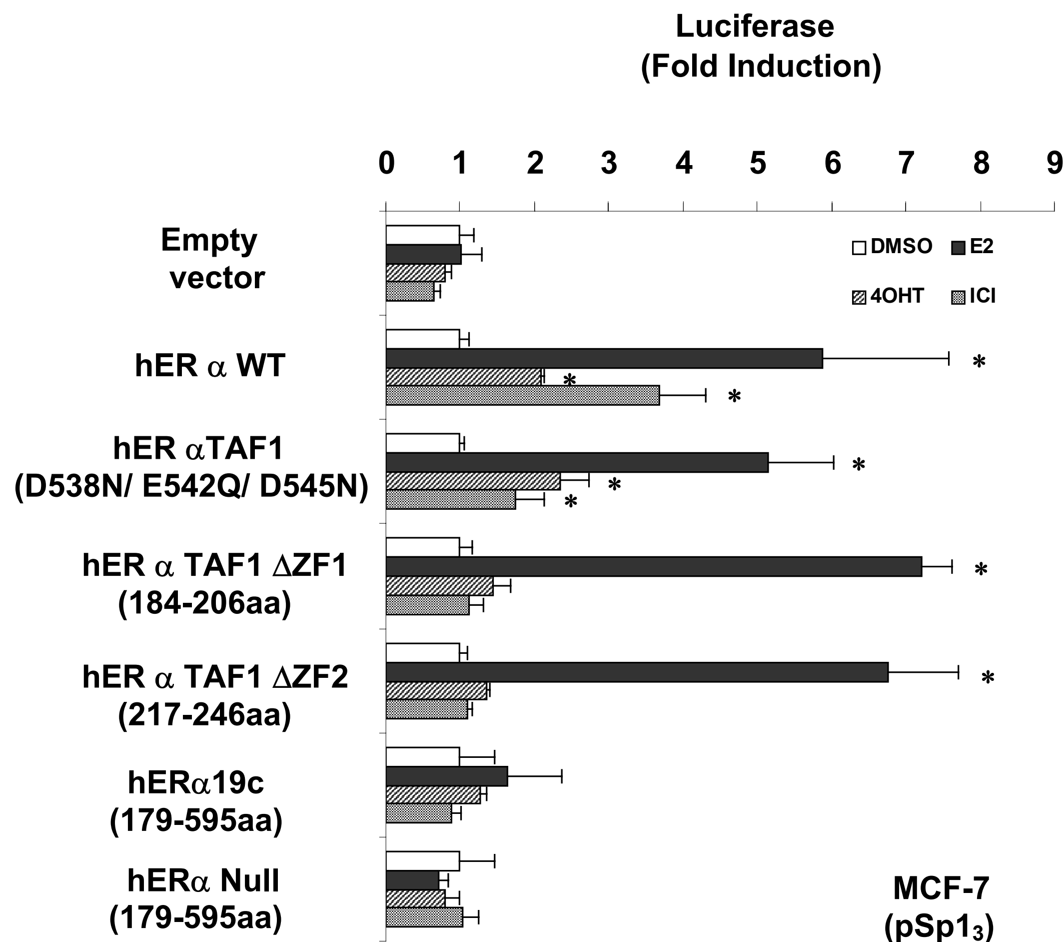


Fig. 27. Activation of hERα/Sp1 by helix 12 and zinc finger mutants of hERα. MCF-7 cells were treated with DMSO, 10 nM E2, 1 μM 4-OHT, or 1 μM ICI 182,780, transfected with several hER point and/or deletion mutants, and luciferase activity was determined as described in *Materials and Methods*. The hER19c and hERNull mutants do not express AF1 (aa 1–178) of hER, and hERnull also contains D538N, E542Q, and D545N point mutations in the AF2 domain of hERα. Results are expressed as means ± for three separate determinations for each treatment group and significant ($P < 0.05$) induction is indicated (*).

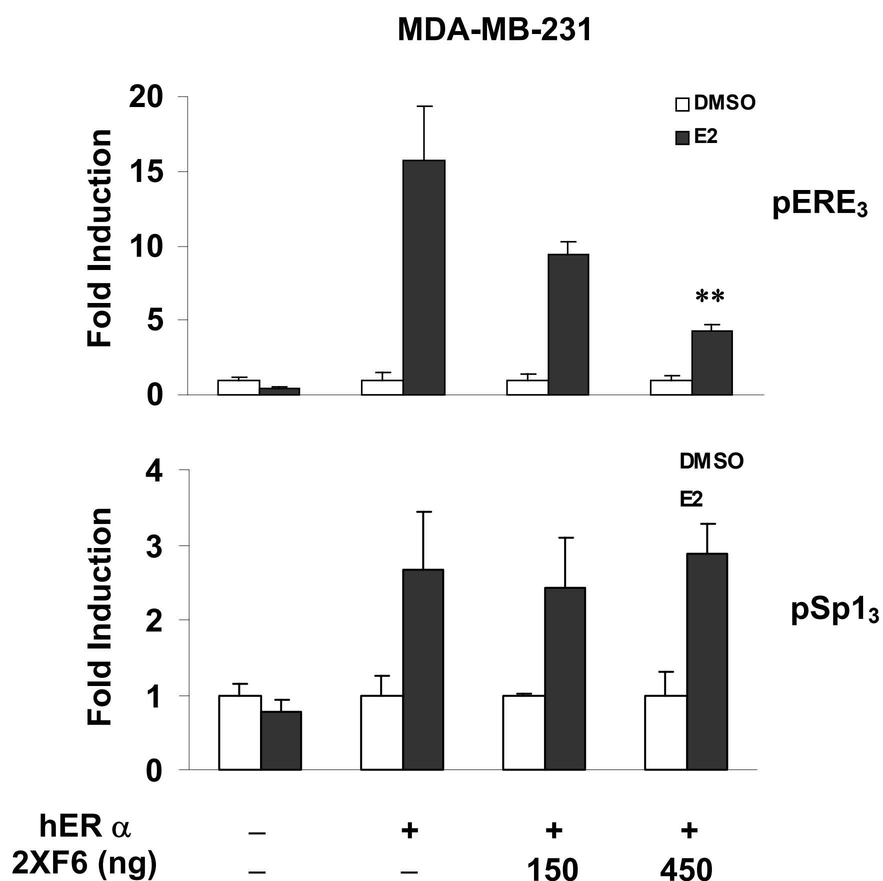


Fig. 28. Inhibition of transactivation by 2XF6 peptide. MDA-MB-231 cells were treated with DMSO or 10 nM E2, transfected with pERE3 or pSp13 and different amounts of 2XF6 expression plasmid, and luciferase activity was determined as described in Materials and Methods. Results are expressed as means ± SD for three replicate determinations for each treatment group, and significant ($P < 0.05$) inhibition of induced activity is indicated (**).

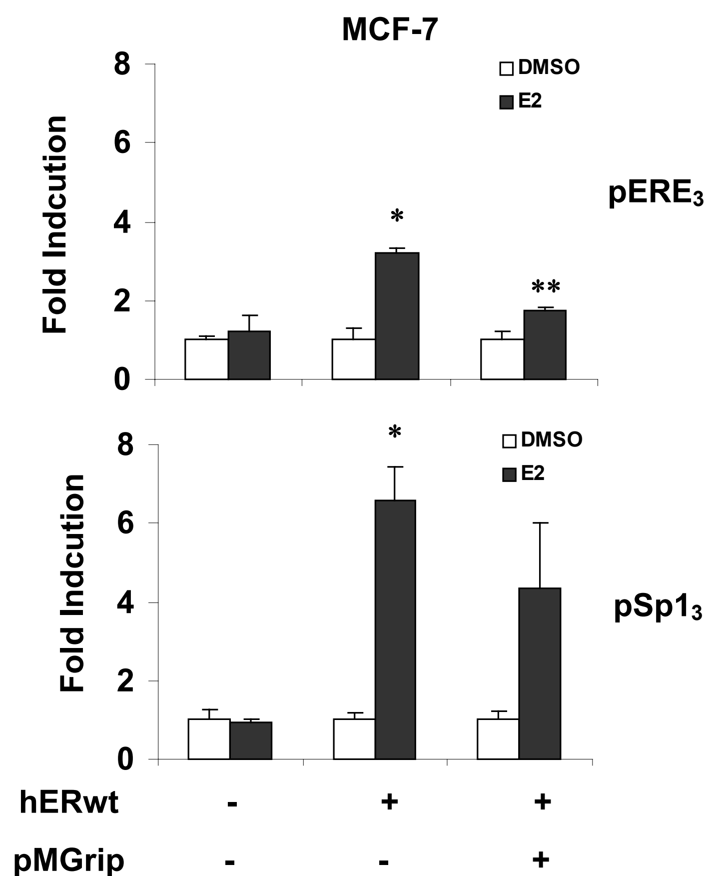


Fig. 29. Inhibition of transactivation by pMGAL4 fusion Grip peptide. MCF-7 cells were treated with DMSO or 10 nM E2, transfected with $pERE_3$ or $pSp1_3$ and different amounts of pMGrip expression plasmid, and luciferase activity was determined as described in Materials and Methods. Results are expressed as means ± SD for three replicate determinations for each treatment group, and significant ($P < 0.05$) inhibition of induced activity is indicated (**).

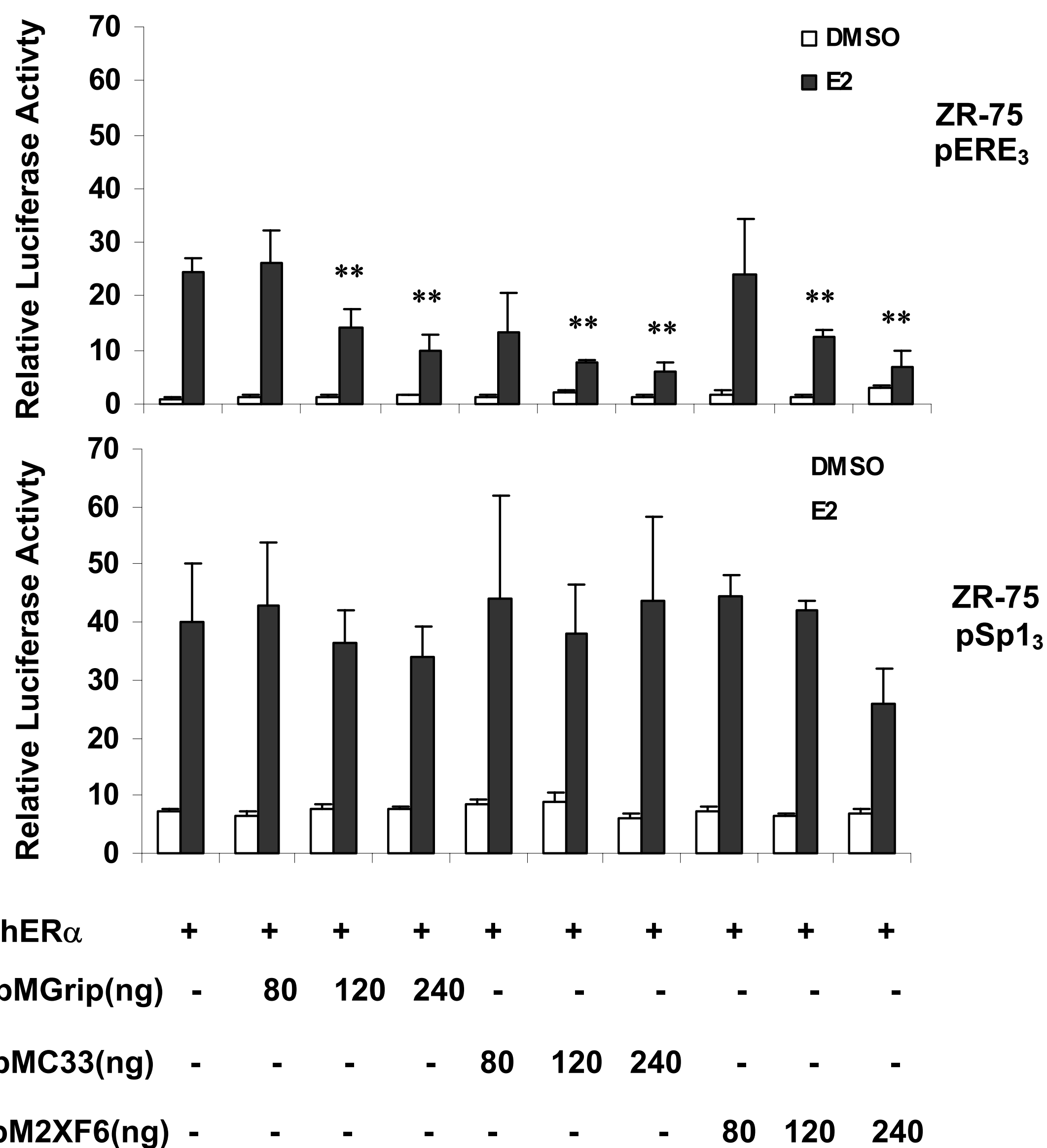


Fig. 30. Inhibition of transactivation by various pMGAL4-fusion peptides. MCF-7 cells were treated with DMSO or 10 nM E2, transfected with $pERE_3$ or $pSp1_3$ and different amounts of pMGAL4 fusion expression plasmid, and luciferase activity was determined as described in Materials and Methods. Results are expressed as means ± SD for three replicate determinations for each treatment group, and significant ($P < 0.05$) inhibition of induced activity is indicated (**).

Coactivators of hERα and other nuclear receptors have been extensively investigated, and these include AF2-dependent steroid receptor coactivators (SRCs) and AF1-dependent p68 RNA helicase (Kumar et al., 1987; Endoh et al., 1999). However, many of these coactivators have not been investigated in breast cancer cells, and their coactivation of hERα/Sp1 through LXXLL-dependent or -independent pathways have not been reported previously. The effects of selected coactivators on hERα/Sp1-dependent transactivation in breast cancer cells transfected with pSp13 and on hERα/ERE-dependent transactivation in Chinese hamster ovary (CHO) cells are summarized in Figures 32, 33, and 34. MCF-7 cells were treated with 10 nM E2, transfected with pSp13 and hERα, and different amounts (10, 50, and 100 ng) of expression plasmids for SRC-1, SRC-2 (glucocorticoid receptor interacting protein 1), SRC-3 (AIB1), and p68 RNA helicase, an AF1-dependent coactivator of ERα (on an ERE promoter; Endoh et al., 1999). E2 induced activity (~2-fold; Figs. 31 and 32); however, cotransfection with SRC-1, SRC-2, SRC-3, or p68 did not enhance activity in this cell line, and similar results were observed for the RNA coactivator SRA or p300 and for higher amounts (500 ng) of transfected coactivators (data not shown). A parallel experiment was carried out in MDA-MB-231 cells (Figure 33), and E2 induced activity (~2-fold), but cotransfection with SRC-1, SRC-2, SRC-3, and p68 did not enhance hERα/Sp1 action. Moreover, many of these coactivators significantly inhibited the induction response in breast cancer cells. As a positive control for coactivation, we also

investigated effects of p160 coactivators and p68 in CHO cells treated with 10 nM E2 and transfected with $pERE_3$ and hERα.

