

**MECHANISMS OF HORMONAL REGULATION OF CAD GENE
EXPRESSION AND INHIBITION BY ARYL HYDROCARBON RECEPTOR
AGONIST IN HUMAN BREAST CANCER CELLS**

A Dissertation

by

SHAHEEN MUNAWAR ALI KHAN

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

December 2005

Major Subject: Genetics

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ABSTRACT

Mechanisms of Hormonal Regulation of CAD Gene Expression and Inhibition by Aryl Hydrocarbon Receptor Agonist in Human Breast Cancer Cells. (December 2005)

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The CAD gene is trifunctional and expresses carbamoylphosphate synthetase/aspartate carbamyltransferase/dihydroorotase, which are required for pyrimidine biosynthesis. CAD gene activities are induced in MCF-7 human breast cancer cells, and treatment of MCF-7 or ZR-75 cells with 17 β -estradiol (E2) resulted in a 3-5 fold increase in CAD mRNA levels in both cell lines. E2 induced reporter gene activity in MCF-7 and ZR-75 cells transfected with a construct containing the growth-responsive -90/+115 (pCAD1) region of the CAD gene promoter, which contains three upstream GC-rich and two downstream E-box motifs. Deletion and mutation analysis of the CAD gene promoter demonstrated that only the GC boxes that bind Sp1 protein were required for E2-responsiveness. Results of gel shift and chromatin immunoprecipitation (CHIP) assays show that both Sp1 and estrogen receptor α (ER α) interact with the GC-rich region of the CAD gene promoter. Moreover, hormone-induced transactivation of pCAD1 was inhibited by cotransfection with dominant-negative Sp1 expression plasmid and small inhibitory RNA for Sp1. These results demonstrate that, in common with

many other genes involved in E2-induced cell proliferation, the CAD gene is also regulated by a nonclassical ER α /Sp1-mediated pathway.

2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) and other aryl hydrocarbon receptor (AhR) ligands suppress several E2-induced responses in the rodent uterus and mammary tumors and in human breast cancer cells. TCDD inhibited hormone-induced activation of CAD mRNA levels and reporter gene activity in MCF-7 and ZR-75 cells transfected with E2-responsive pCAD promoter constructs. E2-mediated transactivation of pCAD constructs with a mutant inhibitory dioxin responsive element DRE (iDRE) were also inhibited by TCDD suggesting that inhibitory AhR-ER α /Sp1 crosstalk was iDRE-independent. It was not possible to determine whether the levels of ER α in cells cotreated with E2 plus TCDD were limiting since the proteasome inhibitor MG132 itself directly decreased CAD mRNA levels. Using fluorescence resonance energy transfer (FRET), it was shown that both E2 and TCDD enhanced AhR-ER α interactions. E2 also induced interactions between ER α and Sp1. However cotreatment with TCDD abrogated this effect. Results of this study demonstrate a unique model of AhR-ER α crosstalk where the liganded AhR inhibits ER α -Sp1 interactions and also recruits ER α to Ah-responsive gene promoters such as CYP1A1.

DEDICATION

I would like to dedicate this dissertation to my mother, Rashida Khan for her unconditional love, support and sacrifices.

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I would like to thank my advisor, Dr. Stephen Safe, for his guidance and support through out my graduate studies. I am deeply grateful for his understanding and patience during difficult times, both inside and outside the laboratory. I truly respect and admire him for his modesty, diligence and his enthusiasm for science. I have learned a lot from him in all these years especially the importance of hard work and commitment to be a successful scientist. I would also like to thank other members of my committee, Dr. Robert Burghardt, Dr. David Peterson and Dr. Gary Kunkel, for all their help, time and guidance during my graduate studies.

I am very grateful to Lorna Safe, for her kindness and help in time of need and her administrative help in the laboratory all these years. I also would like to thank Kim Daniel and Kathy Mooney for their administrative help. I wish to thank all past and present members of Dr. Safe's Lab for friendship, help and training and most importantly, I truly appreciate Dr. Mark Wormke for his friendship, help, discussions and training in the laboratory.

Last but not the least I would like to thank my entire family for their love and support and most of all, I want to deeply thank my mother Rashida Khan, for all the sacrifices she made in her life for her children. This dissertation would have not been possible without her unconditional love, strength and sacrifices. I also want to thank my husband, Alope Mishra, for his love, patience, support and understanding and for always

being there for me. Finally, I would like to thank all my friends for their friendship, love and support.

"We must accept finite disappointment, but never lose infinite hope"

--Martin Luther King Jr.

TABLE OF CONTENTS

	Page
ABSTRACT	iii
DEDICATION	v
ACKNOWLEDGMENTS.....	vi
TABLE OF CONTENTS	viii
LIST OF FIGURES.....	x
LIST OF TABLES	xv
 CHAPTER	
I INTRODUCTION.....	1
1.1 Cancer	1
1.2 Breast Cancer	6
1.3 Transcription	40
1.4 Transcription Factor Sp1	54
1.5 Nuclear Receptor Superfamily	66
1.6 Estrogen Receptors.....	75
1.7 Aryl Hydrocarbon Receptor	101
1.8 CAD	125
1.9 Research Objectives	128
II MATERIALS AND METHODS	132
2.1 Cells, Chemicals and Biochemicals	132
2.2 Plasmids, Oligonucleotides and Cloning	133
2.3 Semiquantitative Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)	136
2.4 Northern Blot Analysis	137
2.5 Transient Transfection Assays	138
2.6 Electrophoretic Mobility Shift Assay (EMSA).....	139
2.7 Chromatin Immunoprecipitation Assay	139
2.8 Coimmunoprecipitation.....	141
2.9 Western Blot Analysis.....	142

CHAPTER	Page
2.10	Fluorescence Resonance Energy Transfer (FRET)..... 143
2.11	Small Inhibitory RNA..... 145
2.12	Real-Time PCR..... 146
2.13	Statistical Analysis..... 147
III	RESULTS..... 148
3.1	Mechanism of Hormonal Regulation of CAD Gene Expression in MCF-7 and ZR-75 Breast Cancer Cells 148
3.2	Molecular Mechanisms of AhR-ER α /Sp1 Crosstalk in Breast Cancer Cells..... 173
IV	DISCUSSION AND CONCLUSIONS..... 200
4.1	Mechanism of Hormonal Regulation of CAD Gene Expression in MCF-7 and ZR-75 Breast Cancer Cells 200
4.2	Molecular Mechanisms of Inhibitory AhR-ER α /Sp1 Crosstalk in Breast Cancer Cells 208
	REFERENCES..... 218
	VITA..... 320

LIST OF FIGURES

FIGURE	Page
1. Estimated US Cancer Deaths in 2005 in the United States.....	2
2. Multistage model of carcinogenesis.....	5
3. Acquired capabilities of cancer.....	6
4. Schematic representation of the female breast.....	7
5. Potential mechanisms of estrogen-induced carcinogenesis in human breast tissues.....	16
6. Schematic representation of the postulated pathways of estrogen actions on breast epithelial cells.....	18
7. Structures of selected SERMS.....	28
8. Structures of selected antiestrogens.....	29
9. Structures of vitamin D and synthetic retinoic acid analogues.....	34
10. Chemical structures of selected PPAR γ agonists.....	36
11. Core promoter elements for RNAP II.....	42
12. Schematic representation of alternative pathways of PIC assembly.....	47
13. Schematic representation of transcriptional activation of protein coding genes.....	52
14. Structural motifs in Sp proteins.....	58
15. Schematic representation of the functional domains of nuclear receptors.....	70
16. Schematic representation of the DBD of nuclear receptors.....	71

FIGURE	Page
17. The types of DNA response elements used by nuclear receptors.....	74
18. Structural domains of human ER α and ER β	77
19. Classical mechanism of ER action.....	84
20. ERs use two separate pathways for activation of ER/AP-1.....	88
21. DNA-dependent ER α /Sp1.....	89
22. DNA-independent activation of GC-rich promoter by ER α /Sp1.....	90
23. Activation of ER α by kinases.....	94
24. Structures of selected classical (A) and non-classical (B) synthetic ligands of AhR.....	104
25. Structures of selected naturally occurring AhR ligands.....	105
26. Schematic representation of functional domains of AhR and Arnt proteins.....	108
27. Mechanism of transcriptional activation by AhR.....	112
28. Domain structures of mAhRR and mAhR proteins.....	115
29. Structure of SAhRMs.....	124
30. Schematic representation of de novo pyrimidine biosynthesis in mammalian cells.....	126
31. Structural organization and regulation of CAD.....	128
32. Induction of CAD mRNA levels by E2 in MCF-7 cells.....	149

FIGURE	Page
33. Induction of CAD mRNA levels by E2 in ZR-75.....	150
34. Activation of pCAD1 by E2 in MCF-7 and ZR-75 cells.....	152
35. Schematic representation for ER α variants containing deletions and point mutants.....	154
36. Effect of wild type and variant ER α on pCAD1 in MCF-7 and ZR-75 cells.	155
37. Effects of wild type and ER α variants on pCAD2 in MCF7 and ZR75 cells.....	156
38. Deletion analysis of pCAD constructs in MCF-7 cells.....	158
39. Deletion analysis of pCAD constructs in ZR-75 cells.....	159
40. Mutation analysis of pCAD constructs in MCF-7 and ZR-75 cells.....	162
41. Binding of Sp1 to CAD promoter in MCF-7 cells.....	164
42. Binding of Sp1 to CAD promoter in ZR-75 cells	165
43. Binding of USF-1 to CAD promoter in MCF-7 cells.....	167
44. Effects of dominant negative Sp1 on E2-mediated transactivation of pCAD1.....	169
45. Inhibition of hormonal activation of pCAD1 by small inhibitory RNA for Sp1.....	170
46. Analysis of ER α and Sp1 interactions with the CAD gene promoter by ChIP.....	172
47. Regulation of CAD mRNA levels in ZR-75 and MCF-7 cells.....	174

FIGURE	Page
48. Regulation of CAD constructs by TCDD in ZR-75 cells.....	176
49. Regulation of CAD constructs by ICI 182,780 in ZR-75 cells.....	177
50. Regulation of CAD constructs by TCDD in MCF-7 cells.....	178
51. Regulation of CAD constructs by ICI 182,780 in MCF-7 cells.....	179
52. Summary of CAD constructs and their cis-elements used to study inhibitory AhR-ER α crosstalk.....	180
53. Inhibitory AhR-ER α crosstalk in ZR-75 cells transfected with wild-type or mutant CAD constructs.....	182
54. Inhibitory AhR-ER α crosstalk in MCF-7 cells transfected with wild-type or mutant CAD constructs.....	183
55. Summary of chimeric constructs used for FRET studies.....	185
56. Activity of YFP-AhR in MCF-7 and COS-1 cells.....	188
57. Ligand-dependent interactions of YFP-AhR and CFP-ER α in MCF-7 cells.....	189
58. Ligand-dependent interactions of YFP-AhR and CFP-ER α in COS-1 cells.....	191
59. Ligand-dependent interactions of YFP-ER α and CFP-Sp1 in MCF-7 cells.	192
60. Flag AhR-ER α interactions and function.....	194
61. Effects of proteasome inhibitor MG132 on CAD mRNA levels in ZR-75 cells.....	195

FIGURE	Page
62. Analysis of protein interactions with CAD and CYP1A1 promoters by ChIP.....	197
63. Effects of E2 on TCDD-induced transactivation of pDRE ₃ in MCF-7 and ZR-75 cells.....	199
64. Model for Myc-mediated transcriptional activation of CAD gene expression in NIH 3T3 cells.....	204
65. Hormone-induced transcriptional activation of CAD gene involves ER α /Sp1 in MCF-7 and ZR-75 cells.....	205
66. Functional iDREs in promoters of the cathepsin D, c-fos, heat shock protein 27, and pS2 genes.....	210
67. Proposed model for inhibitory AhR-ER/Sp1 crosstalk on CAD gene promoter.....	215

LIST OF TABLES

TABLE	Page
1. Summary of breast cancer risk factors.....	10
2. Summary of breast cancer stages.....	19
3. Summary of the most common types of breast cancer.....	21
4. Summary of chemotherapeutic agents for treatment of breast cancer.....	24
5. Human general transcription initiation factors.....	45
6. Summary of the expression pattern and functional features of Sp1-like/KLF family members.....	55
7. Subfamilies of mammalian nuclear receptors.....	68
8. Sequences of consensus and nonconsensus EREs from estrogen-responsive genes.....	85
9. Summary of tissue-specific extranuclear activities of ER α and their proposed physiological relevance.....	97
10. List of genes regulated by TCDD.....	117
11. Primers for pCAD deletion constructs.....	135
12. Primers for pCAD constructs containing point mutations.....	136
13. Oligonucleotides used for EMSA.....	140
14. Excitation and emission of fluorescent proteins.....	144
15. Sequences for siRNA duplexes.....	146

CHAPTER I

INTRODUCTION

1.1 Cancer

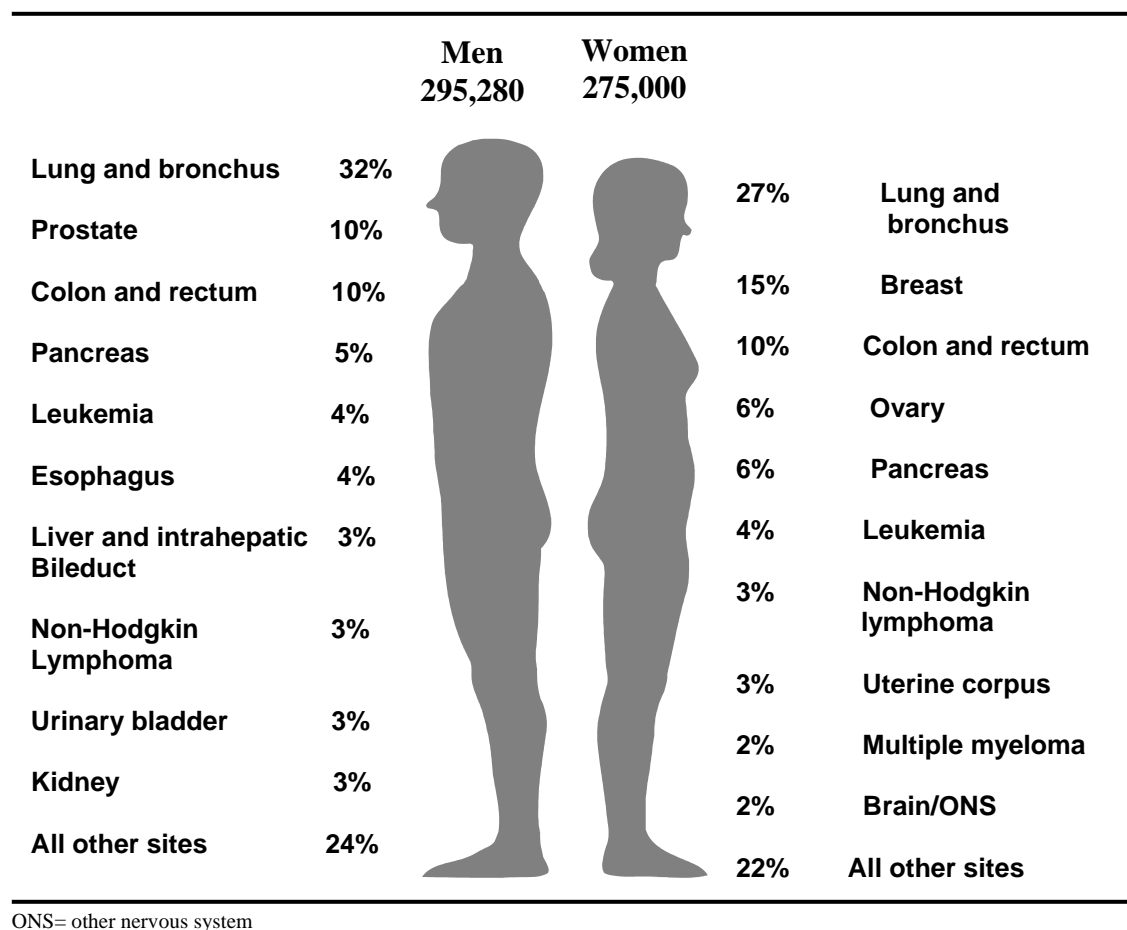
1.1.1 General introduction with statistics

Cancer is a complex disease involving uncontrolled cell growth and metastasis that is caused by the interplay of multiple genes and different environmental factors (Luch, 2005). Cancer cells harbor alterations in key steps that regulate cell proliferation, differentiation, and cell-cell communication and these mutations cause cancer cells to acquire phenotypes associated with the malignant state (Hahn, 2004b). The origin of the word cancer is credited to the Greek physician Hippocrates, considered the "Father of Medicine." Hippocrates used the terms carcinos and carcinoma to describe non-ulcer forming and ulcer-forming tumors.