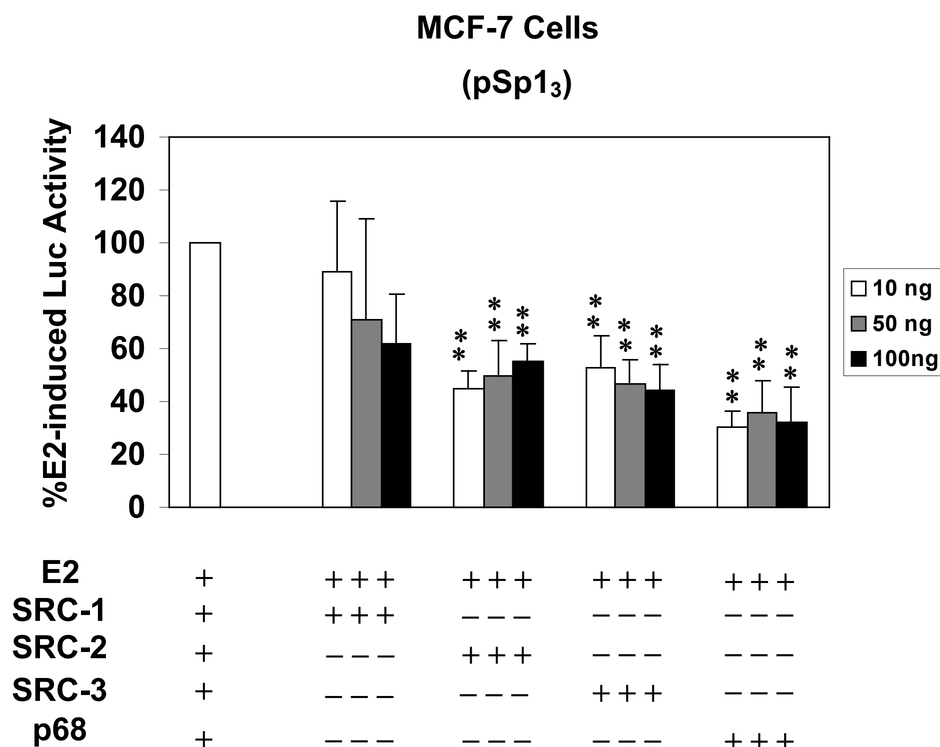


Fig. 31. Coactivator interactions with ERα/Sp1in MCF-7 cells. Cells were transfected with $pSp1_3$, hERα, and different amounts of coactivators SRC-1, SRC-2, SCR-3, and p68 RNA helicase (10, 50, or 100 ng), treated with E2, and luciferase activities were determined as described in Materials and Methods. Significant ($P < 0.05$) coactivation (*) or inhibition (**) of E2-induced activities are indicated; similar results were observed after transfecting higher amounts (500 ng) of each coactivator in both cell lines. Results are expressed as means ± SD for three separate experiments for each treatment group.

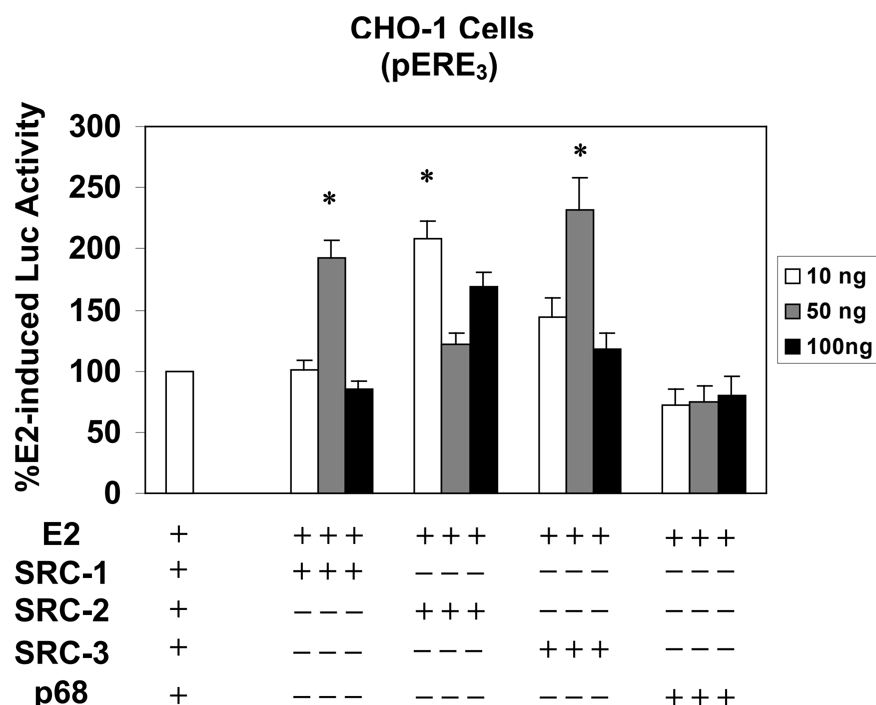


Fig. 32. Coactivator interactions with ERα/Sp1 in MDA-MB-231 cells. Cells were transfected with $pSp1_3$, hERα, and different amounts of coactivators SRC-1, SRC-2, SRC-3, and p68 RNA helicase (10, 50, or 100 ng), treated with E2, and luciferase activities were determined as described in Materials and Methods. Significant ($P < 0.05$) coactivation (*) or inhibition (**) of E2-induced activities are indicated; similar results were observed after transfecting higher amounts (500 ng) of each coactivator in both cell lines. Results are expressed as means ± SD for three separate experiments for each treatment group.

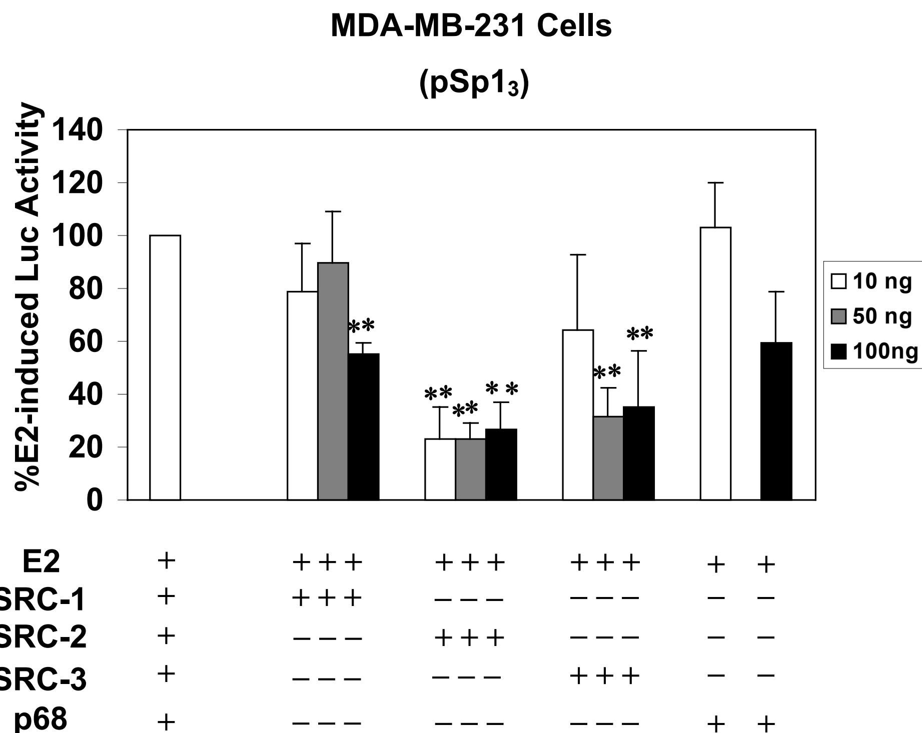


Fig. 33. Coactivation of hERα action in CHO cells transfected with pERE$_3$. Cells were transfected with pERE$_3$ and treated as described above. Significant ($P < 0.05$) coactivation of E2-induced activity is indicated (*). Results are expressed as means ± SD for three separate experiments for each treatment group.

This cell line has frequently been used by other investigators to demonstrate coactivation of hERα using ERE-dependent promoter-reporter constructs. E2 induced luciferase activity (8- to 15-fold) and SRC-1, SRC-2, and SRC-3 (but not p68) enhanced the induction response (Figure 33). Coactivation of hERα/Sp1 by SRCs was not observed in ER-positive or negative breast cancer cell lines; this was consistent with the importance of AF1 for hERα/Sp1-

mediated transactivation (Saville et al., 2000). Surprisingly, we did not observe coactivation of hERα or hERα /Sp1 by the AF1-interacting coactivator p68 in ER-negative or- positive cell lines, suggesting that cell context modulates the effects of p68 as a coactivator.

3.7 Role of the AF1 domain in ERα/Sp1 action

It has been recognized that ERα is subject to phosphorylation, which regulates its transcriptional activity. Phosphorylation of serines 104 and 106 in the AF1 domain by cyclin A/CDK2 complex potentiates hERα-dependent transcriptional activity (Rogatsky et al., 1999) and serine 118 is phosphorylated by MAP kinase (Kato et al., 1995). AKT and p90RSK1 kinases catalyze phosphorylation of serine 167 and regulate AF1-dependent transcriptional activation (Joel et al., 1998; Campbell et al., 2001).

To assess the role of these phosphorylation events in hERα/Sp1-mediated transactivation, hERα mutant containing point mutation(s) on the phosphorylation sites in the AF1 domain were transfected into ER-negative MDA-MB-231 cells along with the $pERE_3$ or $pSp1_3$ constructs. Estrogen induces luciferase activity in MDA-MB-231 cells transfected with $pERE_3$, and hERα wt or hERα mutants containing point mutation in AF1, but not with hERα15c that contains complete deletion of AF1. Increased level of basal transcription were observed in cells transfected with S104A, S106A, S118A, and S167A mutants.

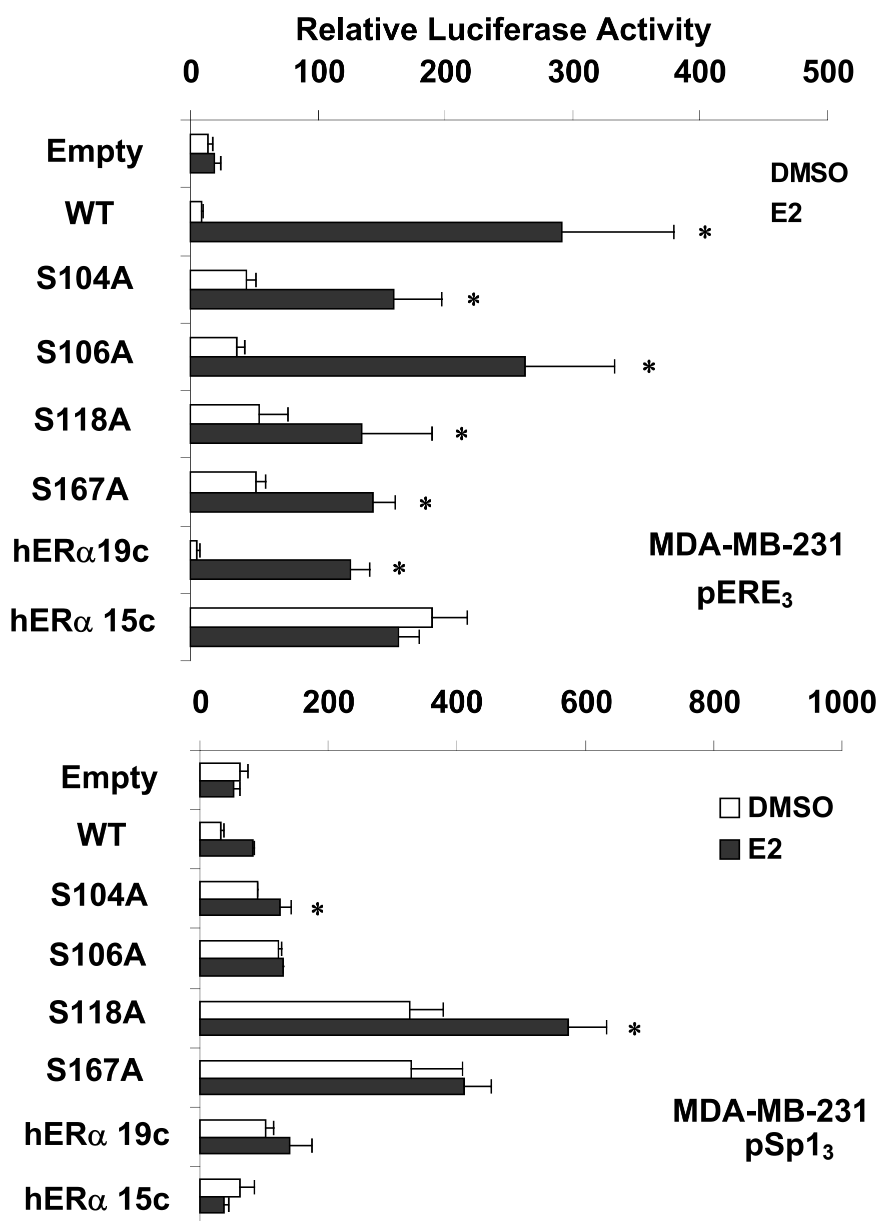


Fig. 34. Effects of hERα point and deletion on ERα/Sp1 action. MDA-MB-231 cells were treated with DMSO or 10 nM E2, transfected with pERE3 (top) or $pSp1_3$ (bottom) and luciferase activity was determined as described in Materials and Methods. Results are expressed as means ± SD for three replicate determinations for each treatment group, and significant ($P < 0.05$) induction is indicated (*).