In the United States 1 in 4 death are caused by cancer and it is the second leading cause of death exceeded only by heart disease. About 76% of cancers are diagnosed at age 55 or older; more than 10 million people are diagnosed with cancer every year worldwide and it is estimated that there will be 15 million new cancer cases every year by 2020. Cancer causes 6 million deaths every year worldwide. Lung, colorectal and stomach cancer are among the five most common cancers in the world for both

This dissertation follows the style of Gene.

men and women (excluding skin cancers other than melanoma). Among men, lung and stomach cancer are the most common cancers worldwide and for women, the most common cancers are breast and cervical cancer (World Health Organization, 2005; International Agency for Research on Cancer, 2005). Figure 1 shows the estimated cancer deaths in the United States in 2005; lung cancer is the most common fatal cancer in both men (32%) and women (27%)(American Cancer Society, 2005a).



ONS= other nervous system

Figure 1. Estimated US Cancer Deaths in 2005 in the United States (American Cancer Society, 2005a).

There is a marked overall difference in the total cancer burden between developed and developing countries. Cancer of the lung, colon, breast, prostate and bladder are greatest in developed countries such as Northern Europe whereas in Sub-Saharan Africa the incidence of these cancers is significantly lower (Parkin et al., 2005). The most common cancers in developing countries are of the cervix, liver, stomach and mouth. Also the incidence and mortality of cancers can vary within the same geographical area and this can be attributed to ethnicity. For example, there is high incidence of oesophageal cancer in parts of Iran and within the United States, incidence and mortality is higher among African Americans than all other racial/ethnic groups (Clegg et al., 2002). There is a 20-fold variation in the incidence of colon cancer worldwide (highest in the United States, lowest in India), and the incidence of breast cancer varies sevenfold within the United States with the highest incidence in Hawaiians and the lowest in Israeli non-Jews (Bingham and Riboli, 2004).

There are various causes and risk factors for development of cancer and these include exposure to chemicals, radiation, viruses, diet, tobacco, genetics and heredity, hormones and other miscellaneous factors such as occupation, environmental pollution, sunlight, radiation, food additives, pesticides, drugs, exercise and stress. Epidemiological evidence indicates that several factors also protect against cancer and these include reduction of smoking, increased consumption of fruits and vegetables, control of infections and reduced exposure to carcinogens (Ames and Gold, 1998; Tominaga, 1999).

1.1.2 Carcinogenesis

Carcinogenesis is a multistage, multimechanistic process and is divided into three stages: initiation, promotion, and progression (Nowell, 1976; Weinstein et al., 1984; DiGiovanni, 1992) (Figure 2). Tumor initiation results from irreversible DNA damage or error-prone DNA repair/replication leading to mutagenesis. In addition, genetic changes associated with tumor initiation include activation of proto-oncogenes such as ras family and loss of function in tumor-suppressor genes such as p53 and Rb. Initiators can be chemical carcinogens such as nitrosamines, ionizing radiation such as x-rays or viruses such as papillomavirus or Epstein-Barr virus.

The next step is tumor promotion that occurs when a single initiated cell undergoes clonal expansion by a combination of growth stimulation and inhibition of apoptosis and produces a larger population of cells that are at risk of further genetic changes (Trosko, 2001). Promoters such as phenobarbital, phorbol ester, polybrominated biphenyl, peroxisome proliferators, etc. are nonmutagenic (Yuspa et al., 1996) and most, if not all, tumor promoting agents reversibly inhibit gap junctional intercellular communication (GJIC) (Yotti et al., 1979). Promoters such as phenobarbital, phorbol ester, polybrominated biphenyl, peroxisome proliferators, etc. are nonmutagenic (Yuspa et al., 1996) and most, if not all, tumor promoting agents reversibly inhibit gap junctional intercellular communication (GJIC) (Yotti et al., 1979). The promotion stage is reversible and is caused by epigenetic mechanisms including alterations in the expression of the genetic information at the transcriptional, translational, or posttranslational level (Hikita et al., 1999).

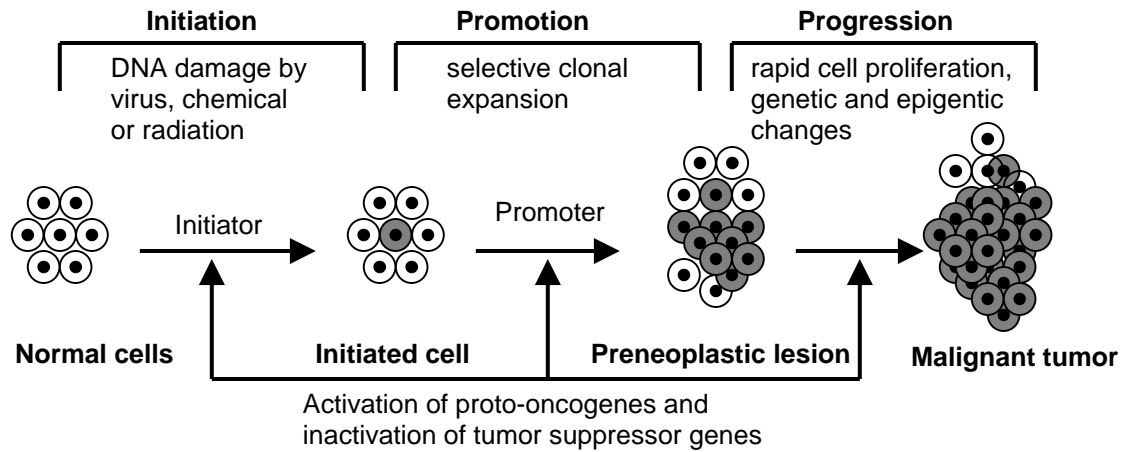


Figure 2. Multistage model of carcinogenesis (Alberts et al., 2002).

The final stage of progression begins when benign initiated cells in a promoted mass accrue additional stable, genetic or epigenetic changes so as to become independent of an endogenous or exogenous tumor promoter due to the permanent genomic change (Vogelstein et al., 1988). The cells proliferate rapidly, are characterized by gross morphological and karyotypic changes and are considered to have been neoplastically transformed (Lengauer et al., 1998). By this time sufficient genomic instability has occurred to allow further genetic and epigenetic changes that, in turn, allow one of these cells to achieve the phenotypes of invasiveness and metastasis. Hanahan and Weinberg have proposed six essential alterations in cell physiology that collectively dictate malignant growth and are acquired by most if not all cancers during their development (Hanahan and Weinberg, 2000). These hallmarks are summarized in Figure 3.

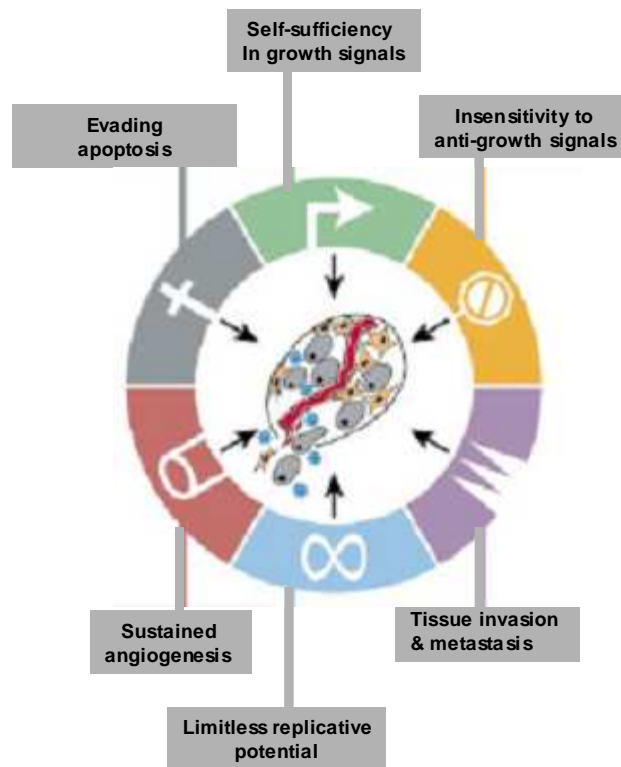


Figure 3. Acquired capabilities of cancer (Hanahan and Weinberg, 2000).

1.2 Breast Cancer

1.2.1 Introduction

Breast cancer is a complex multifactorial malignant disease in which tumors develop from cells of the breast. Figure 4 is a schematic representation of the female breast. Female breast is made up lobules, ducts, fatty and connective tissue, blood vessels, and lymphatic vessels. Lobules are glands that make breast milk and ducts are small tubes that connect lobules to the nipple. Most breast cancers begin in the ducts (ductal carcinoma), some in the lobules (lobular carcinoma), and the rest in other tissues (American Cancer Society, 2005b).

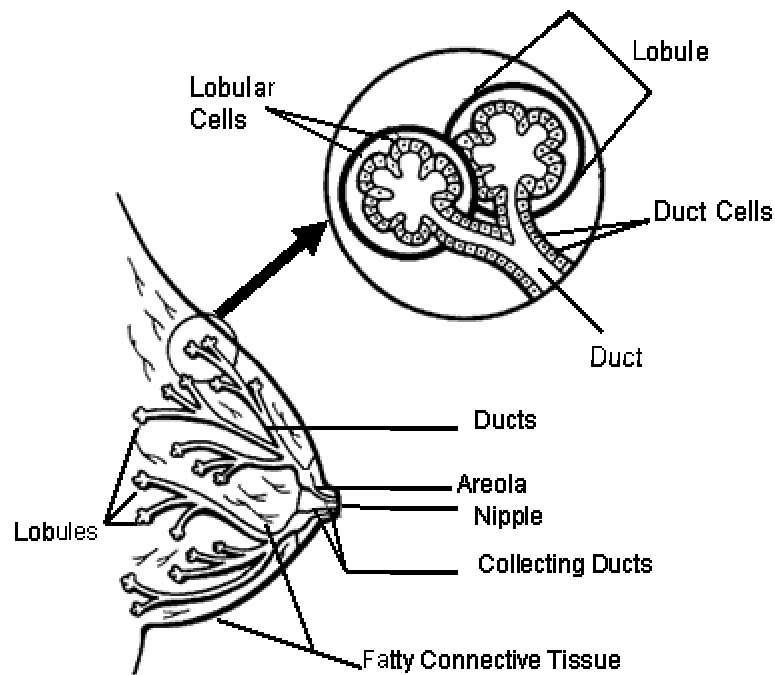


Figure 4. Schematic representation of the female breast (Adapted from American Cancer Society, 2005b).

Breast cancer is the most common cancer diagnosed among women and is the leading cause of cancer deaths in women worldwide (World Health Organization, 2005). It is the second leading cause of death among women in the United States and it is estimated that one in eight women will develop breast cancer over her lifetime (American Cancer Society, 2005b). In the United States incidence rates of breast cancer are generally 20%-40% higher in Caucasian women than in African-American women although the mortality is higher in the latter group (Lacey et al., 2002; Shavers et al., 2003). There is a geographic variation in the incidence and mortality of breast cancer with higher rates in developed countries compared to developing countries (Althuis et al., 2005; Parkin et al., 2005). Studies on migrants have demonstrated that breast cancer

incidence increases in people who move from a region with low breast cancer incidence such as Asian countries to other locations with higher breast cancer incidence such as the United States and other western countries (Ziegler et al., 1993). These geographic variations and international differences can be attributed to the differences in lifestyle such as reproductive and socioeconomic variables, diet and environmental factors (Bray et al., 2004; Gordon, 2003).

1.2.2 Risk factors for breast cancer

There are several different risk factors for breast cancer and there is a strong interplay of genetic and environmental risk factors in the initiation and progression of this disease (Martin and Weber, 2000). Breast cancer risk increases with age and 77% of women diagnosed with breast cancer are older than 50. Risk factors can be broadly classified as genetic, hormonal, environmental and other lifestyle factors. A summary of breast cancer risk factors has been described in Table 1 (Dumitrescu and Cotarla, 2005).

1.2.2.1 Genetic risk factors and family history

Five to ten percent of all breast cancers are associated with the inheritance of mutations in one of the two major breast cancer susceptibility genes BRCA1 and BRCA2 (Peto et al., 1999; Struewing et al., 1996). There is an 80% chance of developing breast cancer during a lifetime in women with an inherited BRCA1 or BRCA2 mutation (Nathanson et al., 2001; Rebbeck et al., 1996). BRCA1 and BRCA2 are tumor suppressor genes and their proteins have been implicated in a multitude of different processes including DNA repair and recombination, cell cycle control, and transcription (Venkitaraman, 2002). Ashkenazi Jewish women are at a much higher risk

of developing breast cancer at an early age (before 40 years) and this is largely due to the high occurrence of three founder mutations in BRCA1 and BRCA2 genes (Berman et al., 1996; Oddoux et al., 1996).

Mutations in other genes such as p53, PTEN, ATM, MSH2/MLH1 and STK11/LKB1 account for a small proportion of heritable breast cancer (de Jong et al., 2002; Olsen et al., 2001; Slattery and Kerber, 1993). Wild type p53 plays an important role in maintaining genomic stability in response to DNA damage by inducing transient G1 arrest or by triggering apoptosis. Germ line p53 mutations are reported in 50% cases of Li-Fraumeni syndrome (childhood cancer) and approximately 50% of patients who survive this disease develop breast cancer by age of 50 (Easton et al., 1995; Malkin et al., 1990). Germ line p53 mutations occur in less than 1% of women with sporadic breast cancer (Patel et al., 1995).

Cowden syndrome is an autosomal dominant disorder, characterized by the development of hamartomas and benign tumors and is caused by mutation in germ line PTEN gene (Hanssen and Fryns, 1995). PTEN is a tumor suppressor gene that encodes a phosphatase protein and women with germ line mutations in PTEN exhibit a 25-50% lifetime breast cancer risk (Hlobilkova et al., 2000; Thull and Vogel, 2004). Peutz-Jeghers syndrome is an autosomal dominant disorder and the relative risk of breast cancer in these families is 20.3% (Boardman et al., 1998). It is caused by germ line mutations in the LKB1 gene that encodes for a serine-threonine kinase (Hemminki et al., 1998). Both PTEN and LKB1 do not seem to play a role in sporadic breast cancer (Bignell et al., 1998).

There are also low penetrant breast cancer susceptibility genes and these include protooncogenes (RAS, HER2 and myc genes), metabolic pathway genes [cytochrome p450 family, GST family and N-acetyl transferase (NAT1 and NAT2)], estrogen receptor (ER), progesterone receptor (PR), androgen receptor (AR), heat shock protein 70 (HSP70), tumor necrosis factor α (TNF α), HLA region and vitamin D receptor (VDR) genes (de Jong et al., 2002). Polymorphisms in these genes have a greater contribution to breast tumorigenesis in combination with other risk factors (Table 1).

Table 1. Summary of breast cancer risk factors (Dumitrescu and Cotarla, 2005).

Factors that increase breast cancer risk	
Well-confirmed factors	Increasing Age Geographical region (USA and western countries) Family history of breast cancer Mutations in BRCA1 and BRCA2 genes Mutations in other high penetrance genes (p53, ATM, PTEN, STK11) History of benign breast disease High mammographic breast density Late age at menopause (> 55) Early age at menarche (< 12) Nulliparity and older age at first birth Hormonal replacement therapy Oral contraceptives recent use Ionizing radiation exposure Environmental factors Obesity in postmenopausal women High alcohol consumption
Probable factors	High saturated fat High socioeconomic status
Factors that decrease breast cancer risk	
Well-confirmed factors	Geographical region (Asia and Africa) Early age of first full-term pregnancy Higher parity Breast feeding (longer duration) Fruit and vegetables consumption Physical activity
Probable factors	Non-steroidal anti-inflammatory drugs Polymorphisms in low-penetrance genes

Fifteen to twenty percent of female breast cancers occur in women with no apparent gene mutations but with a family history of breast cancer and this could be due to other unidentified genetic factors modified by environmental influences (Madigan et al., 1995; Mincey, 2003). The risk of breast cancer increases with the increasing number of relatives with breast cancer in the family and the risk is even higher if a mother or sister has a history of breast cancer (Claus et al., 1994; Webb et al., 2002). History of benign breast disease, particularly atypical hyperplasia or extensive mammographic breast density, is also associated with increased breast cancer risk (Boyd et al., 1995; Byrne et al., 2001; London et al., 1992; Wang et al., 2004; Webb et al., 2002).

1.2.2.2 Hormonal factors

Several studies have shown that prolonged exposure to the hormone estrogen increases the risk of breast cancer. Epidemiological studies have established a strong link between higher risk of breast cancer and reproductive factors that increase the overall number of menstrual cycles such as early menarche (before age 12), late menopause (after age 55), age of women at first birth (over 30-35) and nulliparity (Russo et al., 1992; Talamini et al., 1996). Breast cancer risk is lower in women with multiple pregnancies and women with a pregnancy prior to age 24 (Lambe et al., 1996; Meeske et al., 2004). Women who are above 30-35 years of age at first birth are at higher risk compared to nulliparous women (Albrektsen et al., 1994; Rosner et al., 1994). The protective effects of pregnancy against breast cancer is explained by the induction of complete differentiation of the breast that may markedly reduce the susceptibility of the fully differentiated mammary gland to carcinogens due to, at least in part, by decreasing

proliferative activity of parous epithelium (Russo et al., 2000). Another hypothesis is that the decreased risk may also be due to the altered hormonal environment during pregnancy, and these include specific molecular changes induced by estrogen and progesterone and decreased circulating growth hormone (Russo et al., 2005; Sivaraman and Medina, 2002). Breastfeeding is also protective against breast cancer and this may be due to the reduction of total number of ovulatory menstrual cycles and consequently cumulative ovarian hormone exposure (Enger et al., 1997; Lee et al., 2003; Lipworth et al., 2000; Russo et al., 2000).

Recent studies have shown that the use of hormone replacement therapy and oral contraceptives for long time periods can also increase the risk of developing breast cancer (Althuis et al., 2003; Ewertz et al., 2005). In some studies women exposed to diethylstilbestrol (DES) were also found to be at increased breast cancer risk (Sasco et al., 2003; Titus-Ernstoff et al., 2001).

1.2.2.3 Environmental factors

Ionizing radiation is the most well characterized environmental risk factor for breast cancer. Radiation induced breast cancer risk depends on the various factors including age at exposure (highest before age 30), the status of hormone levels, parity and other genetic disorders (Coyle, 2004; Brody and Rudel). A number of reports showed patients who received radiation therapy for Hodgkin's disease, breast cancer and other illnesses, had an increased risk of breast cancer and women in Japan exposed to atomic bomb radiation also have a high rate of breast cancer (Goodman et al., 1997; Hancock et al., 1993; Land et al., 2003). Other risk factors include solar radiation, light

and chemicals. Solar radiation creates an active form of vitamin D that may lower the risk of breast cancer and studies show that women who work at night are at higher breast cancer risk. This may be due to decreased vitamin D synthesis and suppression of normal nocturnal production of melatonin by the pineal gland, which, in turn, increases the of estrogen release by the ovaries thereby disrupting circadian patterns (Davis et al., 2001; Garland et al., 1990; Grant, 2002; Schernhammer et al., 2001).

Environmental toxic chemicals such as organochlorine compounds (OCs) have been linked to breast cancer. The most prevalent OC residues found in human tissues are dichlorodiphenyldichloroethane (Van Loo et al.), the major metabolite of DDT (dichlorodiphenyltrichloroethane) and PCBs (polychlorinated biphenyls). These compounds have been hypothesized to be factors for breast cancer however the linkage between these compounds is debatable and not supported by most studies or biological plausibility (Laden et al., 2002; Romieu et al., 2000; Safe, 2004).

1.2.2.4 Lifestyle factors

Various lifestyle factors such as diet, exercise, smoking and alcohol consumption are related to an increased risk of developing breast cancer (Key et al., 2003).

Confirmation of the risk of dietary fat intake and breast cancer has not been substantiated in large epidemiology studies (Smith-Warner et al., 2001; Velie et al., 2000), however a dietary pattern of high fiber and low fat intakes is associated with a lower risk of breast cancer in postmenopausal women (Baghurst and Rohan, 1994; Mattisson et al., 2004; Saadatian-Elahi et al., 2004). In some studies protective effects of some vegetable fats, vitamin E, selenium and other antioxidants have been observed

(Gaudet et al., 2004; Gerber et al., 2003). Decreased ovarian hormone levels and decreased risk of breast cancer in populations in Asia is related to their high consumption of soya products containing significant amount of the isoflavones, daidzein and genistein, that act as weak estrogens (Lu et al., 2000a; Mezzetti et al., 1998).

Obesity causes increased levels of estrogen and other hormones and is linked to increased breast cancer risk particularly in postmenopausal women (Lahmann et al., 2004; Lahmann et al., 2003; van den Brandt et al., 2000). There is increasing evidence that exercise reduces the risk of breast cancer (Lagerros et al., 2004) and there is conflicting data regarding smoking and breast cancer risk (Brunet et al., 1998; Hamajima et al., 2002; Hanaoka et al., 2005; Reynolds et al., 2004). Alcohol is a risk factor for many cancers and an increased breast cancer risk has been observed with high alcohol consumption (Hamajima et al., 2002; Lin et al., 2005; Petri et al., 2004; Singletary and Gapstur, 2001). Other factors such as the use of antibiotics, breast implants, non-steroidal anti-inflammatory drugs and induced abortions are still a topic of debate and more studies are required to resolve their contributions, if any, to breast cancer (Beral et al., 2004; Erlandsson et al., 2003; McLaughlin et al., 1998; Sorensen et al., 2005; Velicer et al., 2004; Zhang et al., 2005).

1.2.3 Role of estrogen in breast cancer

Several studies showed that estrogen is capable of initiating and promoting growth of both carcinogen-induced and spontaneous mammary tumor formation in rats (Broerse et al., 1987; Nandi et al., 1995; Noronha and Goodall, 1984). It is also well established that increased exposure to estrogen is an important risk factor for breast

cancer but the role of estrogen in development of breast cancer has been difficult to ascertain (Henderson et al., 1988; Pike et al., 1993). 17β -Estradiol (E2) is the most biologically active estrogen in breast tissue. The tissue concentrations of E2 is significantly higher in the malignant tissues compared to nonmalignant tissues and is similar in pre- and postmenopausal women despite the decrease in the peripheral plasma levels of E2 by 90% after menopause (Pasqualini et al., 1996; van Landeghem et al., 1985). This suggests specific local biosynthesis and accumulation of the potent estrogen by breast cancer tissue itself (Suzuki et al., 2003).

High concentrations of circulating inactive steroids, including androstenedione and estrone sulfate, are considered to be major precursor substrates for local estrogen production (Santner et al., 1984). Three main enzymes that are involved in estrogen biosynthesis are: aromatase that converts androstenedione and testosterone to estrone and E2, respectively; Estrone sulfatase, that hydrolyses estrogen sulphate to estrone and 17β -hydroxysteroid dehydrogenase type 1 (17β -HSD type 1) that preferentially reduces estrone to E2 in tumor tissues. There is substantial information that human breast cancer tissues contain all the enzymes required for the local biosynthesis of E2 from circulating precursors (Dao et al., 1974; Lipton et al., 1987; Pasqualini et al., 1995; Pasqualini et al., 1986). Several mechanisms have been proposed to explain carcinogenicity of estrogens in breast cancer (Figure 5) (Liehr, 2000). One of them is that the estrogen receptor (ER)-mediated activity of E2 is related to induction of genes critical for regulating the cell cycle and stimulating cell proliferation. These hormone-responsive tumor cells may fix any spontaneous or induced DNA damage resulting in accumulation of genetic changes

and thus establishing a potentially malignant tumor (Cavalieri et al., 2000; Feigelson and Henderson, 1996). However not all breast cancers contain ER α , breast cancer can be ER α - positive (+ve) or ER α -negative (-ve) (Russo and Russo, 2004) and it is important to understand how ER α (+ve) and ER α (-ve) breast cancer arise in order to fully understand the initiation and progression of breast cancer. It is suggested that either ER α (-ve) breast cancers result from the loss of the ability of the cells to synthesize ER α during clinical evolution of ER α (+ve) cancers or that ER α (+ve) and ER α (-ve) cancers arise independently and are different entities (Russo et al., 1999; Brown, 2000). It is postulated that terminal ductal lobular unit in the female breast contain at least three cell types, ER α (+ve) cells that do not proliferate, ER α (-ve) cells that can proliferate and a small proportion of ER α (+ve) cells that can proliferate as well.

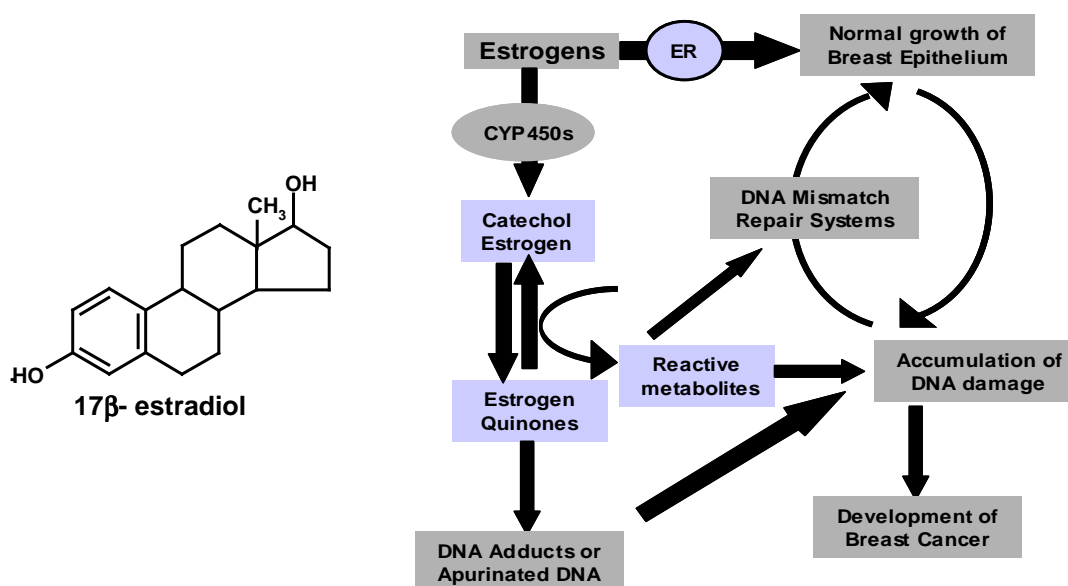


Figure 5. Potential mechanisms of estrogen-induced carcinogenesis in human breast tissues (Russo and Russo, 2004).

Estrogen might stimulate ER α (+ve) cells to produce a growth factor that in turn stimulates neighboring ER α (-ve) cells capable of proliferating. Similarly, ER α (+ve) cells can proliferate and become the stem cells of ER α (+ve) tumors. Also ER α (+ve) cells can convert to ER α (-ve) cells (Figure 6). The role of ER β in breast cancer progression is not yet clear. Hormone receptor status such as ER α and progesterone receptor (PgR) status are important indicators for prescribing hormone and endocrine therapy. Patients with ER or PgR (+ve) tumors tend to have better prognosis and are more likely to respond to hormone therapy compared to patients with ER or PgR (-ve) tumors. Studies in women with early stage breast cancer receiving no adjuvant therapy showed a higher 5-year disease free survival rates in patients with ER α (+ve) tumors compared to patients with ER α (-ve) tumors (Crowe et al., 1991; Fisher et al., 1988).

Another mechanism involves oxidative catabolism of estrogens by various cytochrome p450 complexes. Hydroxylation at the C-16 α position and C2 or C-4 position of E2 are the major pathways that result in formation of hydroxylated estrogens (catechol estrogens). The catechol estrogens can be easily oxidized to DNA-reactive quinones and semiquinones that can cause oxidative stress and genomic damage directly (Ashburn et al., 1993). Reactive free radicals may be produced in the process of oxidation resulting in formation of DNA adducts that can cause additional DNA damage thereby initiating carcinogenesis (Cavalieri et al., 1997; 2000). Estrogen also can stimulate production of autocrine and paracrine growth factors from the epithelium and stroma in the breast that can further contribute to breast cancer progression. Clearly more than one pathway is involved in initiation and progression of estrogen-mediated

breast carcinogenesis and more studies are required to further elucidate the mechanisms underlying the carcinogenicity of estrogen in breast cancer.

1.2.4 Stages of breast cancer

One of the important factors in considering treatment option is the stage of breast cancer, which describes the severity of the original (primary) tumor and the extent of metastasis to other tissues (American Cancer Society, 2005b). The most common system used to describe the stage of a tumor is the American Joint Committee on Cancer (AJCC) TNM system. This staging system classifies cancers based on their T, N, and M stages where **T** stands for **tumor** (its size and how far it has spread within the breast and to nearby organs), **N** stands for spread to lymph **nodes** and **M** is for **metastasis** (AJCC) (Singletary et al., 2002).

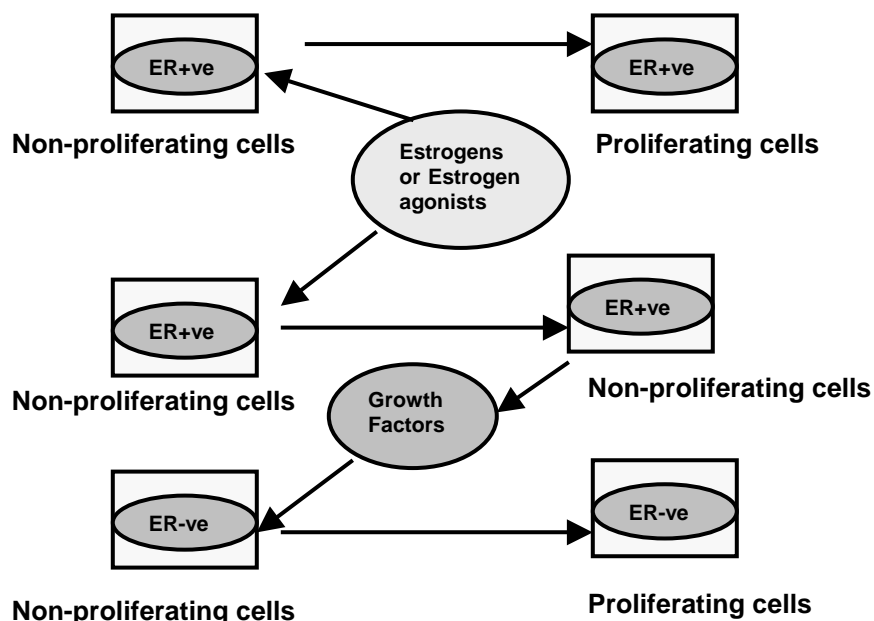


Figure 6. Schematic representation of the postulated pathways of estrogen actions on breast epithelial cells (Russo and Russo, 2004).

Breast cancer has been grouped into five stages based on the description of stages outlined by the AJCC (Singletary et al., 2002) (American Cancer Society, 2005b) (Table 2). The 5-year survival rate shown in Table 2 refers to the percentage of patients who live at least 5 years after their cancer is diagnosed. Stage IIIC was defined only a few years ago and therefore survival rates are not yet available for this stage.

Table 2. Summary of breast cancer stages (modified from American Cancer Society, 2005b).

Stage of tumor	Size (diameter) of the tumor	Spread of the tumor to axillary lymph nodes	Metastasis (M)	5-year relative Survival rate
0	Tiny clusters of cells within the breast duct or within the lobules	No spread	None	100%
I	2 cm or less in diameter	No spread	None	98%,
IIA	No tumor is found in the breast	Tumor is identified in 1 to 3 axillary lymph nodes	None	88%,
	Less than 2 cm	Has spread to 1 to 3 axillary lymph nodes		
	Between 2 and 5 cm	No spread		
IIB	Larger than 2 cm in diameter and less than 5 cm	Has spread to 1 to 3 axillary lymph nodes	None	76%
	Larger than 5 cm and the tumor does not grow into the chest wall	No spread		
IIIA	Any size	Has spread to the axillary lymph nodes and to axillary tissues	None	56%
IIIB	Any size and has attached itself to the chest wall	Has spread to the pectoral (chest) lymph nodes	None	49%
IIIC	Any size	Has spread to 10 or more nodes in the axilla or to 1 or more lymph nodes on the same side as the breast cancer	None	Not available
IV	Any size	May or may not have spread to axillary lymph nodes	Has spread to other parts such as bone, lung, liver, and brain	16%.

1.2.5 Treatment of breast cancer

The incidence of breast cancer appears to be increasing worldwide but the mortality rates are now declining in at least some western countries (Peto et al., 2000). This decrease in mortality is most likely due to increased use of screening for early disease and the effective treatment including widespread administration of adjuvant systemic therapies. Treatment of breast cancer except lobular carcinoma in situ (LCIS) includes the treatment of local disease with surgery, radiation therapy (RT), or both, and the treatment of systemic disease with cytotoxic chemotherapy, biological or hormonal therapy. Treatment decisions are based on a number of prognostic and predictive factors. These factors include tumor histology, clinical and pathologic characteristics of the primary tumor, axillary node status, hormone receptor status, level of HER2 expression, presence or absence of detectable metastatic disease, comorbidity, patient's age and menopausal status. Patient preference is also important in situations in which survival rates are equivalent among the available treatment options (NCCN clinical practical guidelines in oncology). Table 3 lists the most common types of breast cancer.