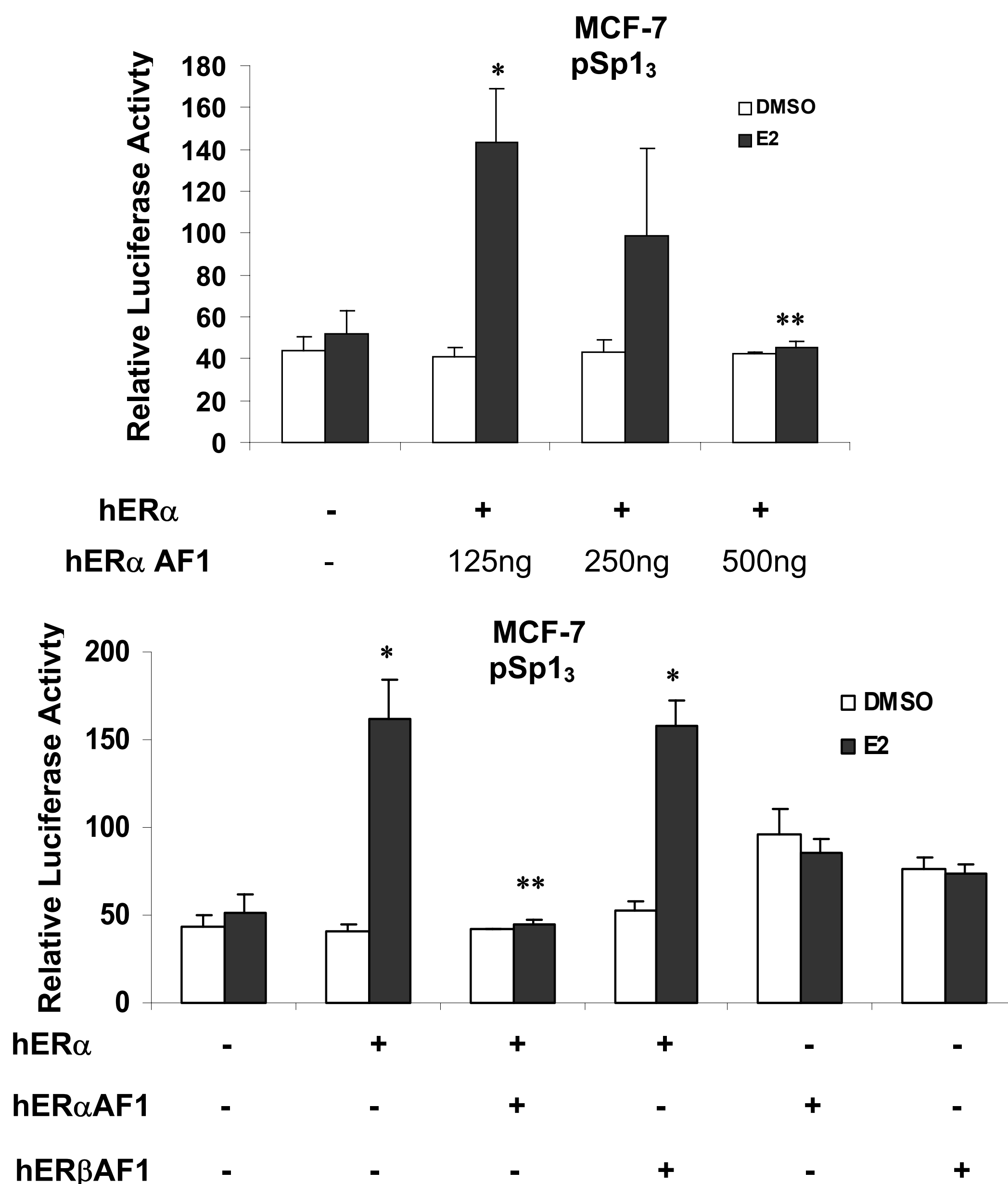


Fig. 35. Effects of hERα point mutations in the AF1 domain on ERα/Sp1 action in MCF-7 cells. Cells were treated with DMSO or 10 nM E2, transfected with pSp13 and luciferase activity was determined as described in Materials and Methods. Results are expressed as means ± SD for three replicate determinations for each treatment group, and significant ($P < 0.05$) induction is indicated (*).

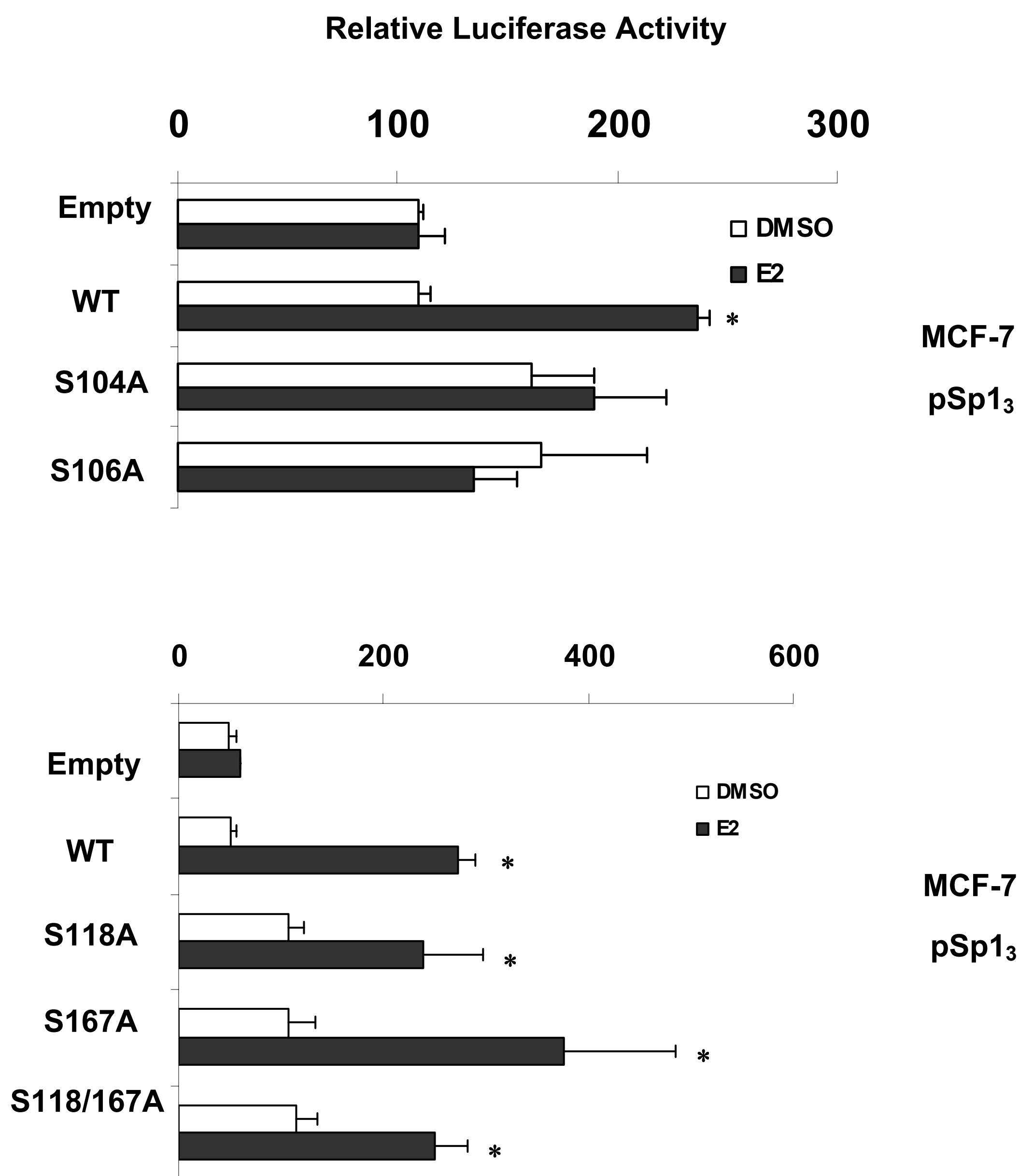


Fig. 36. Effect of hERα AF1 or hERβ AF1 peptide in hERα/Sp1-mediated transactivation. Cells were treated with DMSO or 10 nM E2, transfected with pSp1$_3$ and hERα AF1(1-182aa) or hERβ AF1 (1-98aa) and luciferase activity was determined as described in Materials and Methods. Results are expressed as means ± SD for three replicate determinations for each treatment group, and significant ($P < 0.05$) induction is indicated (*) or inhibition (**).

In addition, hERα 15c mutant constitutively activated $pERE_3$ reporter gene activity in ligand-independent manner (Fig. 34). In contrast, estrogen only activated luciferase activity in cells transfected with $pSp1_3$ and wild type hERα or S118A. Higher basal activities have also been observed in cells transfected with S104A, S106A, S118A, and S167A mutant (Fig. 34).

We have also tested the transcriptional activity of these mutants in MCF-7 cells under the same experimental condition. Consistently, estrogen did not induce luciferase activity in MCF-7 cells transfected with $pSp1_3$ and S104 A or S106A whereas significant induction was observed in cells transfected with S118A, S167A, and S118A/S167A mutant containing double point mutations (Fig. 35).

To further assess the role of the AF1 domain of hERα in hERα/Sp1-mediated transactivation, hERαAF1 (1-182aa) peptide was generated and increasingly overexpressed in cells transfected with $pSp1_3$ and wild type hERα. Overexpression of the AF1 peptide inhibited luciferase activity in cells transfected with $pSp1_3$ and wild type hERα whereas hERβ AF1 peptide (1-98aa) did not exhibit any inhibitory effect in hERα/Sp1-mediated transactivation (Fig. 36). These results summarized in Figs. 34-36 confirm the important role of the AF1 domain of hERα in ERα/Sp1 action.

3.8 Role of AF2/Hinge (DEF) region for activation of hERα/Sp1 by estrogen and antiestrogens

The requirements for other regions within the DEF domains of hERα for activation of hERα/Sp1 by estrogens and antiestrogens have also been investigated in MCF-7 cells. E2, 4-OHT, and ICI 182,780 did not induce luciferase activity in MCF-7 cells transfected with $pSp1_3$, whereas a 2.5- to 4.5-fold induction was observed by all three compounds in cells cotransfected with hERα (Fig. 37). E2 did not induce activity in MCF-7 cells transfected with hERα (Δ271–300) or hERα (Δ265–330), which contain deletions of the hinge (D) or hinge (D) plus helix 1 of the E domain. ICI 182,780 and 4-OHT were also inactive in cells transfected with hERα (Δ265–330), whereas induction by the antiestrogens was observed in cells transfected with hERα (Δ271–300). Estrogen/antiestrogen-dependent activation of hERα/Sp1 was also investigated in MCF-7 cells transfected with a series of C-terminal deletion mutants, namely hERα (Δ538–595), hERα (Δ554–595), and hERα (Δ579–595). These mutants contain deletions of helix 12 (E) and the C-terminal F domain (538–595), the F domain (554–595) alone, and the β-strand region of the F domain (579–595). In MCF-7 cells, both 4-OHT and ICI 182,780 induced luciferase activity in cells transfected with these hERα deletion mutants, whereas E2 was inactive. The failure of E2 to induce transactivation in cells transfected with $pSp1_3$ and hERα (Δ579–595) suggests that the C-terminal aa 579–595, which contains a

QKYYIT β-strand motif (Schwartz et al., 2002), may be critical for transcriptional activation by E2 but not 4-OHT or ICI 182,780.

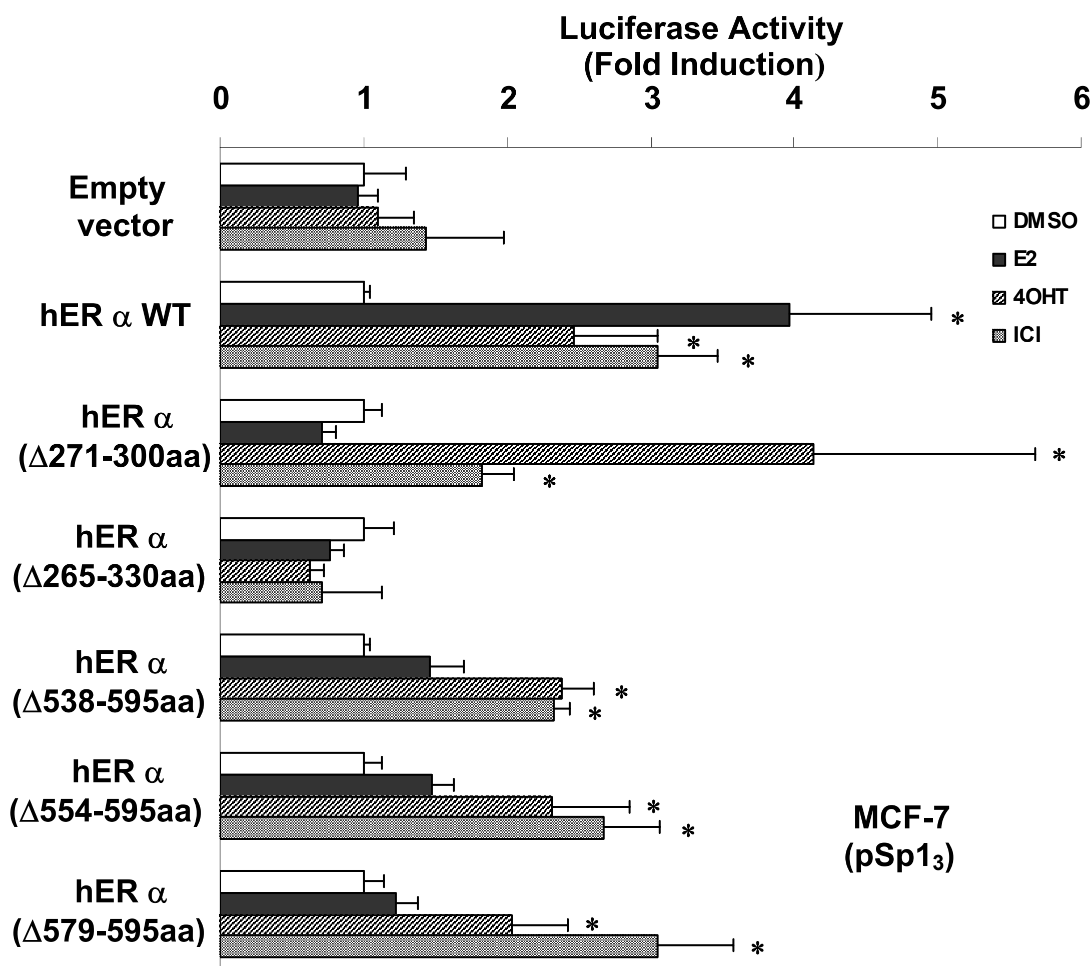


Fig. 37. Effects of DEF domain mutants of hERα on hormone and antiestrogen-Induced transactivation. MCF-7 cells were transfected with pSp13 and wild-type or variant hERα, and induction of luciferase activity by 10 nM E2, 1 μM 4-OHT, or 1 μM ICI 182,780 was determined as described in Materials and Methods. Significant ($P < 0.05$) induction is indicated by an asterisk.

transactivation in cells cotransfected with $pERE_3$ and hERα (Δ271–300) or

We further confirmed the F domain requirement for hormonal activation of hERα/Sp1 by examining a similar series of hERα deletion mutants in MDA-MB-231 cells cotransfected with $pSp1_3$ or $pERE_3$ (Fig. 38). E2 induced hERα (Δ554–595), confirming results of previous studies in other cell lines showing that the hinge region and F domain are not necessary for hormonal activation of

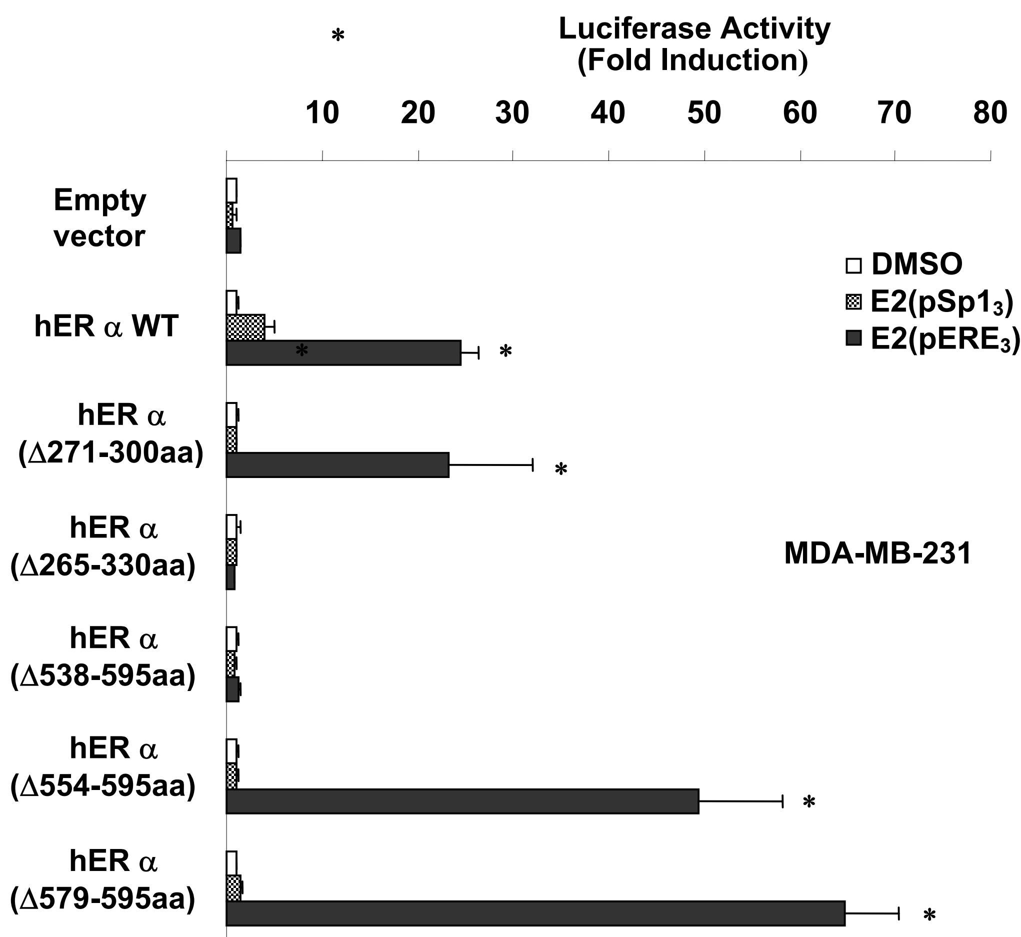


Fig. 38. Hormonal activation of $pERE_3$ or $pSp1_3$ in MDA-MB-231 cells. Cells were treated with DMSO or 10 nM E2, transfected with wild-type or variant hERα and $pERE_3$ or $pSp1_3$, and luciferase activity was determined as described in Materials and Methods. Significant ($P < 0.05$) induction is indicated by an asterisk.