1.2.5.1 Surgery and radiation

Several surgery procedures including breast-conserving surgery (lumpectomy) (surgical removal of a cancerous lump in the breast along with a small margin of the surrounding normal breast tissue, mastectomy (complete breast removal), sentinel lymph node biopsy (SLNB)(removal of first one to three lymph nodes in the lymphatic chain) and axillary dissection (operation in which 20-30 lymph nodes are removed) are used in breast cancer therapy (Sakorafas, 2001). Radiation therapy uses high-energy rays to

damage the genetic material and inhibit tumor cell growth. The option of surgery and radiation is primarily based on the stage of the breast cancer. Surgery is a standard treatment for stage 0 DCIS cancers. For smaller tumors lumpectomy is preferred followed by radiation therapy to reduce the risk of local recurrence although in some cases women choose to prefer mastectomy. The risk of invasive breast cancer greatly increases if DCIS is left untreated. Patients with stage 0 LCIS have very low risk of developing invasive carcinoma and can be managed with observation alone; however, some women prefer bilateral mastectomy as a risk reduction strategy. Stages II and I are the “early” stages of invasive carcinoma and are considered operable. Together they constitute 75%-80% of all cases of breast cancer. Primary treatment options for stage I and stage II cancers are total mastectomy with axillary lymph node dissection or breast-conserving surgery, axillary dissection, and breast irradiation (Arriagada et al., 1996; Fisher et al., 2002; Veronesi et al., 2002).

Table 3. Summary of the most common types of breast cancer (American cancer society, 2005b).

Type of breast cancer	Description
Ductal carcinoma in situ (DCIS)	Begins in the lining of the milk ducts of the breast and has not invaded the surrounding fatty breast tissue. It is the most common type of noninvasive breast cancer.
Lobular carcinoma in situ (LCIS)	Begins in the lobules where breast milk is produced but they do not penetrate through the wall of these lobules.
Invasive ductal carcinoma	Begins in the milk ducts of the breast and then breaks through the wall of the duct and invades the fatty tissue of the breast. This is the most common form of breast cancer, accounting for 80% of cases.
Infiltrating (invasive) lobular carcinoma	Begins in the lobules of the breast and has spread to surrounding tissues or the rest of the body. It accounts for 10-15% of breast cancers.

Although mastectomy is appropriate for some patients, breast conservation has become the preferred method of treatment for many patients (Singletary, 2001). Radiation is used after lumpectomy or mastectomy, either alone or in combination with chemotherapy, to reduce the risk of breast cancer recurrence. Stage III breast cancers are defined as locally advanced breast cancers in the AJCC system. Stage IIIA tumors are divided in to operable (T3N1M0) and inoperable (TanyN2M0) cancers based on their lymph node status. Stage IIIA operable tumors are treated in the same fashion as stages II and I. In case of inoperable stage III tumors, preoperative chemotherapy is used to shrink the size and this followed by local treatment which involves either total mastectomy with axillary lymph node dissection, with or without delayed breast reconstruction, or lumpectomy and axillary dissection (Favret et al., 2001; Pisansky et al., 1996). Radiation is an important component of multimodal therapy in treatment of stage III tumors (Recht et al., 2001). In stage IV breast cancer, the cancer has spread elsewhere in the body and local treatments like surgery or radiation do not work; however surgery and radiation is used in some cases to relieve pain or other symptoms (Brito et al., 2001; Hortobagyi et al., 1995).

1.2.5.2 Chemotherapy

Chemotherapy is the use of anti-cancer drugs to treat breast cancer. It can be used alone as the main treatment or in combination with radiation or other breast cancer therapies. Adjuvant chemotherapy is the use of systemic therapy to treat microscopic metastasis following surgery and is generally used for patients with early stage breast cancer to reduce the odds of recurrence and death (Bonadonna et al., 1995). Neoadjuvant

therapy (also known as preoperative or induction therapy) is the use of chemotherapy before surgery and is used to treat patients with stage II and stage III operable breast cancer to shrink the size of the tumor (Fisher et al., 1998a; Nabholz et al., 2002; Scholl et al., 1995). Chemotherapy is the main treatment for patients with stage IV breast cancer and is often used in combination with hormone therapy or immunotherapy. For women with ER-negative and PR-negative tumors, symptomatic visceral metastasis, or hormone refractory disease, chemotherapy is the first-line treatment and may include use of sequential single agents or combination chemotherapy. In patients with metastatic or recurrent breast cancer whose tumors overexpress HER2, selected chemotherapeutic agents are considered in combination with trastuzumab (Slamon et al., 2001). Table 4 describes several classes of chemotherapeutic agents used for treatment of breast cancer.

1.2.5.3 Taxanes

The taxanes, paclitaxel (taxol) and docetaxel, are plant alkaloids and are among the most promising new agents for treatment of breast cancer. Taxol was isolated from the Pacific yew (*Taxus brevifolia*), and docetaxel is a semi-synthetic taxane analogue from the European yew (*Taxus baccata*) (Gligorov and Lotz, 2004). Their unique mechanism of action involves binding and stabilizing microtubules, thereby preventing their depolymerization and thus inhibiting cell division. Taxanes can be combined with almost all active chemotherapeutic agents commonly used for breast cancer therapy. Moreover these compounds have improved outcomes in metastatic, adjuvant and neoadjuvant settings and both taxanes exhibit substantial antitumor activity in treatment of anthracycline-resistant breast carcinoma (Piccart et al., 2001; Ravdin et al., 1995).

1.2.5.4 Antiestrogens

The steroid hormone E2 mediates a broad spectrum of physiologic functions ranging from regulation of the menstrual cycle and reproduction to the modulation of bone density, cholesterol transport and cognitive function in elderly women. Earlier work by Beatson and others also showed a connection between ovarian hormonal function and breast cancer and these observations were the initial basis for the concept of antihormone therapy.

Table 4. Summary of chemotherapeutic agents for treatment of breast cancer (Miller and Sledge, 2002).

Class	Mechanism of action
Alkylating agents Cyclophosphamide Thiotepa Ifosfamide	Forms DNA adducts and interferes with DNA replication (Chabner and Roberts, 2005).
Platinum based compounds Cisplatin Carboplatin	Forms DNA adducts and thereby disrupts DNA structure and function (Fuertes et al., 2003).
Anthracyclins Doxorubicin Epirubicin	Intercalates within DNA and inhibits topoisomerase II. The main side effect associated with the use of Doxorubicin is cardiac toxicity thereby limiting its use (Hortobagyi, 2000).
Vinca alkaloids Vincristine Vinblastine Vinorelbine	Inhibits microtubule synthesis and cell division.
Antimetabolites	
5-fluorouracil (5-FU)	Inhibits thymidylate synthetase, the key enzyme that promotes the de novo synthesis of thymidylic acid which leads to the formation of dTTP (Parker and Cheng, 1990; Wurzer et al., 1994).
Methotrexate (MTX)	It is a folic acid analogue and inhibits the enzyme dihydrofolate reductase (DHFR) activity thereby blocking DNA synthesis (Huennekens, 1994).
Capecitabine	It is an oral fluoropyrimidine that generates 5-fluorouracil preferentially at the tumor site.
Gemcitabin (dFdC).	Causes cell death due to DNA chain termination (Plunkett et al., 1995).

Ovarian ablation including oophorectomy and ovarian irradiation has been used for more than a century in the treatment of breast cancer (Conte et al., 1989; Prowell and Davidson, 2004). A meta-analysis by the Early Breast Cancer Trialists Collaborative Group of 12 properly designed randomized trials reported significant greater disease-free and overall survival rates for women under the age of 50 receiving ovarian ablation as a single adjuvant therapy and this was independent of nodal status (Ovarian, 1996).

In addition chemical suppression of ovarian estrogen production has been accomplished via the administration of luteinizing hormone releasing hormone (LHRH) analogues which have been used for breast cancer chemotherapy (Clarke, 1998; Prowell and Davidson, 2004). Goserelin (Zoladex) is a LHRH analogue approved by the United States Food and Drug Administration (FDA) for the treatment of premenopausal women with advanced breast cancer. Ovarian suppression is a preferred option treatment over ovarian ablation due to lower morbidity. There is also a lower likelihood of permanent amenorrhea and potential for restoration of fertility. Several clinical trials combining the antiestrogen tamoxifen with ovarian ablation/suppression showed that combination therapy is superior to monotherapy (Klijn et al., 2001).

Unlike estrogens, which are uniformly agonists, and antiestrogens, which are uniformly antagonists, selective estrogen receptor modulators (SERMs) are a new category of therapeutic agents that display an unusual tissue-selective pharmacology (Figure 7). They are agonists in some tissues (bone, liver, and the cardiovascular system), antagonists in other tissues (brain and breast), and mixed agonists/antagonists in the uterus. An ideal SERM will mimic estrogen's critical benefits for the bones and heart but

will act as antiestrogens in the breast and uterus, thus avoiding estrogen's harmful effects in these tissues (Lewis and Jordan, 2005). Tamoxifen (Nolvadex) is the first SERM used in treatment of breast cancer that acts as an antagonist in breast cancer tissue (Cole et al., 1971; Jordan, 1976) but preserves bone density and lowers serum cholesterol levels (Bertelli et al., 1988; Powles et al., 1996; Zidan et al., 2004). Studies with tamoxifen showed response rates of about 70 % in patients with advanced breast cancer whose tumors express both ER and PgR whereas less than 10% response rate was observed in patients with ER and PgR negative breast cancer (Ravdin et al., 1992). In patients with ER-positive breast cancer, tamoxifen reduces the annual recurrence rate by 50% and annual death rate by 28% (Wickerham, 2002).

Tamoxifen is the most widely used hormonal treatment for all stages of breast cancer including advanced breast cancer, as an adjuvant treatment of primary breast cancer and adjuvant therapy in premenopausal and postmenopausal women with node-negative disease. It is the first line treatment for women with hormone-responsive breast cancer. The Breast Cancer Prevention Trial demonstrated that tamoxifen can be used as a preventive therapy because it reduced the incidence of ER-positive breast cancer by about 50% (Fisher et al., 1998b; Fornander et al., 1989). Thus, in adjuvant settings tamoxifen has been approved for the prevention of breast cancer in high-risk women. However, the major problems associated with the prolonged use of tamoxifen is the increased risk of endometrial cancer, thrombosis and development of resistance to tamoxifen (Cuzick et al., 2003; Fisher et al., 1994; O'Regan and Jordan, 2002). Resistance to tamoxifen can be either intrinsic, where patients with advanced ER-

positive disease fail to respond to tamoxifen or acquired, where ER-positive tumors in patients that initially respond subsequently progresses to a resistant phenotype. The possible causes for intrinsic and acquired resistance have been attributed to the pharmacology of tamoxifen, alterations in the structure, expression and function of the ER, interactions with the tumor environment and genetic alterations in the tumor cells (Clarke et al., 2003; Jordan, 2004). For example alterations in the ER signal transduction pathways and altered levels and/or activities of coactivators and corepressors can convert the inhibitory tamoxifen-ER complex to a growth stimulus resulting in acquired resistance (Lewis and Jordan, 2005).

In addition, ligand-independent ER action by aberrant expression/activation of growth factor receptors or aberrant growth factor signaling, can also contribute to tamoxifen resistance (Nicholson et al., 2005). For example overexpression of HER2 and p160 coactivator, AIB1, in breast tumors have been associated with tamoxifen resistance (Shou et al., 2004). Overexpression of the HER-2/neu gene is a frequent event in about 30% of breast cancer and overall survival rates of breast and ovarian cancer patients whose tumors overexpress HER-2/neu are significantly lower than those of patients whose tumors do not overexpress HER-2/neu (Muller et al., 2004; Slamon et al., 1989). It is suggested that high levels of HER2 results in activation of MAPK that activates AIB1 and enhances the AIB1-mediated ER activity. This results in increase in estrogenic activity of tamoxifen (List et al., 2001; Osborne et al., 2003). This has led to further developments of SERMS such as toremifene, droloxifene, idoxifene, raloxifene, and arzoxifene (Figure 7). These compounds exhibit minimal activity for treatment of

tamoxifen-resistant disease and are not any more effective than tamoxifen as a first-line treatment for breast cancer (Jordan, 2004; Robertson, 2004). However, some of these compounds such as raloxifene increases bone mineral density in postmenopausal women and have minimal side effects in the uterus (Cummings et al., 1999; Delmas et al., 1997). Moreover the results from MORE (The Multiple Outcomes of Raloxifene Evaluation trial) showed that raloxifene reduced the risk of invasive breast cancer by 72% and reduced the risk of ER-positive invasive breast cancer by 84% compared with placebo (Cauley et al., 2001). This led to the initiation of STAR trial to compare tamoxifen and raloxifene for the reduction in the risk of breast cancer. Raloxifene is currently available for prevention of osteoporosis.

Fulvestrant (Faslodex, formerly ICI 182,780) is a potent steroidal antiestrogen that mediates its effects by downregulating ER expression without exerting any partial ER agonist activities (Robertson et al., 2005; Robertson et al., 2001) (Figure 8).

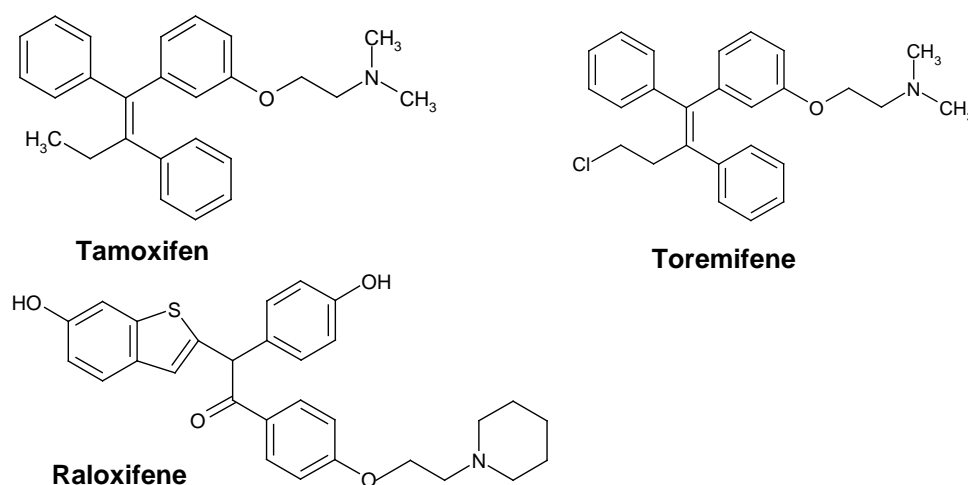


Figure 7. Structures of selected SERMS (pearce and Jordan, 2004).

Fulvestrant represents an additional treatment option for postmenopausal women with advanced breast cancer whose disease progresses after tamoxifen therapy (Howell et al., 2004; Osborne et al., 2002).

Aromatase inhibitors (AIs) form another class of compounds that act by inhibiting estrogen biosynthesis. Anastrozole, letrozole, and exemestane are three commercially available aromatase inhibitors approved by the US Food and Drug Administration for treatment of hormone receptor-positive breast cancer in postmenopausal women (Figure 8) (Choueiri et al., 2004). AIs act by inhibiting cytochrome P450-dependent aromatase activity that converts testosterone to estrogen and androstendione to estrone in the final steps of estrogen biosynthesis.

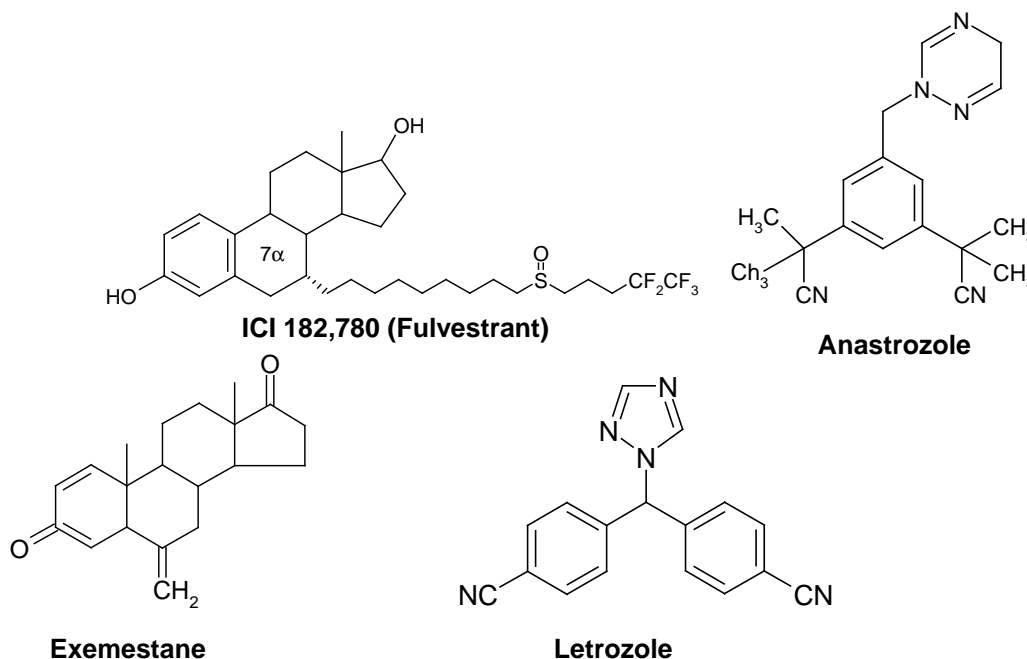


Figure 8. Structures of selected antiestrogens (O'Regan and Jordan, 2002).