ERα/pERE (Kumar et al., 1987; Schwart etal., 2002; Nichols et al., 1998). E2 did not induce transactivation in MCF-7 or MDA-MB-231 cells cotransfected with $pSp1_3$ and hERα (Δ554–595). Thus, hormonal activation of hERα/Sp1 by E2 was dependent on the hinge (D) and F domains of hERα, whereas these same regions of hERα were not required for activation of $pERE_3$. Peptides targeted to different regions of hERα block hormone-induced transactivation of ERE-dependent promoters/genes (Chang et al., 1999a; Schaufele, et al., 2000). This has been extensively investigated with peptides containing LXXLL motifs that block coactivator interactions with ERα (Chang et al., 1999a;Schaufele, et al., 2000) and inhibit hormone-induced activation in cells
transfected with $pERE_3$ (Fig. 39). Hormone-induced transactivation in MDA-MB-231 cells transfected with $pERE_3$ was not significantly decreased after cotransfection with the Fβ strand peptide containing aa575–595 from the F domain of hERα fused to the DBD of the yeast GAL4protein. In contrast, the F domain peptide blocked hormone-induced transactivation in MDA-MB-231 cells transfected with $pSp1_3$, whereas the 2XF6 peptide was inactive, and similar results were obtained with other peptides containing LXXLL sequences (data not shown). These results are consistent with the activity of wild-type and variant hERα constructs and confirm that the F domain of hERα is also essential for E2-dependent activation of hERα/Sp1.

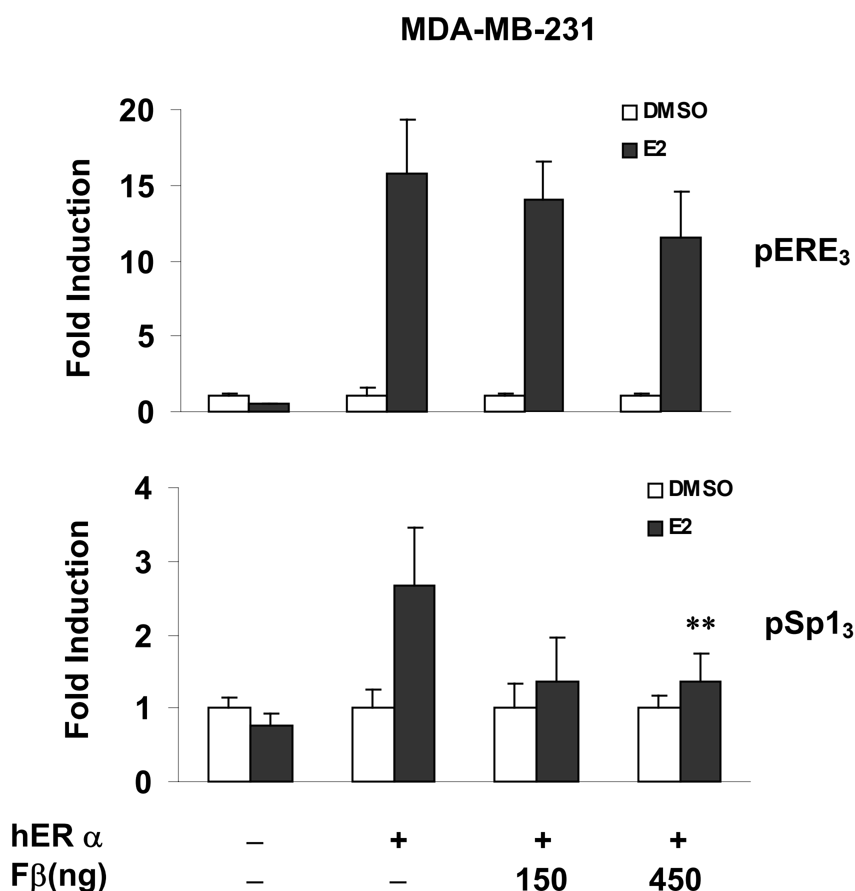


Fig. 39. Inhibition of transactivation by Fβ peptide. MDA-MB-231 cells were transfected with $pERE_3$ or $pSp1_3$, treated with DMSO or 10 nM E2, and cotransfected with Fβ (F domain) peptide, and luciferase activity was determined as described in Materials and Methods. Significant ($P < 0.05$) decreases in hormone-induced activity by Fβ peptide is indicated (**).

3.9 Screening of putative coactivator of ERα/Sp1

In order to identify putative coactivators for ERα/Sp1-mediated transactivation, several coactivators reported to enhance receptor-mediated transactivation have been tested in cells transfected with $pSp1_3$, wild type hERα, and increasing amounts of transfected-coactivator expression plasmid.

It has been reported that the cyclin A/cdk2 complex phosphorylated serines 104 and 106 in the AF1 domain of hERα and these modifications enhanced transcriptional activity of hERα independent of AF2 function (Trowbridge et al., 1997 and Rogatsky et al., 1999). In addition, cyclin A/cdk2 and cyclin E/cdk2 complexes also phosphorylated glucocorticoid receptor to enhance its transcriptional activity (Krstic et al., 1997). The results shown in Fig. 40 indicated that overexpression cyclinA or cyclin E did not enhance transcriptional activity in MDA-MB-231 cells transfected with $pSp1_3$ and hERα; basal transcriptional activity was increased by cyclin A and E expression and this decreased the overall fold induction level.

Ets-1 transcriptional factor, generally known as a target of MAP kinase signaling, exhibited AF2-independent coactivation of several nuclear receptor (Tolon et al., 2000). Both MCF-7 and ZR-75 cells were transfected with $pSp1_3$ and increasing amounts of transfected Ets-1 expression plasmid. Increased amount of Ets-1 expression enhanced both the basal and E2-induced reporter gene activity in cells transfected with $pSp1_3$ and hERα without any significant changes in overall fold-induction (Fig. 41).

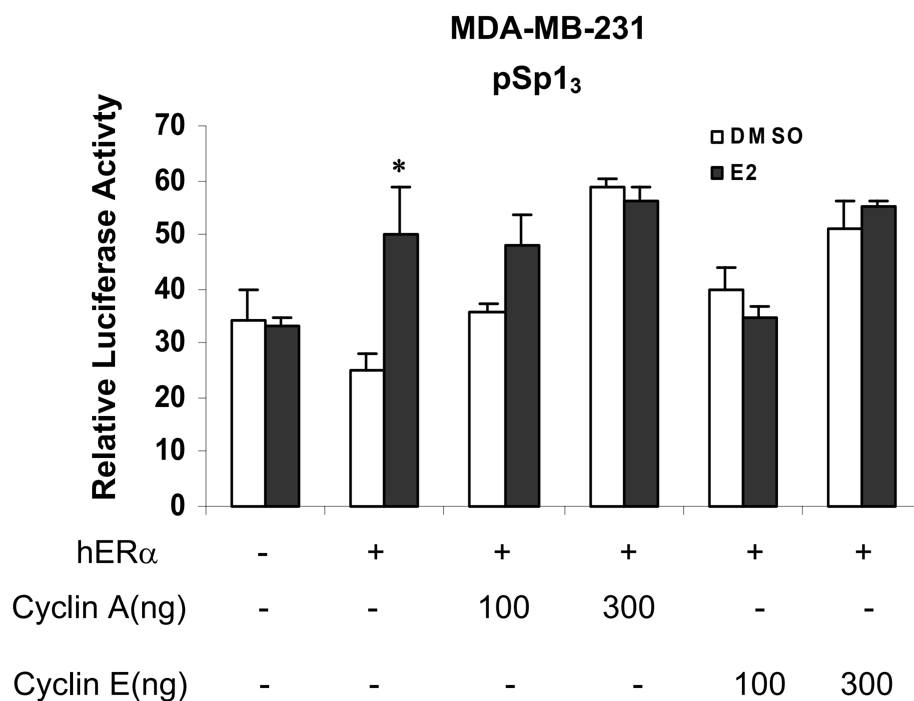


Fig. 40. Coactivation of hERα/Sp1-mediated transactivation by cyclin A or cyclin E. MDA-MB-231 cells were transfected with pSp1$_3$, hERαexpression plasmid and increasing amount of cyclin A or cyclin E expression plasmid and luciferase activity was determined as described in Materials and Methods. Significant ($P < 0.05$) induction is indicated by an asterisk.

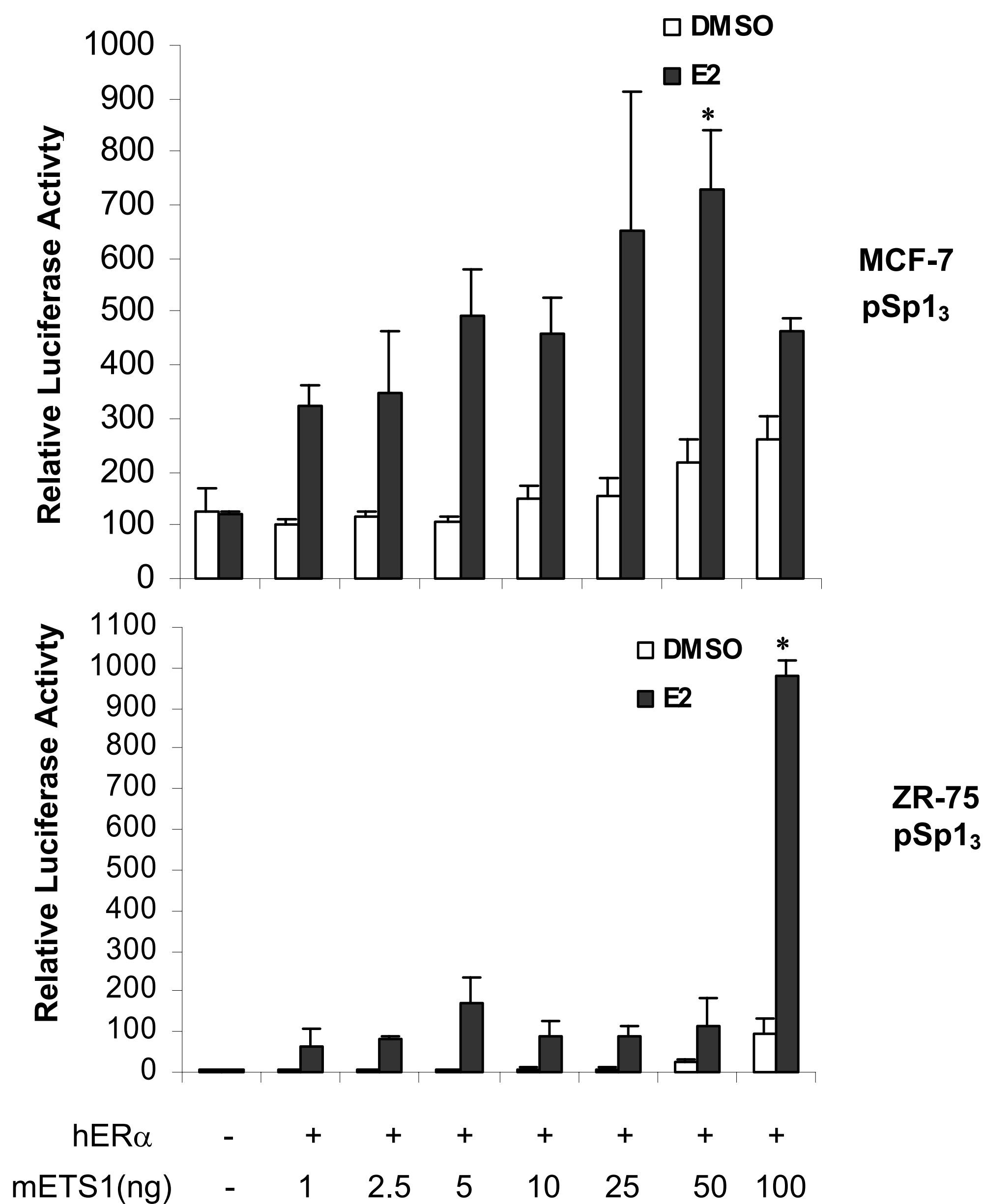


Fig. 41. Coactivation of hERα/Sp1-mediated transactivation by mETS-1. MCF-7 and ZR-75 cells were transfected with pSp1_3, hERαexpression plasmid, and increasing amount of mouse ETS-1 expression plasmid and luciferase activity was determined as described in Materials and Methods. Significant ($P < 0.05$) induction is indicated by an asterisk.