Anastrozole and letrozole are nonsteroidal aromatase inhibitors that bind reversibly to aromatase whereas exemestane is a steroidal inhibitor that competitively binds to the substrate-binding site of the enzyme forming irreversible covalent bonds that result in permanent enzyme inactivation (Choueiri et al., 2004; Osborne and Tripathy, 2005). AIs have been used in treating advanced breast cancer but their utility in treating early disease has not been completely evaluated. Side effects associated with AIs are the increased risk of osteoporosis, and/or bone fracture (Nabholtz et al., 2003).

1.2.5.5 Herceptin

HER2 or c-erbB2 oncogene encodes a 185-kDa transmembrane cell surface receptor with intrinsic tyrosine kinase activity showing homology to the epidermal growth factor receptor (EGFR or c-ErbB) and is involved in normal cell proliferation and tissue growth. Multiple studies demonstrated that HER2 amplification/overexpression occur in 25–30% of human breast cancers and is associated with poor prognosis, short survival and recurrences (Slamon et al., 1987; Stefano et al., 2004; Yamashita et al., 2004). It has been reported that patients with elevated HER2 levels show poor response to antiestrogens or chemotherapy (Jukkola et al., 2001; Lipton et al., 2002; Rosenthal et al., 2002; Slamon et al., 1987). HER2/neu has been used as both a prognostic marker for node positive patients and predictive marker for response to adjuvant chemotherapy, endocrine therapy (tamoxifen), and herceptin treatment (Yamauchi et al., 2001). Herceptin (Trastuzumab) is a therapeutic monoclonal antibody that specifically binds to the extracellular portion of HER2 and is now regarded as a therapeutic option for treating HER2-overexpressing metastatic breast cancers (Ross et

al., 2004; Slamon et al., 2001). It has been reported that with increasing amounts of HER2/Neu oncoprotein on the cell membrane, the success of herceptin immunotherapy is increased (Baselga et al., 1998; Masood and Bui, 2002; Molina et al., 2001). There is a moderate response rate in women treated with herceptin as a single agent however in combination with chemotherapy greater response rates and prolonged survival of women with advanced breast cancer is observed (Bell, 2002). Cardiac toxicity is associated with the use of herceptin in combination therapy with anthracyclines (Bell, 2002; Seidman et al., 2002; Slamon et al., 2001). Ongoing clinical trials are examining the potential role of herceptin in neoadjuvant and adjuvant settings for treatment of HER2-positive breast cancer (Baselga et al., 2004; Burstein et al., 2003).

1.2.5.6 Vitamin D analogues

1,25-Dihydroxyvitamin D3 [1,25(OH)₂D₃], the biologically active form of vitamin D that interacts with the vitamin D receptor (VDR), has been recognized as a regulator of proliferation, differentiation, and survival of breast cancer cells (Colston and Hansen, 2002; Welsh, 2004). Various studies suggest a possible role for Vitamin D in prevention and treatment of breast cancer (Lipkin and Newmark, 1999; Shin et al., 2002). Several mechanisms have been proposed to explain the anti-tumor effects of vitamin D and its analogues in breast cancer (Colston and Hansen, 2002). One of them is the induction of apoptosis by decreasing the relative expression of anti-apoptotic (bcl-2/ bcl-XL) to pro-apoptotic family members such as bax and bak and by upregulating apoptotic related proteins such as clusterin, cathepsin B and transforming growth factor β (TGF β) (James et al., 1998; Simboli-Campbell et al., 1997). In addition,

several studies have demonstrated that pretreatment of breast cancer cells with 1,25(OH)₂D₃ or active vitamin D analogues potentiates the effects of TNF α on induction of apoptosis and this is attributed to enhanced accumulation of ceramide and cytosolic phospholipase A₂ (cPLA₂) activation (Mathiasen et al., 2001; Pirianov et al., 1999).

Several studies also show that 1,25(OH)₂D₃ and its analogues inhibit breast cancer cell growth by regulating the expression of certain cell cycle regulators to bring about G₁ arrest. This include increase in expression of cyclin-dependent kinase (cdk) inhibitors such as p21 and p27 and dephosphorylation of the retinoblastoma protein (Fan and Yu, 1995; Hansen et al., 2001; Wu et al., 1997). An additional mechanism by which vitamin D derivatives may influence breast cancer cell growth is through modulation of mitogenic pathways mediated by insulin-like growth factor I (Vink-van Wijngaarden et al., 1996; Xie et al., 1999) and the ER (James et al., 1994; Swami et al., 2000).

The potential anti-tumor properties displayed by naturally occurring 1,25(OH)₂D₃ (Figure 9) are limited by the tendency of this compound to cause hypercalcaemia when administered at high doses. This led to development of synthetic vitamin D analogues that retain the anti-tumor activity but have reduced calcaemic activity. (Colston and Hansen, 2002). Seocalcitol (EB1089), a vitamin D analogue showed markedly reduced calcaemic activity in vivo and also in phase I study of patients with advanced breast cancer (Gulliford et al., 1998). Various other synthetic vitamin D analogues inhibit breast tumor growth in vitro and in vivo and are presently in clinical trials.

1.2.7.7 Retinoids

Retinoids are a group of natural and synthetic vitamin A analogs that are known to possess antiproliferative, differentiative, immunomodulatory, and apoptosis-inducing properties. Retinoids bind to various retinoid acid binding proteins in the cytosol, which control their availability for interactions with nuclear retinoid receptors, the retinoic acid (RA) receptors (RARs) and retinoid X receptors (RXRs). These receptors form RXR–RAR heterodimers, as well as RXR–RXR homodimers and bind to the retinoic acid response elements (RAREs) in the upstream region of various genes (Toma et al., 2005; Yang et al., 2002). Retinoids suppress breast cell growth as well as tumor growth and development in several animal models by inhibiting mitogenic pathways and inducing genes linked to growth inhibition, differentiation or apoptosis (Yang et al., 1999). In several human clinical breast cancer trials naturally occurring retinoids were found to be highly toxic and most current trials are using synthetic retinoids. LGD1069 (targretin), an RXR-selective retinoid and fenretinide {N-[4-hydroxyphenyl] retinamide (4-HPR)} (Figure 9), a selective activator of RAR, are the most widely used retinoids in chemoprevention clinical trials (Decensi et al., 2003).

Fenretinide has very low toxicity associated with its use and in a phase III secondary prevention trial with this compound there was a trend in reduction of second breast malignancies in premenopausal but not in postmenopausal women (Camerini et al., 2001; Menard et al., 2001). LGD1069 effectively suppressed ER-negative tumor development in mouse mammary tumor virus-erbB2 transgenic mice with minimal toxicity (Wu et al., 2002).

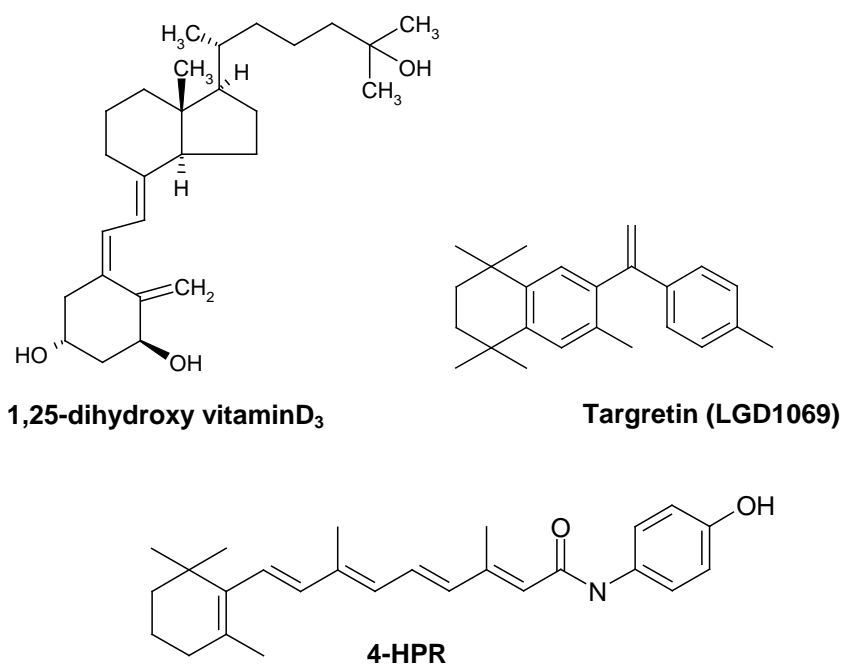


Figure 9. Structures of vitamin D and synthetic retinoic acid analogues.

Bexarotene is a RXR-selective retinoid that exhibited antitumor activity in a breast cancer preclinical trial, but the efficacy for treatment of patients with refractory metastatic breast cancer was limited in a multicenter phase II study (Esteva et al., 2003).

1.2.5.8 PPAR γ ligands

The peroxisome proliferator-activated receptors (PPARs) are ligand-activated transcription factors and belong to nuclear hormone receptor (NHR) superfamily. PPAR γ plays an important role in diverse metabolic pathways including differentiation of adipocytes and macrophage foam cells, control of peroxisome lipid metabolism, maintenance of insulin sensitivity, atherosclerosis, and hypertension (Chen et al., 1993; Dreyer et al., 1992). Upon ligand binding, PPARs heterodimerizes with one of the retinoid X receptors (RXRs) and binds to DNA sequences called PPAR response

elements (PPREs) in the promoters of target genes and thereby act as transcriptional regulators (Jowharji et al., 1992; Osumi et al., 1996).

Naturally occurring PPAR γ ligands include several polyunsaturated fatty acids (PUFAs) (Issemann et al., 1993; Thoennes et al., 2000) and eicosanoids such as 15-deoxy- $\Delta^{12,14}$ -prostaglandin J₂ (PGJ₂). In addition, there are variety of synthetic ligands including non-steroidal anti-inflammatory drugs (NSAIDs) such as indomethacin and ibuprofen, and thiazolidinediones (TZDs) such as ciglitazone, troglitazone, pioglitazone, rosiglitazone and LY171.833 (Lehmann et al., 1995). Pioglitazone and rosiglitazone have been used clinically for treatment of insulin-resistant type II diabetes (Girnun and Spiegelman, 2003; Theocharis et al., 2004) (Figure 10). The endogenous ligand(s) for PPAR γ is still unclear although PGJ₂ may be the most potent endogenous ligand of PPAR γ (Forman et al., 1995; Murphy and Holder, 2000).

PPAR γ is expressed in several breast cancer cell lines such as MCF-7, BT474, T47D, and MDA-MB-231 cells and in human primary and metastatic breast adenocarcinomas (Clay et al., 1999; Grommes et al., 2004; Kilgore et al., 1997; Mueller et al., 1998). Several studies have shown that PPAR γ ligands inhibit proliferation of breast cancer cell lines and breast cancer tissue cultured from patients and this has been associated with cell cycle arrest in G1 phase, induction of apoptosis, enhanced expression of cyclin-dependent kinase inhibitors p21 and p27 (Clay et al., 1999; Elstner et al., 1998; Forman et al., 1995) and downregulation of cyclin D1 expression (Qin et al., 2003; Wang et al., 2001a; Yin et al., 2001).

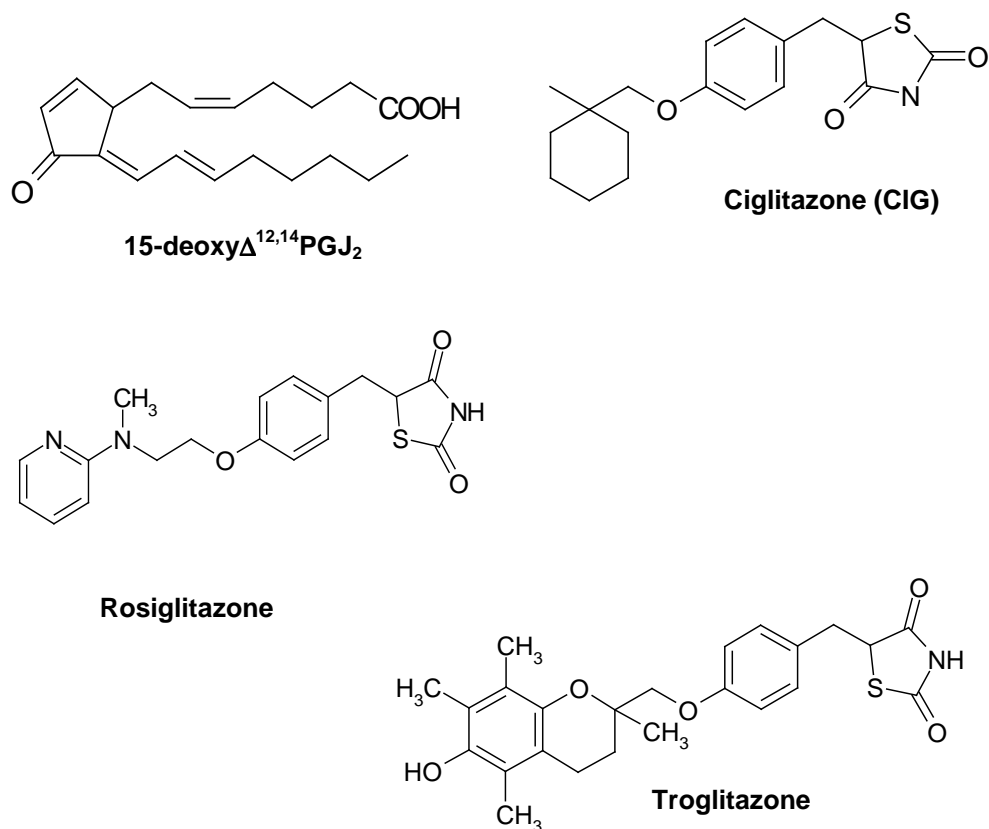


Figure 10. Chemical structures of selected PPAR γ agonists (Theocharis et al., 2004).

PGJ₂ and TGZ inhibit proliferation of both estrogen receptor-negative breast cancer cell line MDA-MB-231 and estrogen receptor-positive MCF-7 breast cancer cells and induce morphological changes associated with apoptosis, including cellular rounding, blebbing, the production of echinoid spikes, blistering and cell lysis in both the cell lines (Clay et al., 1999). Pighetti et al. (2001) reported that troglitazone (TGZ) treatment resulted in regression or stasis of total tumor volume in 40–50% of the dimethylbenzanthracene (DMBA)-induced mammary tumors in rats. Elstner and co-workers showed that combination of an RAR agonist, all-*trans*-retinoic acid (ATRA) and

TGZ caused irreversible inhibition of MCF-7 cell growth and induced apoptosis associated with a dramatic decrease of bcl-2 protein levels (Elstner et al., 1998). Furthermore the same group demonstrated significant inhibition of MCF-7 tumor growth in triple immunodeficient mice by TGZ alone, and combined administration of TGZ and ATRA resulted in apoptosis and fibrosis of these tumors without any toxic side-effects (Elstner et al., 1998). TGZ also prevented formation of preneoplastic mammary lesions in DMBA-treated murine mammary glands (Mehta et al., 2000) and the selective PPAR γ agonist GW7845 delayed tumor formation in a mouse mammary carcinogenesis model (Yin et al., 2005).

PGJ₂ blocked phosphorylation of epidermal growth factor receptor 2 (ErbB2) and ERbB3 and interfered with ErbB signaling pathways in MCF-7 cells and this was accompanied with dramatic growth-suppressive effects, accumulation of cells in the G0/G1 and a marked increase in apoptosis (Pignatelli et al., 2001). Moreover, Patel et al. showed that the activation of PPAR γ by rosiglitazone upregulated PTEN expression and this resulted in reduced proliferation of MCF-7 cells (Patel et al., 2001).

A phase II study on the use of troglitazone for treating patients with advanced refractory breast cancer failed to show any clinical benefits. However, the study was incomplete because troglitazone was withdrawn from commercial availability (Burstein et al., 2003). This is the only published clinical trial using a PPAR γ ligand for treatment of breast cancer. A recent study showed that somatic mutations of the PPAR γ gene is a very rare event in human malignancies suggesting that PPAR γ is unlikely to act as a tumor suppressor gene and thus can be a stable and suitable target for T2D cancer

therapy (Ikezoe et al., 2001; Posch et al., 2004). However, a better understanding of mechanisms underlying the antineoplastic effects of PPAR-gamma ligands will be needed before they may be useful in the treatment of breast cancer patients.