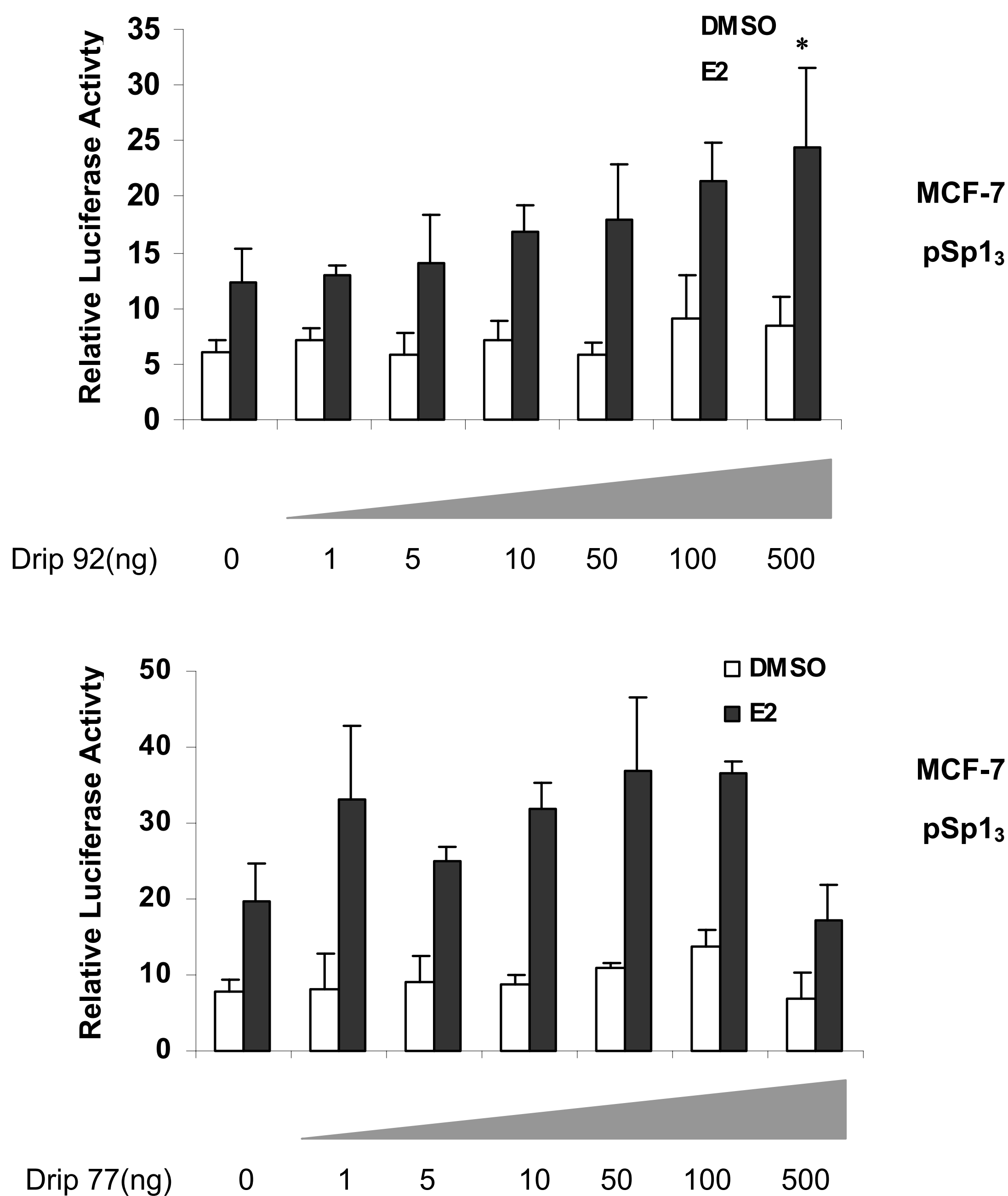


Fig. 42. Coactivation of hERα/Sp1-mediated transactivation by Drip proteins. MCF-7 cells were transfected with pSp1_3, hERαexpression plasmid, and increasing amount of Drp92 or Drip72 expression plasmid and luciferase activity was determined as described in Materials and Methods. Significant ($P < 0.05$) induction is indicated by an asterisk.

DRIPs were first identified as Vitamin D receptor interacting proteins which coactivate several nuclear receptors in cell culture and in cell free system (Rachez et al., 1999). To assess the role of DRIPs in ERα/Sp1-mediated transactivation, two DRIP proteins, Drip 92 and Drip 77, have been investigated. Drip 92 slightly increased E2-induced reporter gene activity with the highest amount of transfected plasmid (500ng). However, Drip72 did not enhance the luciferase activity under the same conditions. The results shown in Fig. 42 suggest that Drip92 possibly acts as weak coactivator of ERα/Sp1-mediated transactivation whereas Drip72 did not exhibit coactivator activity.

3.10 Detection of direct physical interactions between ERα and Sp1 proteins in vivo by FRET

To investigate ERα/Sp1 protein-protein interactions in vivo, we first generated various CFP and YFP fusion constructs expressing CFP-YFP chimera, CFP-hERα, YFP-hERα, and CFP-Sp1 (Fig. 43). First, transcriptional activities of CFP-hERα and YFP-hERα were assayed in a transient transfection system. ER-negative MDA-MB-231 cells were transfected with $pERE_3$ or $pSp1_3$ along with CFP-hERα, YFP-hERα or unfused hERα. Both CFP-hERα and YFP-hERα were active and the levels of E2-induced transactivation between unfused hERα and fused hERα were similar in cells transfected with $pERE_3$ or $pSp1_3$ (Fig. 44).

Overexpression of CFP-Sp1 alone increased the basal level of transactivation in cells transfected with $pSp1_3$ and this increase was reduced by cotransfection of dominant negative Sp1 (Sp1DN) expression plasmid (Fig. 45 (top)). When cells were cotransfected with CFP-Sp1 and YFP-hERα, both basal and E2-induced level was significantly increased without changing the fold-induction (Fig. 45 (bottom)). Thus, CFP-hERα, YFP-hERα, and CFP-Sp1 fusion proteins are transcriptionally active and give results comparable to those obtained with hERα or Sp1 proteins.

To establish FRET in our system, the CFP-YFP chimera was generated and used as a positive control. Cotransfection of CFP empty and YFP empty was used as negative control. Strong FRET signal from cells transfected with CFP-YFP chimera construct whereas a minimal FRET signal was detected from cells transfected with CFP empty and YFP empty constructs (Fig. 46).

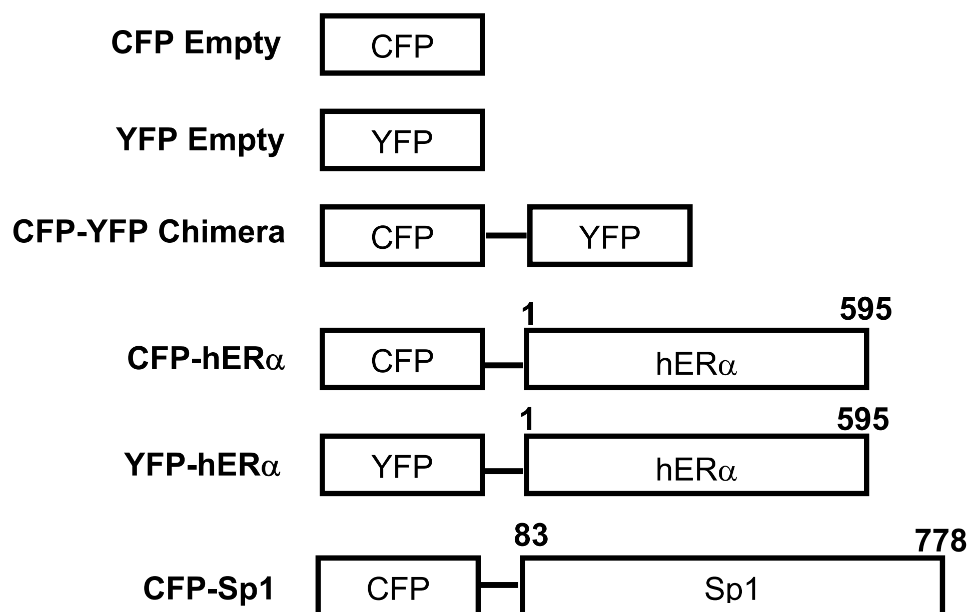


Fig. 43. CFP and YFP fusion proteins used for FRET studies.

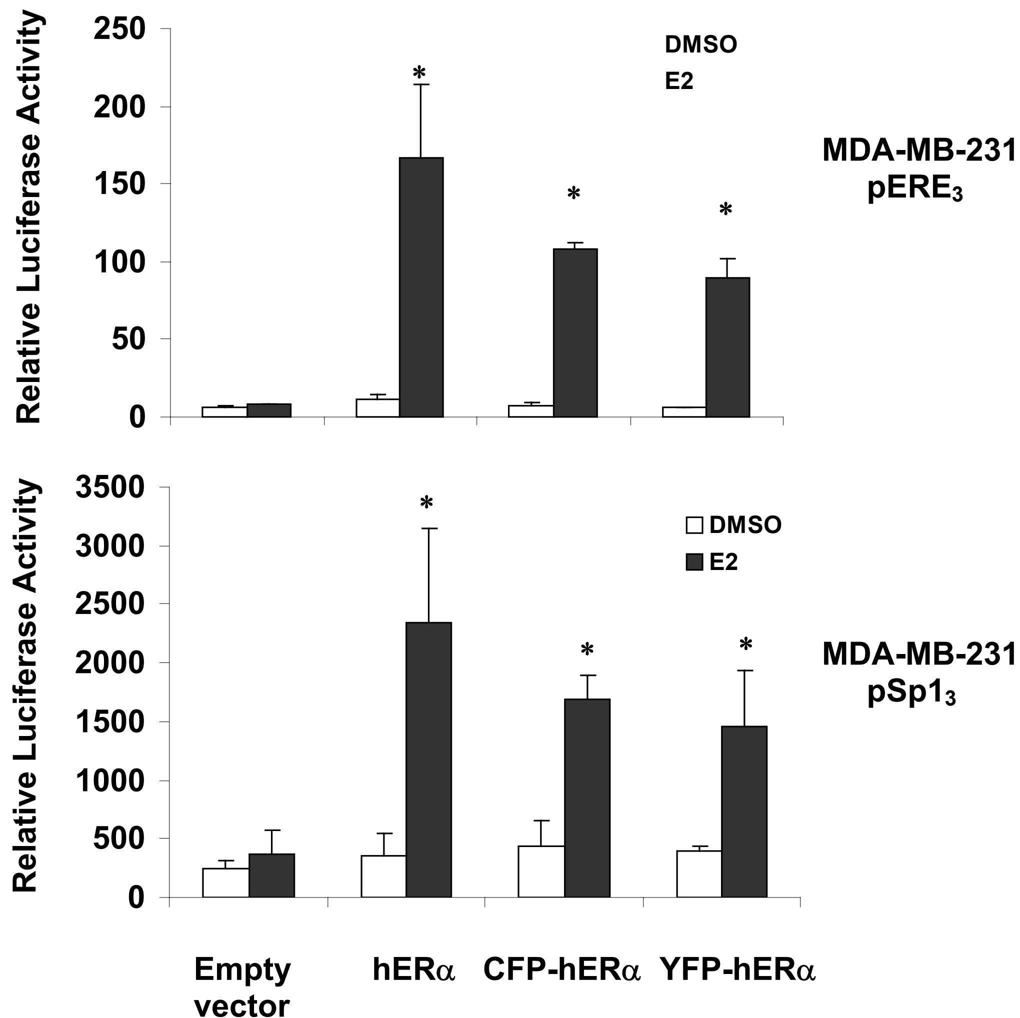


Fig. 44. Effects of CFP-hERα and YFP-hERα on activation of $pSp1_3$ and $pERE_3$. MDA-MB-231 cell were cotransfected with $pSp1_3$ or $pERE_3$ and CFP or YFP fusion construct and treated with DMSO or 10 nM E2. Luciferase activities were determined as described in Materials and Methods. Significant ($P < 0.05$) induction (*) is indicated.

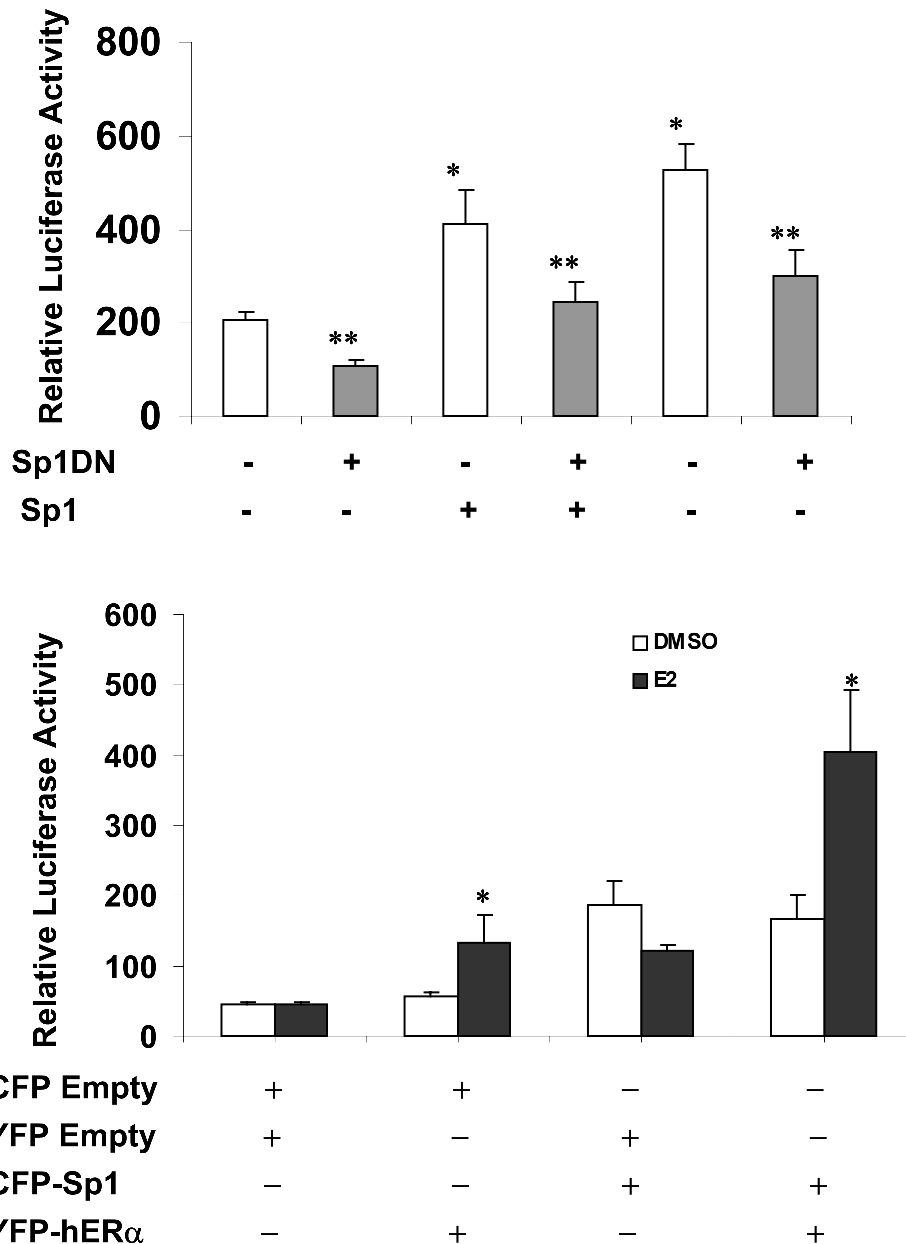


Fig. 45. Effects of CFP-Sp1 and YFP-hERα on activation of $pSp1_3$. ZR-75 cells were transfected with unfused Sp1 construct and Sp1 dominant negative (Sp1DN) or CFP-Sp1 and Sp1DN (top). Cells were treated with DMSO or 10 nM E2 after transfection with $pSp1_3$ and CFP-Sp1, YFP-hERα or both fusion constructs (bottom). Luciferase activities were determined as described in Materials and Methods. Significant ($P < 0.05$) induction (*) of activity by E2 or inhibition of this activity (**) is indicated.

After subtraction of background signal, the ratio of positive control FRET signal to negative FRET signal is ≅ 2.01 where it is assume that FRET Efficiency is equal to 50% maximum and used as a standard for further calculation of FRET efficiency.

In order to detect ligand-dependent protein-protein interactions, cells were transfected with CFP-hERα and YFP-hERα constructs and then images were acquired under the same conditions for a negative and a positive control. After treatment with E2 for 8 min, translocation of CFP-hERα and YFP-hERα into the nucleus was observed and acquired images showed stronger FRET signal in E2-treated cells when compared to DMSO-treated cells (Fig. 47).