Other targeted therapies for treatment of breast cancer that are underway include anti-angiogenesis factors that inhibit VEGF signaling pathways, matrix metalloproteinase inhibitors, proteasome inhibitors, COX-2 inhibitors, cell cycle inhibitors, tyrosine kinase inhibitors and epidermal growth factor receptor blockers (Awada et al., 2003). The role of selective aryl hydrocarbon receptor modulators (SAhRMs) in treatment of breast cancer will be discussed in section 1.7.9.

1.2.6 Genomic analysis in breast cancer

Breast cancer is a complex heterogeneous disease and evaluation of a small panel of markers provides only limited prognostic information. However, high-throughput gene expression profiling such as microarrays is a promising new technology capable of simultaneously measuring thousands of genes that can help to construct molecular fingerprints of individual tumors. These fingerprints have been applied to the classification of breast cancers, to prognosis, and to prediction of response to treatment (Chang et al., 2005; Esteva and Hortobagyi, 2004). Perou and colleagues were the first to classify invasive breast carcinoma into five subtypes based on their distinct gene expression profiles using cDNA microarray (Perou et al., 2000). These included luminal cell-like group (subtypes A & B), a basal cell-like group lacking ER expression, an ErbB2-positive group and a normal-epithelial group. In a subsequent study it was shown that luminal-type had a more favorable prognosis compared to the basal-like group

(Sorlie et al., 2001). Another group used a different DNA microarray platform and identified a 'poor prognosis signature' that included 70 genes involved in regulation of the cell cycle, in invasion, in metastasis, and in angiogenesis (van 't Veer et al., 2002). This signature was validated by the same group in a study of 295 patients with primary breast cancer, where they found that at 10 years, probability of remaining free of distant metastases was 50.6% in the group with a poor-prognosis signature and was 85.2% in the group with a good-prognosis signature. This signature has a strong independent prognostic value that is currently being validated for clinical and diagnostic applications (Sorlie et al., 2003; van de Vijver et al., 2002). In addition, several clinical trials are evaluating the predictive value of gene expression profiles in patients with early-stage breast cancer.

Chang and colleagues have shown that gene profiling can be used in accurately predicting response to neoadjuvant docetaxel (Chang et al., 2003) and another group recently showed that gene expression profiles could predict complete pathologic response to neoadjuvant paclitaxel, fluorouracil, doxorubicin, and cyclophosphamide (Ayers et al., 2004). Genome-wide approaches have great potential in unraveling insights into tumor biology that can accurately predict the outcome and prognosis of individual breast cancer patients, however, the optimum use of such techniques in clinical studies requires further optimization and standardization of reporting procedures, validation of gene sets across platforms and replication of results in carefully planned prospective studies (Ahmed and Brenton, 2005; Gradishar, 2005).

1.3 Transcription

1.3.1 Introduction

The flow of genetic information in all living cells, from bacteria to humans, occurs from DNA to RNA (transcription) and from RNA to protein (translation). This is the central dogma of molecular biology. Transcription is a process by which an RNA molecule is produced from the coding region of DNA and this process is catalyzed by the multisubunit enzyme called RNA polymerase. In eukaryotes there are three different RNA polymerases that transcribe different classes of genes; RNA polymerase I which transcribes 5.8S, 18S and 28S ribosomal RNA (rRNA) (Comai, 2004; Hannan et al., 1998; Larson et al., 1991), RNA polymerase II (RNAP II) that transcribes all protein coding genes in addition to some of the small nuclear RNAs (snRNAs) involved in RNA splicing and RNA polymerase III that transcribes transfer RNA (tRNA), 5S rRNA, Alu-RNA, some snRNA and small cellular RNAs (scRNA)(Bogenhagen et al., 1980; Chedin et al., 1998; Paule and White, 2000; Schramm and Hernandez, 2002; Sharp et al., 1981).

Regulation of gene expression is critical in development, growth, survival and maintenance of cellular and organism homeostasis. A large number of human diseases including cancer involve the dysregulated expression of genes (Villard, 2004).

Transcription initiation is one of the important steps in controlling eukaryotic gene expression and insight into mechanisms of transcription is critical in understanding the development and progression of cancer. The following sections will review the mechanism of RNA polymerase II mediated transcription.

1.3.2 Core promoter and other DNA elements

The core promoter is the site of action of the RNA polymerase II transcriptional machinery and is defined as the minimal stretch of contiguous DNA sequence that is sufficient to direct accurate initiation of transcription by the general (or basal) RNA polymerase II machinery (Butler and Kadonaga, 2002). Various sequence motifs that are commonly found in core promoters include the TATA box, initiator (Inr), TFIIB recognition element (BRE) located upstream of some TATA boxes (Evans et al., 2001; Lagrange et al., 1998), and downstream core promoter element (DPE) (Figure 11). The core promoter may contain some, all or none of these motifs.

The TATA box (also named the Goldberg-Hogness box) was the first core promoter element identified in eukaryotic protein-coding genes (Breathnach and Chambon, 1981). It has a consensus sequence of TATAAA and is located 25 to 30 bp upstream in higher eukaryotes and 40 to 120 bp upstream in yeast from the initiation start site (Wobbe and Struhl, 1990). The TATA box is the binding site for the TATA-binding protein (TBP) that directs the assembly of the pre-initiation complex (Patikoglou et al., 1999). There are many other upstream promoter sequences which differ from the consensus TATA sequence but still can function as TATA boxes and can interact with TBP (Singer et al., 1990). However many promoters do not contain TATA-boxes or even TATA-like sequences; for example a database analysis of human genes revealed that TATA boxes were present in only 32% of 1031 potential core promoters (Suzuki et al., 2001) suggesting the importance and presence of other elements in RNAP II core promoters such as Inr, DPE and others.

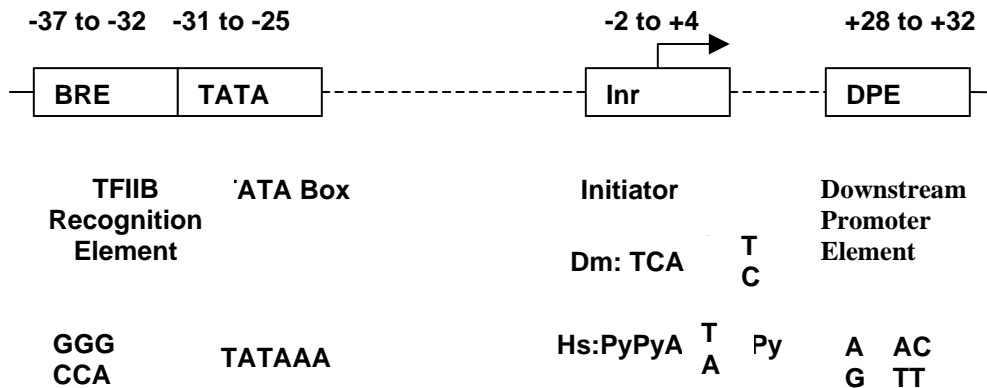


Figure 11. Core promoter elements for RNAP II. The DPE consensus was determined with *Drosophila* core promoters. The Inr consensus is shown for both humans (Hs) and *Drosophila* (Dm) (Butler and Kadonaga, 2002).

The Initiator (Inr) element consists of transcription initiation site and studies have shown that it is functionally similar to the TATA box and can function independently of TATA box (Smale and Baltimore, 1989). Inr is found in both TATA containing as well as TATA-less core promoters (Corden et al., 1980; Javahery et al., 1994; Smale et al., 1998). The TFIID complex is essential for Inr activity (Smale and Kadonaga, 2003) and studies of TATA-Inr spacing revealed that the two elements act synergistically when separated by 25–30 bp but act independently when separated by more than 30 bp (O'Shea-Greenfield and Smale, 1992). The DPE element on the other hand is located precisely at +28 to +32 relative to the A₊₁ nucleotide in the Inr motif and is typically found in TATA-less promoters acting in conjunction with the Inr (Burke et al., 1998; Kutach and Kadonaga, 2000). The DPE element binds TFIID and mutation of either the DPE or the Inr results in loss of TFIID binding and basal transcription activity from the

DPE core promoter elements (Burke and Kadonaga, 1996). In addition there are 0.5–2 kbp stretches of DNA called CpG islands in the human genome that are associated with approximately half of the promoters for protein-coding genes (Suzuki et al., 2001). These usually lack TATA boxes, DPE elements, or Inr elements and are characterized by the presence of multiple binding sites for the transcription factor Sp1 (Macleod et al., 1994). They also possess multiple transcription start sites that span a region of 100 bp or more and are often located 40–80 bp downstream of the Sp1 sites. Sp1 has been suggested to direct the basal machinery to form a preinitiation complex and mediate transcription initiation in these promoters (Blake et al., 1990; Smale et al., 1990).

Other *cis*-acting DNA sequences that regulate RNAP II transcription include the enhancers or upstream activating sequences, silencers, and boundary/insulator elements. Enhancers bind to activators and activate transcription whereas silencers bind to the repressors and repress transcription. These elements can be located many kbp away from the transcription start site and regulate the transcription of target genes.

Boundary/insulator elements appear to prevent the spreading of the activating effects of enhancers or the repressive effects of silencers or heterochromatin (West et al., 2002).

1.3.3 Eukaryotic transcription factors

Eukaryotic transcription by RNAP II requires the close concert of large number of proteins collectively termed transcription factors (Hahn, 2004a; Lee and Young, 2000). Transcription factors are generally divided into three groups: (1) general/ basal transcription factors which are ubiquitous and include RNA polymerase II (Pol II) and a set of accessory general transcription initiation factors (GTFs)(Orphanides et al., 1996);

(2) gene-specific transcription regulators such as activators and repressors that modulate the rate of transcription of specific target genes in a tissue- and developmental stage-specific manner or in response to physiological or environmental stimuli; (3) transcription cofactors/co-regulators (coactivators and corepressors) or mediator complexes which interact with regulators and mediate the effects of regulators on the general/basal transcription machinery either via direct physical interactions with GTFs and/or RNAP II or indirectly through modification of chromatin structure.

1.3.4 Pre-initiation complex (PIC) formation and Initiation of transcription

A breakthrough in understanding the mechanism of transcription initiation followed the discovery that purified mammalian RNAP II would selectively initiate transcription from template DNA when supplemented with a crude cell extract (Weil et al., 1979). This led to identification of the GTFs (Orphanides et al., 1996) and subsequent studies revealed that the basal transcription by RNAP II requires a coordinated assembly of a stable pre-initiation complex (PIC) composed of DNA, RNAP II, and six GTFs (Dvir et al., 2001). GTFs include transcription factor (TF) IIA, TFIIB, TFIID, TFIIE, TFIIIF, and TFIIH. TFIID is composed of TBP plus about fourteen TBP-associated factors (TAFs). Table 5 summarizes the subunit composition and function of human GTFs. Moreover the crystal structures and models of RNAP II (Cramer et al., 2000; Westover et al., 2004), structures of several GTFs and structures of RNAP II interacting with GTFs combined with biochemical and genetic studies have provided novel insights into the mechanism of transcription (Bushnell et al., 2004; Chen and Hahn, 2003; Chung et al., 2003).

Table 5 Human general transcription initiation factors (Martinez et al., 2002).

Factor	Number of subunits	Mass in (Kda) of subunits	Function
TF IID TBP	1	38	Core promoter recognition (TATA box); TFIIB recruitment
TFIID TAFIIS	14	250, 150, 140, 135, 100, 80, 55, 43, 31, 30, 28, 20, 18, 15	Core promoter recognition/selectivity (non-TATA elements); regulate TBP functions; coactivators; protein kinase, histone acetyltransferase, and H1 ubiquitylase activities (TAF250).
TFIIA	3	34 (α), 19 (β), 12 (γ)	Stabilization of TBP-DNA and TAF-DNA interactions; anti-repression and coactivator functions.
TFIIB	1	35	Stabilization of TBP-DNA interactions; recruitment of Pol II-TFIIF; start site selection by Pol II.
TFIIF	2	74(RAP74), 30 (RAP30)	Recruitment of Pol II to promoter DNA-TBP-IIB complex; destabilization of non-specific Pol IIDNA interactions; facilitates Pol II elongation.
Pol II	12	220-10 (RPB1-12)	RNA polymerase (pre-mRNA synthesis); recruitment of TFIIIE; role of its CTD domain in interactions with mediator complex, elongator complex and pre-mRNA processing factors.
TFIIIE	2	57 (α), 34 (β)	TFIIH recruitment, modulation of TFIIH helicase ATPase and kinase activities; facilitates promoter melting.
TFIIH	9	89 (ERCC3/XPB), 80 (ERCC2/XPD), 41 (CDK7), 38 (cyclin H), 34, 32 (MAT1), 62, 50, 44 (hSSL1).	Promoter melting (open complex); helicase (XPB and XPD); CTD kinase (CDK7); role in nucleotide excision repair.

The first step in PIC assembly is nucleated by binding of the TBP, a subunit of TFIID, to the TATA box (Woychik and Hampsey, 2002). The core domain of TBP binds the minor groove of the TATA box as a molecular saddle and induces a sharp bend in the DNA toward the major groove by partial unwinding of base pairs (Kim et al., 1993). TBP is essential for initiation of transcription by all three RNAP I, II, and III (Hernandez, 1993). However, at TATA-less promoters with Inr or DPE elements, basal transcription

is facilitated by interaction of other factors such as TAFs and initiator-dependent cofactors (TICs) (Burke and Kadonaga, 1996; Smale, 1997). The binding of TFIID to the core promoter is stabilized by TFIIA that binds to the DNA sequences upstream of the TATA element (Sun et al., 1994; Tan et al., 1996). This complex is further stabilized by association with TFIIB that contacts both TBP and DNA sequences and help recruit RNAP II and other factors to the complex (Butler and Kadonaga, 2002; Nikolov et al., 1995; Tsai and Sigler, 2000).

There are two models proposed for the recruitment of TFIIF, RNAP II, TFIIE and TFIIH following the formation of TF IID-IIA-IIB-DNA complex (Figure 12). The first model is based on the in vitro experiments with purified factors which suggests sequential recruitment of TFIIF plus unphosphorylated RNAP II, TFIIE and TFIIH resulting in fully assembled or complete PIC formation. Figure 12 is a schematic representation of PIC formation. The alternative model suggests the possibility that the TFIID/TBP-IIA-IIB-DNA complex is recognized in a single step by a pre-assembled Pol II holoenzyme. This is based on the discovery that the hypophosphorylated form of Pol II in yeast and mammalian cells, can be found associated with a variety of factors in large holoenzyme complexes in solution (Hengartner et al., 1995; Koleske and Young, 1994; Ossipow et al., 1995) . The holoenzyme generally includes TFIIF, TFIIE, TFIIH, and components of the multi-subunit cofactor complex called Mediator (Malik and Roeder, 2000; Thompson et al., 1993). Mediator is an essential component of the RNAP II general transcriptional machinery and plays a crucial part in the activation and repression of eukaryotic mRNA synthesis (Conaway et al., 2005b; Myers and Kornberg,

2000). In either case, once the PIC complex is formed, the next step is to form an open complex. TFIIH has two ATP-dependent DNA helicases of opposite polarity (XPB and XPD) and a cyclin-dependent protein kinase (cdk7-cyclin H) (Tirode et al., 1999).