Based on the FRET conditions established above, ligand-dependent CFP-hERα/YFP-hERα and CFP-Sp1/YFP-hERα interactions were analyzed and FRET efficiency was calculated for each treatment. The results (Fig. 48) indicated that E2, 4OHT, and ICI treatment increased FRET efficiency in cells transfected with CFP-hERα and YFP-hERα. The highest FRET efficency was observed after treatment with 4OHT and the order of FRET efficiency was 4OHT > E2 > ICI.

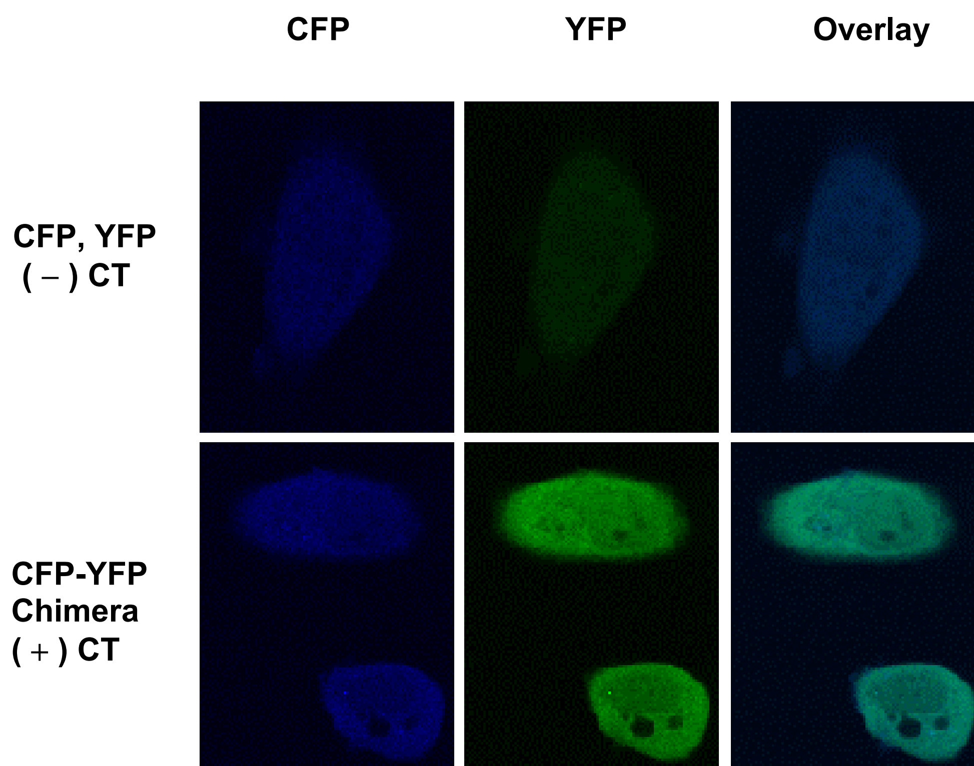


Fig. 46. Visualization of FRET in MCF-7 cells. Cells were transfected with CFP and YFP empty constructs (top) as a negative control or CFP-YFP chimera fusion construct as a positive control (bottom). The higher FRET signal was observed from cells transfected with CFP-YFP chimera construct. The conditions for acquiring images were described in Materials and Methods.

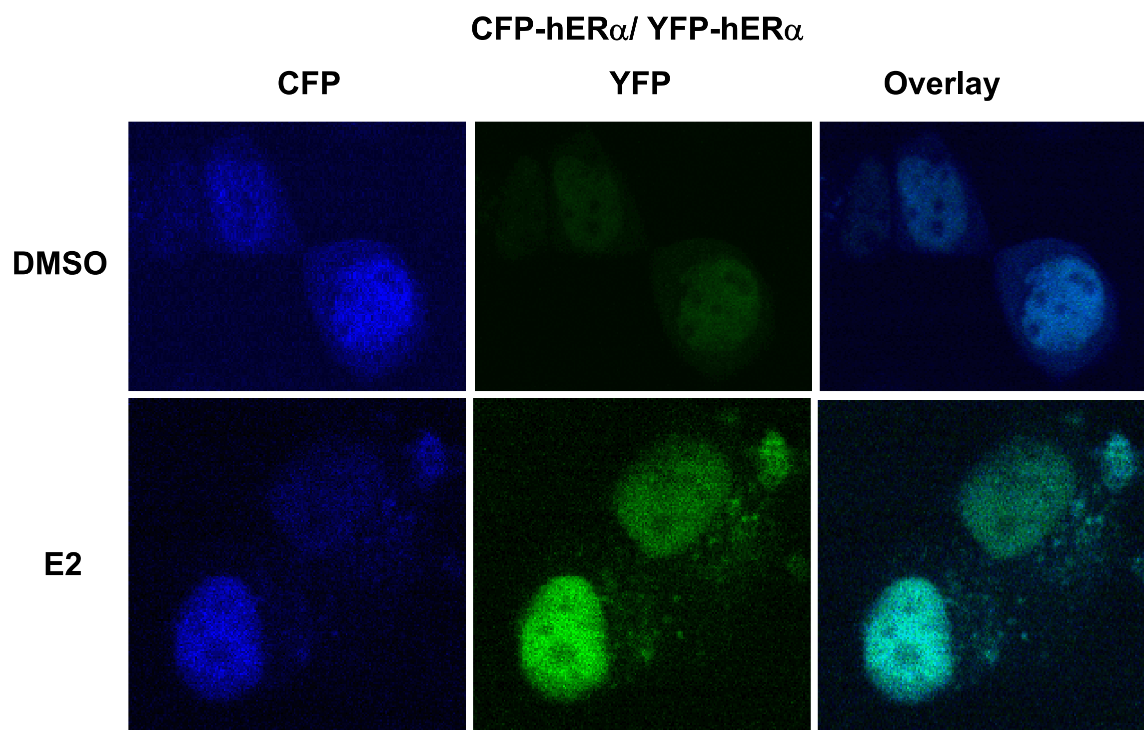


Fig. 47. Representative FRET images from cells transfected with CFP-hERα and YFP-hERα. Images were acquired 8 mins after treatment with DMSO or E2 (10 nM). The colocalization of CFP-hERα and YFP-hERα and translocation of fusion proteins to the nucleus after treatment with E2 were observed. The higher FRET signal was detected from cells treated with E2. The conditions for acquiring images were described in Materials and Methods.

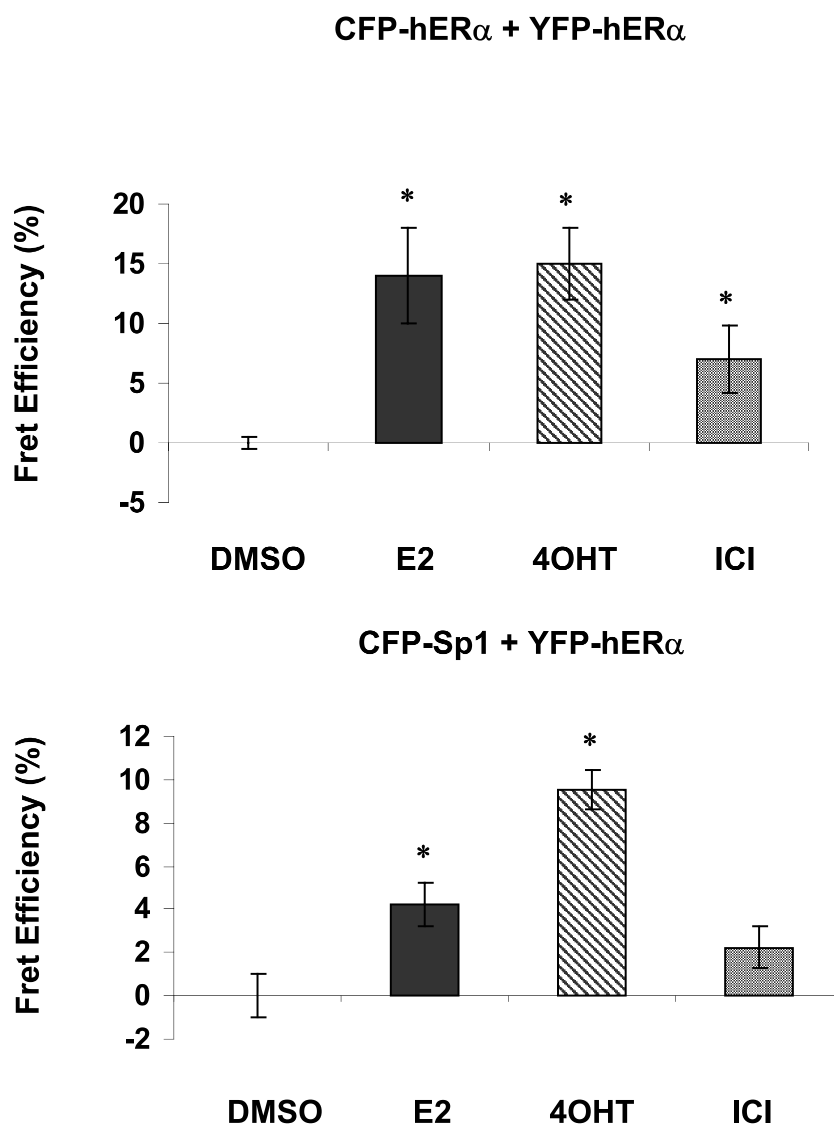


Fig. 48. FRET efficiency of CFP-hERα/YFP-hERα and CFP-Sp1/YFP-hERα. MCF-7 cells were transfected with each CFP/YFP fusion construct set. Images were acquired between 8-18mins after each ligand treatment. 10-15 images were acquired per treatment and each image contains 1-5 cells to be analyzed. The subtraction of background signal from the images was described in Materials and Methods.

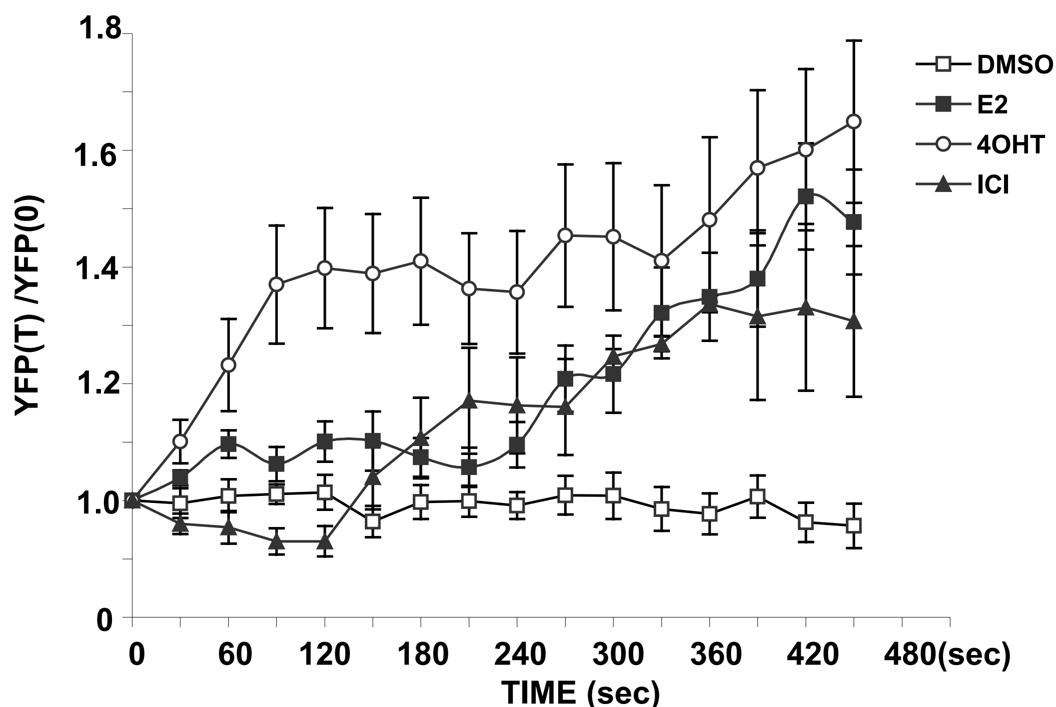


Fig. 49. YFP intensity (YFP(t)/YFP(0)) changes over time following ligand treatment. Images were acquired every 30 sec after each ligand was added. Data represents values from at least 13 cells per treatment.

However, in cells transfected with CFP-Sp1 and YFP-hERα, E2 and 4OHT except ICI significantly increased FRET efficiency and the order of FRET efficiency is 4OHT> E2 > ICI. Furthermore, after ligand treatment, YFP signal intensity changed over time and was measured to confirm ligand-dependent protein interactions between hERα and Sp1. As shown in Fig. 49, DMSO treatment did not change YFP intensity over time. However, E2, 4OHT, and ICI treatments increased YFP intensity.

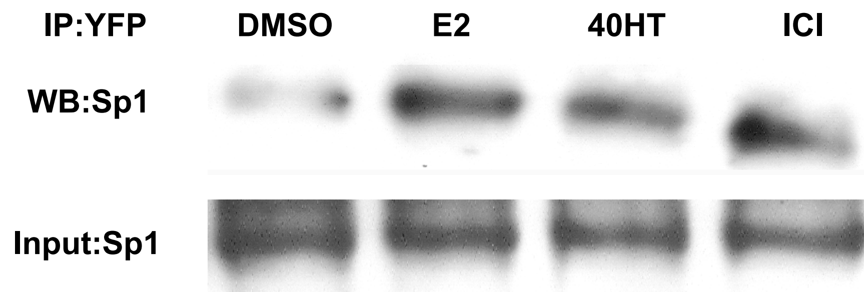
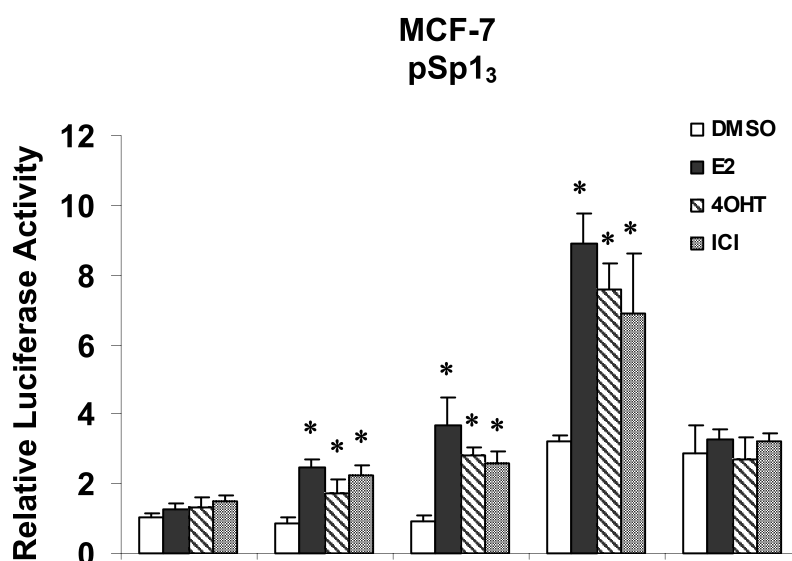


Fig. 50. Coimmunoprecipitation of ligand-dependent hERα/Sp1 protein complex. Whole cell extracts from cells transfected with YFP-hERα and Sp1 were isolated and immunoprecipitated with anti-YFP antibody and the cell extracts were analyzed for immunoreactive Sp1 protein by Western blot analysis with Sp1 antibody described in Materials and Methods.

Consistent with the previous observation, 4OHT induced the most significant changes in YFP intensity and the order of YFP intensity change was followed by E2 and ICI, respectively. Thus, these experiments suggest that ERα interactions with Sp1 interactions are enhanced in ligand-dependent manner in living cells. ERα/Sp1 interactions were not only investigated by FRET analysis but also by coimmunoprecipitation. MCF-7 cells were transfected with YFP-hERα and Sp1 expression plasmids and their ligand-dependent interactions were investigated Immunoprecipitation of whole cell lysate with anti-YFP antibody, followed by Western blot analysis with Sp1 antibodies. As shown in Fig. 50, all ligands, including E2, 4OHT, and ICI, enhanced hERα/Sp1 interactions in ligand-dependent manner and these results are consistent with the FRET data, showing interactions of these proteins In addition, coexpression of YFP-hERα and CFP-Sp1 expression plasmid significantly increased both the basal and estrogen- or antiestrogen-induced luciferase activities in cells

transfected with $pSp1_3$ compared to the luciferase activity in cells transfected with only CFP-Sp1, YFP-hERα, or hERα (Fig. 51). These results also suggest that Sp1 is involved in both in estrogen- and in antiestrogen-induced ERα/Sp1 action.



CFPSp1	–	–	–	+	+
hERα	–	+	–	–	–
YFPhERα	–	–	+	+	–

Fig. 51. Effects of CFP-Sp1 and YFP-hERα on antiestrogen-induced activation of $pSp1_3$ in MCF-7 cells. Cells were transfected with hERα, CFP-Sp1, YFP-hERα or both fusion constructs along with $pSp1_3$ and then treated with 10 nM E2, 1 μM 4-OHT, 1 μM ICI 182,780 (ICI). Luciferase activities were determined as described in Materials and Methods. Significant ($P < 0.05$) induction (*) was indicated.

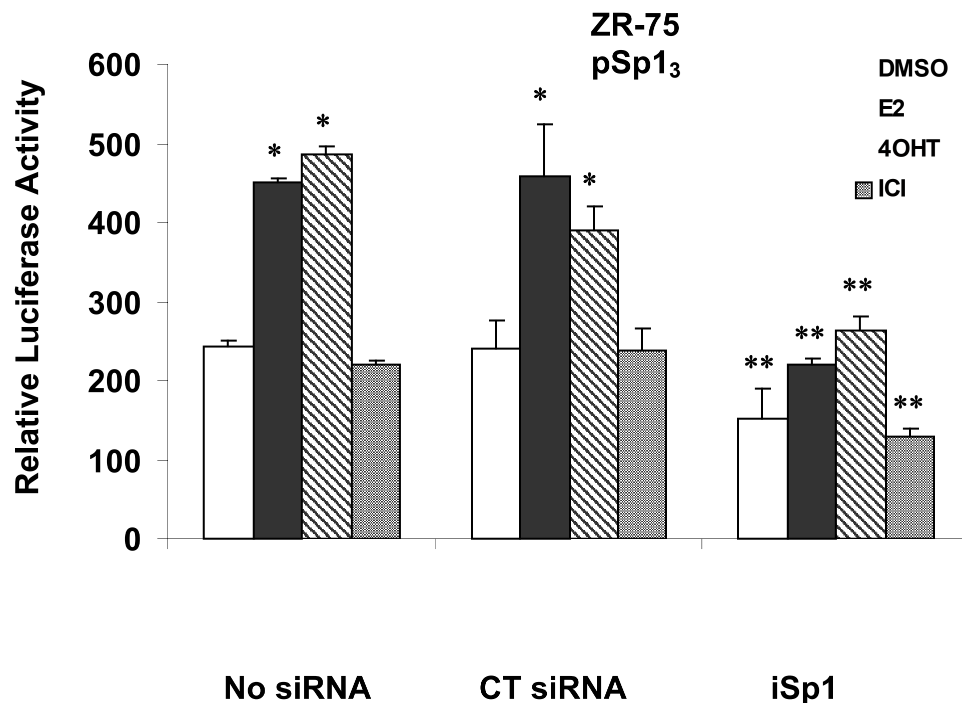


Fig. 52. Effects of siRNA for Sp1 (iSp1) and control scrambled siRNA (CT siRNA) on luciferase activity in MCF-7 cells. Cells were transfected with pSp13 and treated with 50 nM E2, 1 μM 4-OHT, 1 μM ICI 182,780 (ICI). Luciferase activity was determined as described under Materials and Methods. Significant ($p < 0.05$) induction by E2 is indicated by an asterisk and inhibition of the induced responses is also indicated(**).

Finally, the role of Sp1 in ERα/Sp1 action was further investigated by using small interfering RNA (siRNA) for Sp1 protein. To achieve the highest level of induction in the absence of exogenous hERα expression, ZR-75 cells were transfected with $pSp1_3$ along with scrambled siRNA (CT siRNA), Sp1 siRNA (iSp1) or without siRNA (CT) by using LipofectAMINE Plus Reagent. The results (Fig. 52) show that E2 or 4OHT significantly induced luciferase activity whereas luciferase activity induced by ICI was not observed in cells transfected with CT siRNA or CT. However, iSp1 transfected cells significantly decreased

E2- or 4OHT-induced luciferase activity, suggesting the critical role of endogeneous Sp1 protein in ERα/Sp1 action.

CHAPTER IV

DISCUSSION AND CONCLUSIONS*

4.1 The role of zinc finger domain in ERα/Sp1 and ERα/AP1-mediated transactivation

Development of selective ER modulators (SERMs) for treatment of breast cancer and other hormone-related problems is dependent on their tissue-specific activation or inhibition of ER-mediated genes/responses (Mcdonnell and Norris, 2002; Smith and O'Malley, 1999; Jordan, 2001; Fuqua et al., 2001; Krishnan et al., 2000). There are an increasing number of factors that regulate cell context-dependent ER action, and these include relative expression of ER subtypes and a complex network of nuclear proteins that uniquely interact with specific surfaces or domains of ERα, ERβ, and other coregulatory proteins (Horwitz et al., 1996; Glass et al., 1997; Edwards, 2000; McKenna et al., 1999; Robyr et al., 2000., Lemon and Freedman, 1999; Klinge, 2000). The classical mechanism of ER activation involves ligand-dependent formation of ER dimers that bind consensus or nonconsensus EREs and recruit SRCs and other nuclear proteins that facilitate interactions with basal transcription factors (Tsai and O'Malley, 1994; Beato et al., 1995; Mangelsdorf et al., 1995; Enmark and Gustafsson, 1996; Perlmann and Evans, 1997;

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Horwitz et al., 1996; Glass et al., 1997; Edwards, 1999; McKenna et al., 1999; Robyr et al., 2000., Lemon and Freedman, 1999; Klinge, 2000). In contrast, nonclassical pathways that involve ligand activation of ER/Sp1 and ER/AP1 do not require interactions of ER with promoter DNA but with other DNA-bound transcription factors, namely Sp1 and c-Jun, respectively. Research in this laboratory has identified a number of E2-responsive genes regulated by ERα/Sp1 in breast cancer cells (Porter et al., 1997; Sun et al., 1998; Duan et al., 1998; Qin et al., 1999; Dong et al., 1999; Xie et al, 1999; Xie et al., 2000; Castro-Rivera et al., 2001; Khan et al., 2003; Ngwenya and Safe, 2003; Stoner et al., 2000 and 2004). This suggests that the nonclassical pathways for activation of ERα are important in breast cancer cells and may play a significant role in cell context-dependent regulation of genes by E2 and SERMs.

For the last 10 years, not only ER but also many other nuclear receptors that interact with Sp family proteins have been identified. For examples, the progesterone receptor (PR) coimmunoprecitates with Sp1 and p300, and this PR/Sp1 complex is involved in the activation of p21 promoter (Owen et al., 1998). Androgen receptor also forms a complex with Sp1 to activate p21 promoter in LNCaP-FGC cells (Lu et al., 2000). Retinoic acid receptor heterodimer/Sp1 complex induces transglutaminase gene activation in bovine aorta endothelial cells (BACE) via GC-rich element in the gene promoter (Shimada et al., 2001). IkappaB alpha gene is activated via GC-rich sites by peroxisome proliferator-activated receptors α (PPARα) in a DNA binding-

independent manner (Delerive et al., 2002). The thromboxane receptor gene is transcriptionally suppressed by PPAR γ/Sp1 complex in vascular smooth muscle cells (Sugawara et al., 2002). Thus, biochemical interactions between NRs and Sp family proteins may play important roles in the expression of a variety of genes containing GC-rich sites on their promoters.

Previous studies showed that both estrogens and antiestrogens activated a construct containing a GC-rich promoter (pSp1) in breast cancer cells and this response was AF1 dependent (Saville et al., 2000). Moreover, the DBD of hERα was not required for activation by E2, whereas deletion of this region resulted in loss of transactivation by 4-OHT/ICI 182,780 (Saville et al., 2000; Porter et al., 1996). In this research project I have further investigated the effects of slective mutations (deletions and point mutations) of zinc fingers 1 and 2 on estrogen and antiestrogen activation of hERα/Sp1 and hERα/AP1 in breast cells. As expected, the zinc finger deletion mutants of hERα, hERβ, and MOR did not bind [^{32}P]ERE in gel mobility shift assays or activate an ERE promoter (Figs. 17 and 18). However, E2 activated pSp1_3, pADA, and pRAR in MCF-7 and MDA-MB-231 cells transfected with wild type hERα (and MOR) and both zinc finger mutants (Figs. 19, 20, 21 and 22), and similar results were obtained in MCF-7 cells transfected with wild-type and zinc finger mutants of hERαTAF1 (Fig. 28). In contrast, minimal responses were observed for hERβ/Sp1 (data not shown) as previously reported (Saville et al., 2000). Zinc fingers 1 and 2 are important for DNA binding, and the D box region of zinc

finger 2 plays a role in ERα homodimerization (Kumar et al., 1987; Schwabe et al., 1990). The DBD of ERα is also an important determinant not only for antiestrogen activation of ERα/Sp1 but also ERα/AP1. Deletion of either of these zinc fingers or the entire DBD did not affect E2-induced AP1-luciferase activity (Fig. 25 A) but decreased or eliminated antiestrogen-induced AP1-luciferase activity (Fig 25 B).

It was previously reported that point mutations in zinc finger 1 of ERα either decreased (E207G/G208S), eliminated (K201A), or did not affect (E207A/G208A) ICI 182,780 activation of ERα/AP1 in TSA cells, whereas an A227T mutation in zinc finger 2 resulted in loss of ICI 182,780 inducibility through an AP1 element (Jakacka et al., 2001). E2 decreased activation of ERα/AP1 in MCF-7 and TSA cells, and this was also observed in all but one (K210A) of the DBD point mutants (Jakacka et al., 2001). I also investigated activation of hERα/Sp1 by these zinc finger point mutants in breast cancer cells. Unlike the results with hERα point mutants on hERα/AP1, minimal transactivation was observed in cells transfected with 207AA or 207GS after treatment with E2, 4-OHT, or ICI 182,780 (Fig. 26) whereas E2, but not antiestrogens, induced significant activation of ERα/Sp1 in cells transfected with A227T or K210A. Previous studies indicated that deletion of the DBD of hERα did not affect activation of hERα/Sp1 by E2 in breast cancer cells (Fig. 19, 20, 21, and 22). These results suggest that, under the different promoter context,

point mutation(s) in the DBD may cause the distinct conformational changes that ultimately affect the overall transactivation function of hERα whereas deletion of zinc finger or entire DBD did not induce this effect. My results also indicate that both zinc fingers of hERα and hERαTAF1 are required for the activity of antiestrogens in hormone-dependent activation of hERα/Sp1 (Fig. 28). Thus, depending on the type or the location of mutation(s) in the DBD, hERα mutants exhibit distinct transcriptional activation patterns with different promoter. The results clearly show that there are significant differences between hERα/Sp1 and ERα/AP1 and their requirements for regions within the DBD for activation by E2 and SERMs. This implies that cell context-specific interactions of other nuclear proteins with the DBD region of hERα may be important for ligand-dependent activation of hERα/Sp1 and hERα/AP1. DBD-interacting proteins that coactivate hERα and other hormone receptors have been reported (Moilanen et al., 1998; Saville et al., 2002) and current studies are focusing on investigating potential coactivators of hERα/Sp1.

Finally, we investigated how a histone deacetylase inhibitor affects hERα/Sp1 action and compared this responses to the effects on hERα/ERE action. Interestingly, the results (Fig. 27) show different levels of sensitivity to treatment with HDAC inhibitors; both basal and E2-induced luciferase activities were greatly increased in MCF-7 cells transfected with $pSp1_3$ whereas only E2-induced luciferase activity was increased in cells transfected with $pERE_3$, suggesting that different levels of promoter-specific nucleosomal accessibility

exist in vivo by histone acetylation/deacetylation processes (Kuo and Allis, 1998; Struhl, 1998; Workman and Kingston, 1998). These results are consistent with previous data showing that classical ERE-mediated transactivation was significaltly increased with HDAC inbitbitor treatment using cells stably transfected cells with the vitellogenin-CAT reporter construct (Mao and Shapiro, 2000).

4.2 The role of AF2-helix 12 interactions in ERα/Sp1-mediated transactivation

Previous studies with AF1 deletion mutants of hERα showed that AF1 domain of aa51-117 were critical for ERα/Sp1-mediated responses (Saville et al., 2000); however, contributions of the DEF domains have not been determined. The AF2 domain of hERα and other nuclear receptors is required for ligand-dependent activation of hERα through classical DNA-dependent pathways, and this activation process involves recruitment of AF2-interacting coactivators (Horwitz et al., 1996; Glass et al., 1997; Edwards, 1999; McKenna et al., 1999; Robyr et al., 2000., Lemon and Freedman, 1999; Klinge, 2000). NR box (LXXLL) motifs in SRCs and other coactivators specifically interact with helix 12 of hERα. D538N, E542Q, and D545N mutations in helix 12 give hERαTAF1 and these mutations abrogate interactions with most AF2-interacting coactivators and decrease transactivation from ERE promoters (Chang et al., 1999a;Schaufele et al., 2000; Tzukerman et al., 1994; Mcdonnell et al., 1995). Maximal ERα/AP1 activation by E2 requires intact activation

surfaces of both AF1 and AF2, and AF2-dependent responses require helix 12 and the corresponding NR box interacting sites. Moreover, the AF1 domain of ERα inhibits antiestrogen-induced ERα/AP1 action (Webb et al., 1999). In contrast, both estrogens and antiestrogens activated hERα/Sp1 and hERαTAF1/Sp1, and overexpression of the NR box peptides, Grip and 2XF6 (Chang et al., 1999a) derived from SRC-2, did not affect activation of hERα/Sp1 but inhibited ERα on an ERE promoter (Figs. 29 and 30). In addition, these peptides, including C33, also inhibited ERα/ERE-mediated transactivation but not ERα/Sp1-mediated transactivation in ZR-75 cells (Fig. 31). These results imply that regions of AF2 that interact with coactivators through their NR boxes are not necessary for hERα/Sp1 action, and this is supported by studies showing that prototypical AF2-interacting SRCs did not enhance hERα/Sp1-mediated transactivation in breast cancer cells transfected with pSp13 (Figs. 32 and 33). Interestingly, p68, an AF1-interacting protein, also did not enhance hERα/Sp1 activation of a GC-rich promoter in breast cancer cells, indicating that AF1-dependent p68 coactivation of hERα previously observed in COS-1 and HeLa cells transfected with an ERE promoter is also dependent on cell context (Endoh et al., 1999). Results obtained with hERαTAF1, the SRCs, and peptide competition experiments clearly define that some mechanistic differences between hormone-dependent activation of hERα/Sp1 and hERα are due, in part, to helix 12 of hERα.