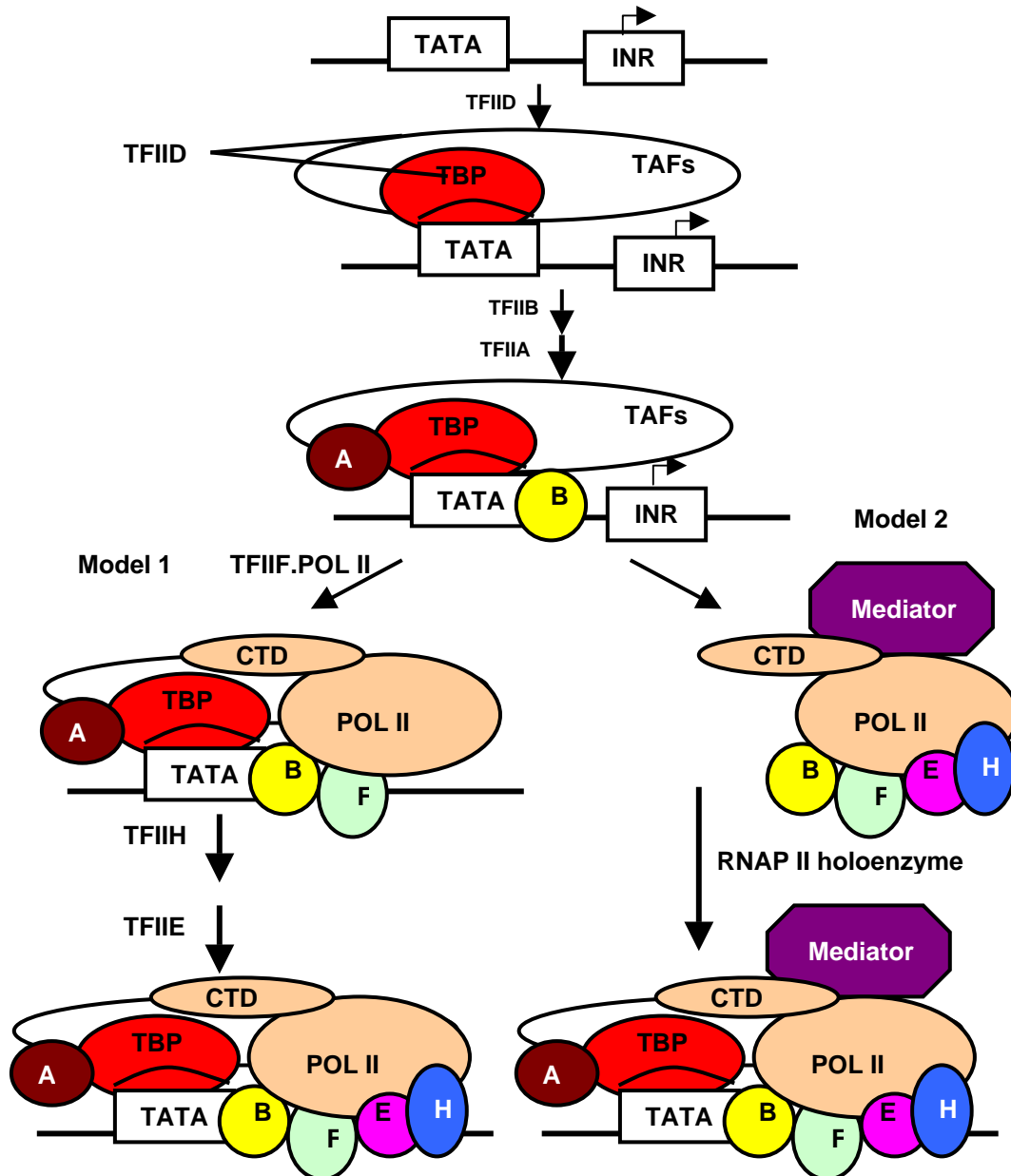


Figure 12. Schematic representation of alternative pathways of PIC assembly (Martinez et al., 2002).

TFIIIE activates the helicase activity associated with TFIIH. The helicase once activated catalyzes ATP-dependent unwinding of the DNA template at the transcriptional start site and promoter melting to form the open complex (Douziech et al., 2000; Kim et al., 2000; Kim et al., 1997). Once the open complex has formed, initiation of transcription begins with synthesis of the first phosphodiester bond of RNA. In many systems, RNAP II synthesizes multiple short RNAs (of three to ten bases), termed abortive products, before it initiates productive synthesis of full-length RNAs (Holstege et al., 1997).

The next step after initiation is phosphorylation of the CTD (carboxy-terminal domain), the largest subunit of Pol II, which facilitates promoter clearance and progression into the elongation phase of transcription by disrupting interactions of the CTD with components of the PIC (Liu et al., 2004). Following this RNAP II, TFIIB and TFIIF dissociate from the promoter leaving the remaining general factors at the promoter serving as a scaffold for the formation of the next transcription initiation complex (Yankulov and Bentley, 1997; Yudkovsky et al., 2000; Zawel et al., 1995). At this point RNAP II enters the stage of transcription elongation and elongation factors that promote productive RNA chain synthesis, RNA processing and RNA export are recruited (Orphanides and Reinberg, 2000; Sims et al., 2004; Svejstrup, 2004). Once the promoter is cleared, the next round of transcription can be reinitiated. Reinitiation of transcription is much faster process relative to the initial round, and is responsible for the bulk of transcription in the cell (Jiang and Gralla, 1993; Ranish et al., 1999). The final step in the transcription cycle is transcript termination followed by mRNA processing which

involves the addition of a 5'-cap, the excision of introns by splicing factors, and the addition of a 3'-poly(A) tail (Proudfoot et al., 2002). Transcript elongation, RNA processing and termination events all happen co-transcriptionally and is coupled to the progress of RNAP II through the gene (Bentley, 2002). Once mRNA is processed it is transported to the cytoplasm for protein synthesis.

1.3.5 Chromatin modifying complexes

DNA in eukaryotes is packaged into nucleoprotein complex known as chromatin. The fundamental repeat unit of chromatin is the nucleosome, which is comprised of an octamer of four core histones, H2A, H2B, H3 and H4 surrounded by 146 bp of DNA (Luger et al., 1997). Nucleosomes are further coiled or folded in to higher order compacted chromatin structures (Georges et al., 2002; Gilbert et al., 2005). It is necessary to open the compacted chromatin into relative extended state to initiate transcription. This is accomplished by chromatin modifying complexes that can be divided in to two classes (Li et al., 2004; Martinez, 2002). The first one contains ATP-dependent chromatin remodeling complexes such as yeast SWI/SNF complex (Smith and Peterson, 2005). These enzymes use the free energy derived from the hydrolysis of hundreds of molecules of ATP per minute to disrupt the chromatin structure. This class of ATPases has been further subdivided into at least three major subfamilies: the SWI2/SNF2, Mi-2/CHD, and ISWI families, as well as a potentially new family of Ino80-like complexes. All of these complexes contain an ATPase subunit that is essential for remodeling along with additional subunits that affect regulation, efficiency, and specificity. They modify chromatin structure in a non-covalent manner, presumably

by modifying the histone-DNA contacts within individual nucleosome, resulting in either localized disruption of the histone-DNA contacts or mobilization of the nucleosomes on the chromatin fiber (Vignali et al., 2000). Human homologs of SWI/SNF complex include BRG1 and BRM and has been found to activate a subset of IFN- α inducible genes (Huang et al., 2002c).

The second class is histone-modifying complexes, which add or remove covalent modifications, such as acetylation, methylation, phosphorylation, and ubiquitination from histones (Strahl and Allis, 2000). Histone acetylation is the best-studied histone modification and is carried out by histone acetyl transferases (HATs) complexes. HATs acetylate the ϵ -amino groups of conserved lysine residues within the histone tails (Roth et al., 2001). HATs are usually associated with multi-protein complexes including those that contain the catalytic HAT subunit Gcn5, such as the SAGA, SLIK/SALSA, ADA, STAGA, and TFTC complexes; complexes containing catalytic subunit p300/CBP (human) and human and mouse catalytic subunit SRC1, SRC3 and TIF2 (Torok and Grant, 2004).

It has been suggested that HAT activity increases the accessibility of DNA to transcription factors by lowering the positive charge, decondensing the compaction of nucleosomal array, disrupting histone-histone interactions between neighboring nucleosomes (Garcia-Ramirez et al., 1995), or by recruiting additional transcription factors through the histone code mechanism (Agalioti et al., 2000; Wolffe and Hayes, 1999). Hyperacetylation of histones is well correlated with transcriptionally active and open chromatin structures. HATs can acetylate substrates other than histones, including

DNA-binding activators such as p53 (Gu and Roeder, 1997), GATA-1; architectural proteins such as HMG-I or HMG-17 (Herrera et al., 1999); GTFs such as TFIIE and TFIIIF (Imhof et al., 1997) and others. On the other hand hypoacetylation of histones is carried out by histone deacetylases (HDACs) and is associated with transcriptional repression and silencing (Ito et al., 2000). HDACs remove the acetyl group from the histones comprising the nucleosomes resulting in tighter wrapping of the DNA. HDACS are associated with multi-protein co-repressor complexes such as Sin3 and NurD (Ayer, 1999). In addition to histones, HDACs have been shown to deacetylate other proteins including p53, E2F, α -tubulin and MyoD (Hubbert et al., 2002; Juan et al., 2000).

1.3.6 Transcriptional activation of eukaryotic genes

RNAP II and GTFs are capable of carrying out basal transcription, however, gene-specific transcriptional activation requires sequence-specific DNA binding proteins called transcriptional activators that bind to the upstream activating sequences (UAS) or enhancers. A typical activator contains a DNA binding domain (DBD) in addition to a separable activation domain (AD) to stimulate transcription (Ptashne and Gann, 1997). One of the important functions of activator is to recruit chromatin-modifying complexes such as SWI/SNF and SAGA to promoters. These complexes disrupt the chromatin structure making the core promoter more accessible for RNAP II and GTFs as well as other transcriptional regulators. Several lines of evidence indicate that an important aspect of activator function is to stimulate the recruitment of the basic transcription machinery (i.e., RNAP II and GTFs) by directly interacting with a component(s) of this machinery (Kuras and Struhl, 1999; Li et al., 1999). For example

TFIID has been implicated as a target for activators such as Sp1, CREB, VP16 and myc (Chiang and Roeder, 1995; Kuras and Struhl, 1999; Orphanides et al., 1996; Ptashne and Gann, 1997). Activator VP16 has also been shown to interact with TFIIB (Hall and Struhl, 2002; Roberts et al., 1993). Interactions of the activation domain of transcription activator E2F1 with two GTFs, the TBP and TFIIF have also been characterized (Pearson and Greenblatt, 1997). Moreover, activators increase the overall elongation rate of polymerase, possibly by stimulating the rate of promoter escape or polymerase II processivity (Blau et al., 1996; Brown et al., 1998). Activator GAL4-VP16 stabilizes the transcription reinitiation intermediate suggesting a role for some activators in promoting high levels of transcription (Yudkovsky et al., 2000). Figure 13 is a schematic representation of transcriptional activation of protein coding genes.

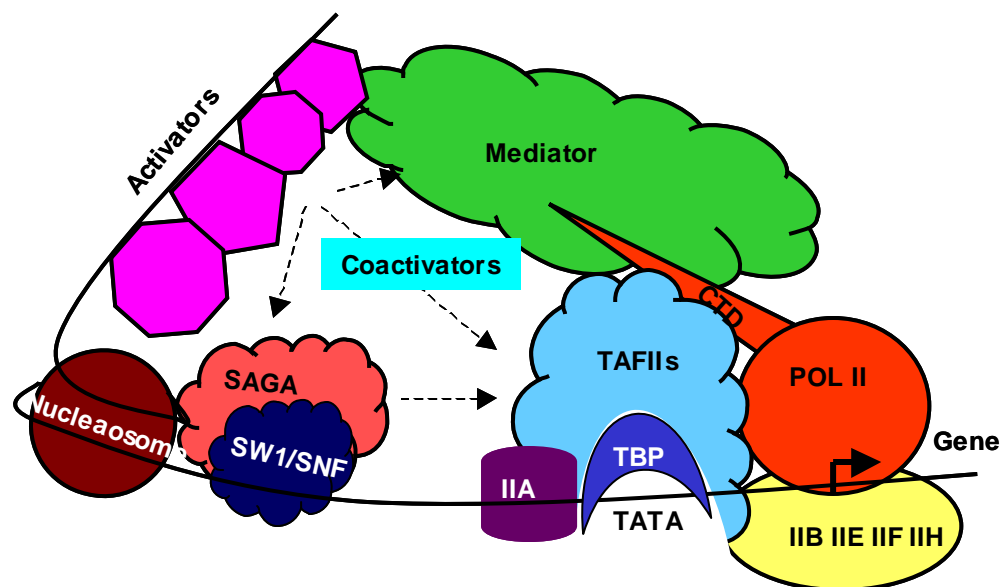


Figure 13. Schematic representation of transcriptional activation of protein coding genes (Martinez et al., 2002).

1.3.7 Mediator complex and its interaction with activators

The Mediator complex consists of 25–30 proteins, and it is conserved throughout eukaryotes (Malik and Roeder, 2005). Mediator was first discovered and purified to near homogeneity from *Saccharomyces cerevisiae* by Kornberg and coworkers (Kim et al., 1994). Yeast Mediator is a stable multi-subunit complex of more than 20 proteins that include various SRB and MED proteins. Subsequently, multiprotein mammalian Med-like complexes have been isolated and include the thyroid hormone receptor-associated proteins/SRB-Med containing cofactor (TRAP/SMCC), activator-recruited factor-large (ARC-L), vitamin D receptor-interacting proteins (DRIP), positive cofactor 2 (PC2), cofactor required for Sp1 activation (CRSP), mouse Med and rat Med complexes (Conaway et al., 2005a).

Mammalian Mediator complexes seem to affect several steps during assembly of the pol II preinitiation complex, including recruitment of TFIID (or TBP) and pol II to the core promoter and the other GTFs (Conaway et al., 2005b; Lewis and Reinberg, 2003). Distinct subunits of the Mediator complex within the basic Pol II machinery are essential targets of activator proteins (Fishburn et al., 2005; Klein et al., 2003). For example, interaction between MED23 and activator Elk-1 has been shown to stimulate transcription of the mouse *Egr1* gene at initiation as well as at a step after PIC assembly (Wang et al., 2005). Also, the interactions of VP16 with both MED17 and MED25 has been well demonstrated (Mittler et al., 2003; Yang et al., 2004a).

1.4 Transcription Factor Sp1

1.4.1 *Sp/KLF family of proteins*

Transcription regulation of protein encoding genes in eukaryotes is carried out by a combination of several cis-acting elements and trans-acting transcription factors. G-rich elements such as GC (5'-GGGGCGGGG-3') and GT (5'-GGTGTGGGG-3') boxes are recurring cis-acting elements in promoters and distal regulatory regions of several mammalian genes (Suske, 1999). These elements play an important role in regulation of expression of many ubiquitous, tissue-specific and viral genes. In addition these motifs are involved in the maintenance of the methylation-free status of the CpG islands as shown for APRT gene (Brandeis et al., 1994; Macleod et al., 1994). Sp1 (Specificity protein 1) and other members of the Sp/KLF bind to these GC and GT boxes and regulate transcription.

Sp1 is the first identified and cloned protein of Sp/ Krüppel-like factor (KLF) protein family that currently contains 25 members. Sp/KLF family can be divided in to two major subgroups. The first group contains Sp proteins (Sp1-Sp9) named after Sp1 and is characterized by the presence of Buttonhead (BTD) box CXCPXC, just N-terminal to the zinc fingers domain. The second group is a more heterogeneous group called KLFs that contain 16 proteins (KLF1-KLF16) and do not have a BTD box (Suske et al., 2005). Table 6 outlines the functional properties and expression patterns of SP/KLF family of proteins. The defining feature of Sp/KLF family of proteins is the presence of zinc finger domain in their C-terminal region.

Table 6. Summary of the expression pattern and functional features of Sp1-like/KLF family members (modified from Kaczynski et al., 2003).

Protein	Transcriptional activity (and functional domains)	Expression	Interacting coactivator/corepressor
Sp1/SPR-2	Activator (Q-rich domains)	Ubiquitous	CRSP,p300/CBP P TAFII130
Sp2	Unknown (Q-rich domain)	Unknown	Unknown
Sp3/SPR-2	Activator and/or repressor (Q-rich domains)	Ubiquitous	Unknown
Sp4/HF1B/SPR-1	Activator (Q-rich domains)	Brain-enriched	Unknown
Sp5	Unknown	Ubiquitous	Unknown
Sp6/ KLF14 SP7/OSX/osterix SP8	Activator	Ubiquitous	Unknown
KLF1/EKLF	Activator (acidic domain)	Erythroid mast cells	p300/CBP,PCAF, SWI/SNF andmSin3A
KLF2/LKLF	Activator (acidic domain)	Lung, blood vessels, lymphocyte	Unknown
KLF3/BKLF	Activator/repressor	Erythroid tissue- and brain-enriched	CtBP2
KLF4/GKLF/EZF	Activator and/or repressor (acidic survival domain)	Gut-enriched	p300/CBP
KLF5/CKLF/IKLF/B TEB2	Activator	Gut and epithelial tissues	Unknown
KLF6/COPEB GBF/ZF9/BCD1/CP BP/ PAC1/ST12	Activator	Ubiquitous	Unknown
KLF7/UKLF	Activator (acidic domain)	Ubiquitous	Unknown
KLF8/BKLF3, DXS741, ZNF74	Repressor	Ubiquitous	CtBP2
KLF9/BTEB, BTEB1	Activator/repressor (SID)	Ubiquitous	mSin3A
KLF10/EGRA, TIEG, TIEG1	Repressor (SID, R2, R3)	Ubiquitous	mSin3A
KLF11 FKLF, FKLF1, TIEG2	Activator and/or repressor (SID, R2, R3)	Ubiquitous	mSin3A
KLF12 AP2REP, AP-2rep	Repressor	Brain, kidney, liver and lung	CtBP1
KLF13/BTEB3/ FKLF2/NSLP1/ FKLF-2/ RFLAT1	Activator/repressor (SID, R2 and R3)	Ubiquitous	mSin3A, p300/CBP, PCAF
KLF15/KKLF	Repressor	Ubiquitous	Unknown
KLF16 DRRF, BTEB4, NSLP2	Repressor (SID)	Ubiquitous	mSin3A

This zinc finger domain represents the DNA-binding domain and consists of three zinc fingers of the classical Cys₂-His₂ type. Sp1 is thought to contact DNA with the amino acids KHA in the first, RER in the second and RHK in the third zinc finger. These critical amino acids are conserved in Sp3, Sp4, KLF9/BTEB1, KLF10/TIEG1 and KLF11/TIEG2 proteins and are capable of binding the classical GC-box (5'-GGGGCGGGG-3') with relatively similar affinities (Cook et al., 1998; Hagen et al., 1992; Sogawa et al., 1993). However, the DNA-binding specificity of these proteins is altered if the proteins differ in the key residues. For instance Sp2 protein has a leucine in place of histidine within the first zinc finger and has been shown to preferentially bind a GT rather than a GC-box (Kingsley and Winoto, 1992). In addition there are several other proteins of the family such as KLF1 and KLF3 that contain a leucine instead of a lysine in the third zinc-finger motif and preferentially bind the 5'-CACCC-3' element (Crossley et al., 1996; Miller and Bieker, 1993).