4.3 The role of AF1 domain and its phosphorylation in ERα/Sp1-mediated transactivation

It is well known that phosphorylation of the AF1 domain of hERα regulates hERα transcriptional activity in both ligand-dependent and-independent ways. For examples, serine 118 in the AF1 is phosphorylated either by EGF- and IGF-activated MAP kinase or by E2-activated TFIIH/cyclin dependent kinase complex (Kato et al., 1995; Chen et al., 2000). Both AKT and p90RSK kinases phosphorylate serine 167 to modulate ligand–dependent transcriptional activity of hERα (Campbell et al., 2001; Joel et al., 1998). It was previously reported that serines 104 and 106 phosphorylation occurs by cyclinA/ cdk2 complex to enhance hERα transcriptional activity in both ligand-dependent and-independent manner (Rogatsky et al., 1999).

By using a variety of hERα mutants and AF1 peptide(s), we further analyzed the functional role of AF1 in ERα/Sp1-mediated transativation. As shown in Fig. 35, mutations of these phophorylation sites results in a slight decrease in the fold induction of activation in cells treated with E2 on ERE promoter. In contrast, S104A and S106A mutants did not exhibit ligand-dependent inducibility of ERα/Sp1 and the basal transcriptional activity was significantly increased in both MDA-MB-231 and MCF-7 cells (Figs. 35 and 36). However, other mutants, including S118A, S167A, or S118/167A double mutant, still exhibited their E2-dependent transcriptional inducibility. Interestingly, increased basal transcriptional levels were also observed in cells

overexpressing cyclin A, which stimulate cyclinA/cdk2 kinase activity, and leads to phosphorylation of serine 104 or 106 (Fig 41). These results suggested that both serines 104 and 106 may play critical a role in hERα/Sp1activation via phosphorylation/dephosphorylation processes whereas other phosphorylation sites seem to be indispensable. Furthermore, the results (Fig. 37) show that inhibition of ERα/Sp1-mediated transactivation by hERα AF1 peptide but not by ERβ AF1 peptide are consistent with important role of phosphorylation sites in AF1 for E2-dependent activation of in hERα/Sp1.

Since AF1 is important for ERα/Sp1 activation, we selected a known ERα LBD-independent coactivator, Ets-1, and investigated its possible coactivation of hERα/Sp1. Coactivation of hERα/Sp1 by Ets-1 was both hormone-dependent and-independent (Fig 42). We also tested Drip 92 and 77 proteins, identified as subunits for Sp1 coactivation complex, and Drip92 exhibited weak coactivation only with a high amount of transfected construct (Fig. 43). Identification of ligand-dependent coactivator for hERα/Sp1 are currently being investigated in our laboratories.

4.4 The role of DEF domains in ERα/Sp1-mediated transactivation

We further investigated other regions within the DEF domains required for ligand-dependent activation of hERα/Sp1 (Fig. 37). The antiestrogens 4-OHT and ICI 182,780 activated hERα/Sp1 in MCF-7 cells transfected with hERα (Δ271–300), a hinge region deletion mutant (Fig. 37), whereas E2 was

inactive. In contrast, E2 activated an ERE promoter in MDA-MB-231 cells transfected with hERα (Δ271–300) (Fig.38), and this was consistent with previous reports showing that the hinge region was not required for activation of ERE-dependent constructs (Kumar et al., 1987). Deletion of the hinge region and helix 1 [i.e. hERα (Δ265–330)] resulted in loss of E2 and antiestrogen activation of hERα and hERα/Sp1 (Figs.37 and 38), and the importance of helix 1 within the E domain for activation of ERE-dependent promoters has previously been reported (Kumar et al., 1987). It has been recently showed that E2, 4-OHT, and ICI 182,780 induced interactions of a helix 1-GAL4 chimeric protein with the ligand binding domain (LBD) or ERα in a mammalian two-hybrid assay (Pissios et al., 2000). Thus, helix 1 may stabilize ligand interactions with the LBD, and this process may be functional for both DNA-dependent and -independent mechanisms of ERα action. However, the importance of helices 1 and 2 as interacting domains for other nuclear factors has not been determined.

Activation of hERα/Sp1 by estrogens was also dependent on the C-terminal region of hERα (aa 538–595), which encompassed part of helix 12 within the E domain (aa 538–553) and the F domain (aa 554–595), which potentially contains helix 13 and β-strand motifs based on secondary structure calculations (Schwartz et al., 2002; Montano and Katzenellenbogen, 1995). Helix 12 is required for E2-dependent activation of ERα in cells transfected with an ERE promoter (Tzukerman et al., 1994) (Fig. 38), and similar results were observed for activation of hERα/Sp1 by E2 (Fig. 37). In contrast, both 4-OHT

and ICI 182,780 activated hERα (Δ538–595)/Sp1, and this result coupled with antiestrogen activation of hERαTAF1/Sp1 confirms that helix 12 is not required for this induction response by antiestrogens. The failure of E2 to activate hERα (Δ538–595)/Sp1 was not due to the requirement for helix 12 because activation was also not observed in cells transfected with hERα (Δ554–595) (i.e. F domain deletion) or hERα (Δ579–595) in which only the C-terminal β-strand region of the F domain has been deleted. F domain deletions can modulate activation of ERE promoters by antiestrogens but have minimal effects on E2-mediated transactivation (Montano and Katzenellenbogen, 1995). However, the results in Fig. 37 clearly demonstrate that F domain aa 579–595 are required for activation of hERα/Sp1 by E2. This was also confirmed in selective NR box (2XF6) and F domain (aa575–595) peptide competition studies, which demonstrate preferential inhibition of hERα/Sp1 action in cells transfected with the Fβ expression plasmid (Fig.42). These results demonstrate that E2- and SERM-mediated activation of hERα/Sp1 in breast cancer cells is complex and dependent on multiple overlapping and distinct regions of hERα (Fig. 53). These domains of hERα may impart unique structural features required for hERα/Sp1 action and may also serve as binding sites for essential interacting nuclear coregulatory proteins. Decreased hERα/Sp1-mediated transactivation in cells transfected with the F domain peptide (Fig.42) suggests that this region of hERα may interact with other nuclear coregulatory proteins, and current

studies are focused on identifying F domain-interacting factors and their function in breast cancer cells.

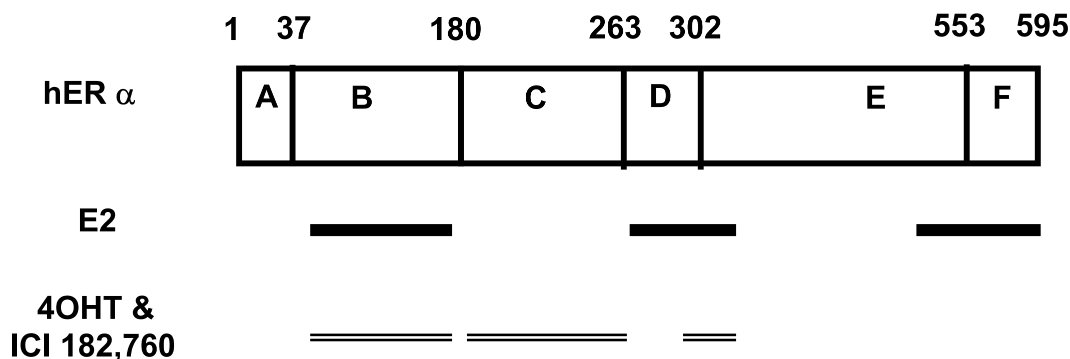

Fig. 53. Summary of domains of hERα required for activation of hERα/Sp1 by E2 and antiestrogens 4-OHT and ICI 182, 780.

4.5 Detection of hERα/Sp1 protein interactions in living cells by Fluorescence Resonance Energy Transfer (FRET)

With advances in fluorescence microscopy and development of multiple color variants of green fluorescence proteins (GFP), derived from the jellyfish Aequoria victoria, protein-protein interactions can be visualized in living cells by FRET and image analysis. FRET is a quantum mechanical process in which energy from an excited donor fluorophore is transferred to a low energy acceptor fluorophore via a long range dipole-dipole interaction; in a nonradiative manner (Day et al., 2001; Gordon et al., 1998; Elangovan et al., 2003; Wallrabe et al., 2003; Miyawaki et al., 1997). The efficiency of energy transfer varies inversely with the sixth power of the distance between the donor and the

acceptor fluorophores (1/r6; r = distance between the donor and the acceptor), which limits FRET to occur in a range of 1-10nm. When FRET occurs, the donor emission is decreased and the acceptor emission is increased; sensitized emission.

The efficiency of energy transfer (FRET efficiency) also depends on the extent of spectral overlap between the donor and acceptor, the quantum yield of the donor, and the relative orientation of the donor and acceptor (Gordon et al., 1998). Although the efficiency of energy transfer can be improved by increasing the spectral overlap between the donor and the acceptor, this increases background FRET signals derived from the donor emission into the acceptor channel (donor bleed through) and from direct excitation of the acceptor by the donor excitation wave length (acceptor bleed through), defined as spectral cross talk (Day et al., 2001). Therefore, it is critical to selectively extract the background signals from sensitized emission of the FRET pair and this requires extensive corrections in order to determine FRET properly.

In order to establish a workable FRET pair, the following conditions also need to be fulfilled; a sufficient separation in excitation spectra between the donor and the acceptor, the spectral overlap between the donor and the acceptor, an appropriate separation in emission spectra to provide independent measurement of the donor or the acceptor fluorescence. A cyan fluorescent protein (CFP) and a yellow fluorescent protein (YFP) have been used together as a sutable FRET pair for detection of intermolecular protein-protein

interactions. Heteromerization of G proteins, dimerization of receptor tyrosine phosphatase, interaction between nuclear transporter factors have all been visualized in living cells through generation of CFP and YFP fusion proteins (Janetopoulos et al., 2001; Damelin et al., 2000; Tertoolen et al., 2001).

However, when intermolecular FRET is measured in living cells via expression of two separate fusion proteins such as a CFP/YFP pair, it is important to consider that either false positive or false negative results can be observed due to the following artifacts. Firstly, formation of mixed complexes between the fluorescence lableled proteins and endogeneous protein partners can interrupt FRET signals; For example, complex formation of YFP-hERα and endogenous ERα in MCF-7 cells competes for potential productive interactions, which can cause a weaker FRET signal. Secondly, overexpression of the fusion proteins can produce false positive because high concentration of the donors and the acceptors as a FRET pair or their ratio difference within a cell can result in increased non-specific interactions. Therefore, control experiments should be performed in parallel studies with other fusion protein mutants that have same biochemical properties such as protein stability and subcellular localization. However, these mutant proteins should not physically interact to assess the contribution of non-specific interactions. Thirdly, false negative also can occur, even when their fusion partners are still interacting, because inappropriate orientation or unfavorable stoichiometry between the fusion proteins may result in an increased distance between the proteins that will interrupt energy transfer

to measure FRET (Day et al., 2001; Zhang et al., 2003). The FRET asay for determing intermolecular protein-protein interactions can be assessed with more sophiscated methods such as the mathemathical processing of the three images (Gordon et al., 1998; Elangovan et al., 2003; Zimmermann et al., 2002) and photobleaching of the acceptor (Miyawaki et al., 2000; Siegel et al., 2000).

The results illustrated in Figs 45 and 46 shows that individual transcriptional activities of CFP and YFP fusion proteins are comparable to those of the unfused proteins such as hERα or Sp1 in cells transfected with constructs contain ERE and Sp1 promoters. Coexpression of CFP-Sp1 and hERα increased both the basal and inducible level of luciferase activity. Thus, the transcriptional activities of the fusion proteins are relatively intact. Acquired images using 2-photon excitation fluorescence microscopy with a three filter set indicated that FRET signal from cells transfected with the CFP-YFP chimera was much higher than that observed in the negative control cells (Fig. 47). In cells cotransfected with CFP-hERα and YFP-hERα, E2 induced translocation of the fusion proteins from the cytoplasm into the nucleus and the stronger FRET signal, indicated ligand-induced ER homodimerization which is consistent with previously described ligand-induced effect on hERα (Kumar and Chambon, 1988; Bai and Giguere, 2003).

In order to accurately determine ligand-dependent interactions of hERα/Sp1 using FRET, we first set the range from minimum to maximum levels of either CFP or YFP expression as the selection criterion. Values from cells

that did not fit this criterion for correcting variations in fluorophore expression level were eliminated. Secondly, we assumed that the ratio of positive control FRET signal to negative control FRET signal, which is ≅ 2.01, would represent 50% FRET efficiency and, based on this assumption, further caculations for measuring FRET efficiency were performed. Moreover, enhanced dimerization between E2-bound CFP-hERα and YFP-hERα has been previously reported (Bai and Giguere, 2003) and this dimerization property was used as another positive control for confirming the method described above for quantifing FRET efficiency. The results shown in Fig 49 indicate that the order of FRET efficiency in CFP-hERα/YFP-hERα or CFP-Sp1/YFP-hERα interactions was 4OHT> E2>ICI; all the ligands induced an increase in protein-protein interactions in vivo. However, the overall FRET efficiencies in CFP-Sp1/YFP-hERα were relatively lower than those in CFP-hERα /YFP-hERα, implicating that the increase size of CFP-Sp1 or unfavorable orientation between the fusion proteins affected the overall FRET efficiency.

To alternatively assess ligand dependent hERα/Sp1 interactions, variations in YFP intensity over time were measured after addtion of each ligand. As shown in Fig. 50, the addition of DMSO did not alter YFP intensity over time whereas all other ligands including E2, 4OHT, and ICI increased YFP intensity with a slightly fluctuating pattern. The final order of YFP intensity 7.5 min after ligand addition was the same as the order of FRET efficiency and this was consistent with ligand-dependent hERα/Sp1 interactions in vivo.

Furthermore, coimmunoprecipitation for YFP-hERα/Sp1 has also been carried out to confirm the ligand-dependent interactions of these proteins in vivo (Fig. 51 (top)). Cells transfected with YFP-hERα and CFP-Sp1 also exhibited significant estrogen-and antiestrogen-induced activation of hERα/Sp1 (Fig. 50 (bottom)).

Finally, transfection of small interfering RNA for Sp1 significantly inhibited endogenous hERα/Sp1 action in ZR-75 cells, suggesting that hERα-mediated transactivation of consensus GC-rich promoter, at least in part, depends on hERα/Sp1 protein-protein interactions. RNA interference is also being used in current studies on the role of Sp3, another GC-rich region binding proteins that can act as a transcriptional repressor or enhancer.

In this study, it has been shown that multiple but distinct domains of hERα are required for hERα/Sp1-mediated transactivation and NR-box motifs (LxxLL) in coactivation do not play a significant role in hERα/Sp1 action compared to their function as coactivators of hERα/AP1 or hERα/ERE. Although it has been shown that many other nuclear receptors interacts with Sp family proteins, this study is the first to observe ligand-dependent hERα/Sp1 protein-protein interactions in living cells.

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