The DBD is the most highly conserved domain among Sp/KLF family members whereas the amino-terminal regions of the Sp1/KLF proteins are much more variable and contain transcriptional activation and repression domains. For example Sp1 is one of the most potent transcriptional activators with two glutamine-rich activation domains whereas KLF11 (TIEG2) functions as a potent transcriptional repressor and possesses three amino-terminal repression domains including the Sin3 interaction domain (SID), which mediates interaction with the corepressor mSin3A (Cook et al., 1999; Zhang et al., 2001). In addition, several Sp/KLF proteins can function as either activators or

repressors, depending on the cell and promoter context. For example Sp3 has both activation and repression domains and thereby can act as an activator as well as a repressor of Sp1-mediated activation depending on the cell and promoter context (Majello et al., 1997). The expression pattern is variable among different family members and is an important aspect in determining the specificity of these proteins. For instance Sp1 and Sp3 are ubiquitously expressed whereas the expression of KLF1/EKLF and Sp4 are restricted to erythroid cells and brain respectively. Also the data from the experiments with knock-out mice have revealed critical roles for most of the members of Sp/KLF family in growth and development (Kaczynski et al., 2003).

1.4.2 Sp subfamily

Sp members of the SP/KLF family consist of 9 Sp proteins, Sp1-Sp9. Sp1 was first identified as the transcription factor that binds to and activates transcription from multiple GC-boxes in the simian virus 40 (SV40) early promoter (Dyran and Tjian, 1983). Sp1 protein was then cloned by Kadonaga and co-workers from HeLa cells (Kadonaga et al., 1987). Sp1 was the first cloned member of Sp proteins and was initially thought to control gene expression of all genes containing GC or GT boxes. However, this view changed after the identification of other Sp family members including Sp3 and Sp4 that also bind GC or GT boxes with the same affinity as Sp1 and regulate gene expression. Within the Sp proteins Sp1, Sp2, Sp3 and Sp4 form a subgroup based on their similar modular structure. Sp1, Sp3 and Sp4 proteins contain two glutamine-rich (Q-rich) transactivation domains A and B that are critical for transcriptional activation while Sp2 has only one glutamine-rich domain (Figure 14).

All four proteins contain serine/threonine-rich sequences that could be a target for post-translational modification. Sp5-8 are low molecular weight proteins and also possess several domains common to Sp1-4. All nine Sp proteins have Btd box immediately N-terminal to the zinc finger domain in addition to Sp box (SPLALLAATCSR/KI) that is located at the N-terminus of the proteins (Harrison et al., 2000). The Btd element has been shown to be involved in synergistic activation by Sp1 or Sp3 with sterol-regulatory element-binding proteins (SREBP) (Athaniyar et al., 1997).

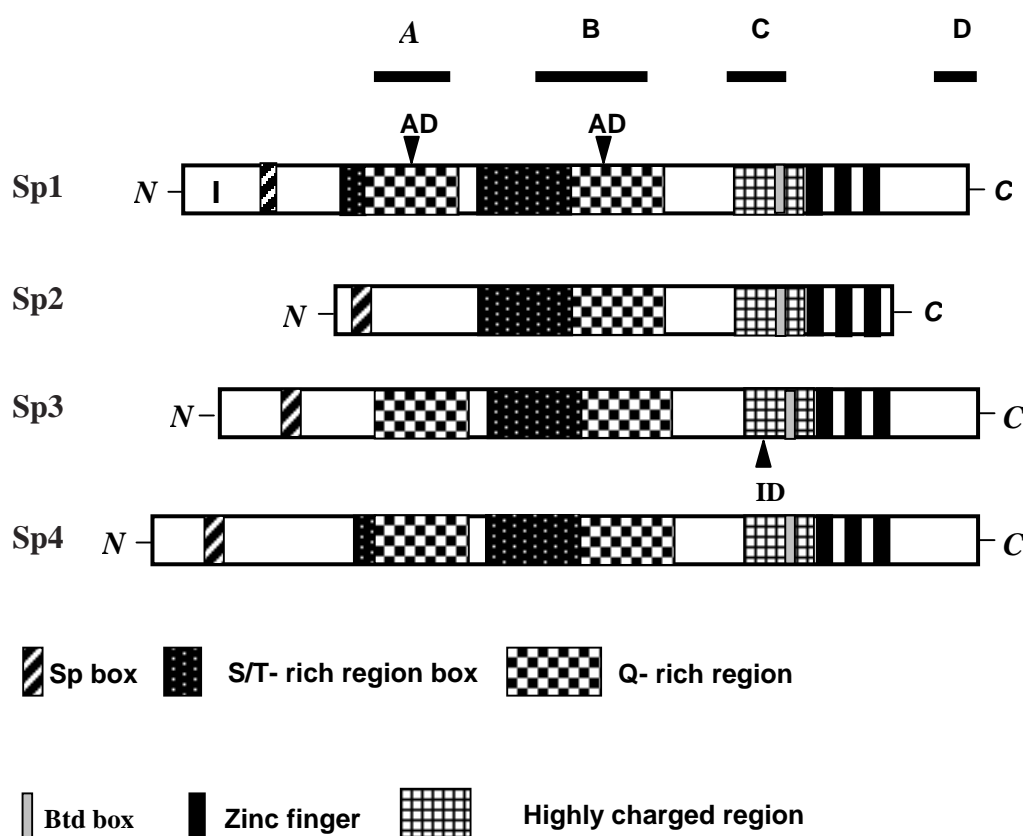


Figure 14. Structural motifs in Sp proteins. Activation (AD) and inhibitory (ID) domains are indicated (modified from Bouwman and Philipsen, 2002).

The Sp-box contains an endoproteolytic cleavage site and is situated close to an N-terminal region of Sp1 that targets proteasome-dependent degradation in vitro (Su et al., 1999). The following sections will review Sp1, Sp2, Sp3 and Sp4 proteins in detail.

1.4.3 Functional properties of Sp1, Sp2 Sp3 and Sp4

1.4.3.1 Sp1

Sp1 regulates transcription of several genes by binding to GC-boxes in their promoter regions. Molecular cloning of Sp1 and subsequent dissection of various functional domains of Sp1 protein revealed that both Q-rich domains act as strong activation domains (Courey and Tjian, 1988). Sp1 can stimulate transcription from both proximal and distal sites and can synergistically activate transcription from multiple binding sites through protein-protein interactions between adjacent Sp1 proteins. Sp1 can form tetramers and this provides further evidence that Sp1 protein-protein interactions are involved in synergistic activation of transcription (Courey and Tjian, 1988). Domain D is required in addition to both transactivation domains for synergistic activation of Sp1 (Pascal and Tjian, 1991).

Sp1-dependent transactivation involves Sp1 protein interactions with several components of basal transcription machinery including TBP (Emili et al., 1994) and specific TAFs. For example DNA binding domain of Sp1 interacts with human TAFII55 (Chiang and Roeder, 1995) and the A-domain of Sp1 interacts with glutamine-rich regions in human TAF130 (Rojo-Niersbach et al., 1999; Saluja et al., 1998). In addition to basal transcription machinery. Sp1 also interacts with human mediator or coactivator complex called cofactors required for Sp1 coactivation or CRSP (Ryu and Tjian, 1999).

CRSP is a 700 kDa multi-protein complex which functions in conjunction with the TBP-associated factors to promote efficient activation of transcription by Sp1 (Naar et al., 1998, 1999; Taatjes et al., 2002; Taatjes and Tjian, 2004).

Sp1 protein can also be modified post-translationally by glycosylation (Jackson and Tjian, 1988) and phosphorylation. Glucose deprivation results in reduced glycosylation of Sp1 and this is associated with an increased susceptibility of Sp1 protein to proteasome-dependent degradation (Han and Kudlow, 1997; Yang et al., 2001). Jackson et al. first reported phosphorylation of Sp1 by a DNA-dependent protein kinase and since then a large number of different kinases that phosphorylate Sp1 including PKA, PKC- ζ , casein kinase II, ERK, cyclin-dependent kinase, and others have been identified (Chu and Ferro, 2005; Jackson et al., 1990). Sp1 phosphorylation has been linked to functional changes in DNA binding and promoter activation and could either increase or decrease expression of the target gene, depending on the cell and promoter context (Chu and Ferro, 2005).

In addition to house keeping genes, Sp1 also regulate transcription of several cell cycle regulated and tissue-specific genes by interacting with other transcription factors and nuclear proteins including AhR, Arnt, GATA-1, GATA-2, GATA-3, NF-YA, VHL, MyoD, HDAC1, PML, HTLF, E2F1, YY1, MDM2, c-jun, AP-2, myc, NFAT-1, HD protein, cyclin A, Oct-1, TBP, HNF3, HNF4, p53, MEF2C, SMAD2, SMAD3, SMAD4, Msx1, several viral proteins (c-rel, p50, p52, rel A, tat, and BPV-E2), E11, Rb, p107 (Rb-like), DNMT1, and ZBP-89 (Safe and Kim, 2004). Most of the interactions of these proteins are mediated by C-terminal domain (C/D) of Sp1. For example both E2F1 and

Sp1 proteins physically interact and are involved in cooperative activation of the thymidine kinase promoter that contains a GC-rich and an E2F1 site separated by 6 bp (Karlseder et al., 1996; Lin et al., 1996). Mutation of either site results in more than 90 percent loss of basal activity in Swiss 3T3 cells.

Sp1 is involved in growth regulation by regulating several growth-promoting genes such as dihydrofolate reductase (DHFR) (Jensen et al., 1997), ornithine decarboxylase promoters (Kumar and Butler, 1998), insulin-like growth factor (IGF)-binding protein 2 (Kutoh et al., 1999), vascular/endothelial growth factor (VEGF) (Milanini et al., 1998) and thymidine kinase (Sorensen and Wintersberger, 1999).

Moreover Sp/KLF family members play a role in the effects of multiple growth-related signaling pathways by mediating effects of growth factors and cytokines, including those involving EGF and erbB receptors, TNF α , PDGF, TGF- β , IGF-I and IGF-II, interferon γ , follicle stimulating hormone, thyroid hormone, estrogen, and androgens (Black et al., 2001a). In addition Sp1, Sp3 and other members of Sp/KLF family are involved in regulation of the growth inhibitory gene p21 (Gartel and Tyner, 1999). p21 gene contains six Sp1 sites in its proximal promoter region and these sites mediate the regulation of p21 in response to multiple stimuli from different signal transduction pathways (Lu et al., 2000b; Wang et al., 2000; Zhang et al., 2000b).

Activation of p21 through the proximal GC-rich elements in the human p21 promoter was primarily dependent on Sp3 and not Sp1 in MG63 cells (Nakano et al., 1997; Sowa et al., 1999a; Sowa et al., 1999b), whereas, in other human cell lines and *Drosophila* cells, Sp1 was a more potent activator (Koutsodontis et al., 2001; Pagliuca et

al., 2000). Thus different Sp proteins bind with different affinities and can activate or repress the p21 promoter depending on cell context and thereby integrate the multiple signaling pathways. The role of Sp1 protein in estrogen-mediated transactivation of genes in breast cancer cells will be discussed in detail section 1.6.5.4.

1.4.3.2 Sp2 and Sp4

Sp2 binds to a GT-box promoter element within the T-cell receptor α promoter *in vitro* (Kingsley and Winoto, 1992). Sp2 represses Sp1- and Sp3-mediated activation of the CTP: phosphocholine cytidyltransferase α promoter in *Drosophila* SL2 cells (Bakovic et al., 2000). Recently Phan et al. (2004) showed that down-regulation of carcinoembryonic antigen-related cell adhesion molecule 1 (*CEACAM1*) gene in prostate cancer is mediated by the transcription factor Sp2 that recruits histone deacetylase (HDAC) to repress transcription of the *CEACAM1* gene (Phan et al., 2004). The transactivation function of Sp4 also resides in the Q-rich domains, however, Sp4 is not able to synergistically transactivate from promoters containing multiple binding sites (Hagen et al., 1995). Sp4 activates promoters in *Drosophila* cells and in several mammalian cell lines (Ahlgren et al., 1999; Lerner et al., 2005; Wong et al., 2001).

1.4.3.3 Sp3

The expression pattern and the structure of Sp3 are very similar to Sp1. In addition Sp3 binds to the GC and GT boxes with similar affinities as Sp1 but there are striking functional differences between the two proteins. Unlike Sp1, Sp3 can function both as an activator or a repressor depending on cell and promoter context. There are multiple isoforms of Sp3 that makes the transcriptional control by Sp3 more complex

(Kennett et al., 1997; Kingsley and Winoto, 1992; Moran et al., 2004; Sapetschnig et al., 2004). Sapetschnig et al. showed that four isoforms of Sp3 are expressed *in vivo*, two long forms and two short forms that differ in the extent of the N-terminal part and are derived from alternative translational start sites (Sapetschnig et al., 2004). The short forms of Sp3 lack the subdomain A of the trans-activation domain and act as repressors or weak activators.

Sp3 stimulates transcription of many promoters including PDGF-B (Liang et al., 1996), thymidine kinase (Birnbaum et al., 1995), p21 (Gartel et al., 2000; Majello et al., 1997; Sowa et al., 1999b), and human $\alpha 2(I)$ collagen (Ihn and Trojanowska, 1997). Transactivation potential of Sp3 appears to be, in part, dependent on the number and arrangement of recognition sites. There are several reports showing that Sp3 can transactivate promoters containing a single Sp-binding site but cannot activate promoters containing multiple Sp-binding sites (Birnbaum et al., 1995; Dennig et al., 1996; Prowse et al., 1997). Whether Sp3 is transcriptionally active or repressive also depends on the cell context. For instance, transfected Sp3 stimulated transcription from the HERV-H long-terminal repeat in the teratocarcinoma cell line NTera2-D1 but acted as a repressor in HeLa human cervical cancer and SL-2 *Drosophila* cells (Sjottem et al., 1996). Moreover, Sp3 has been shown to repress Sp1-mediated transactivation function by competing for the same binding site (Birnbaum et al., 1995; Majello et al., 1995; Yu et al., 2003). It was suggested that since Sp1 and Sp3 compete for the same binding sites, the relative expression of Sp1 and Sp3 within a given cell type should determine the regulation of some target genes. Alterations in the relative abundance of the two proteins

under different conditions have been reported and this was associated with changes in regulation of target genes (Apt et al., 1996; Discher et al., 1998; Hata et al., 1998).

The transactivation potential of Sp3 is due to the two Q-rich domains similar to Sp1 and the inactivity of Sp3 is explained by the presence of an inhibitory domain located between the second glutamine-rich activation domain and the zinc finger region (Dennig et al., 1996; Majello et al., 1997). Mutation of critical KEE triplet within this inhibitory domain results in relief of repression and potentiates the transactivation potential of Sp3. It has been shown that acetylation of the lysine residue within the KEE triplet results in decreased transcriptional activity of Sp3 (Braun et al., 2001). In addition Sp3 is post-translationally modified by the small ubiquitin-like modifier (SUMO) within its inhibitory domain both *in vivo* and *in vitro* (Ross et al., 2002; Sapetschnig et al., 2002). Mutations of amino acids that were essential for SUMO modification were shown to strongly enhance the transcriptional activity of Sp3 suggesting that SUMO modification silences transcriptional capacity of Sp3. Sapetschnig et al. recently showed that unlike Sp1, none of Sp3 isoforms are glycosylated, however, all four isoforms become SUMO-modified *in vivo* as well as *in vitro* specifically and exclusively at lysine residue 551 (Sapetschnig et al., 2004).

1.4.4. Physiological role of Sp proteins

The knockout phenotypes of Sp proteins reveal their critical and distinct functions in growth and development. Sp1-deficient embryonic stem cells (ES cells) are viable with normal growth characteristics and can be induced to differentiate and form embryoid bodies as efficiently as wild type ES cells (Suske, 1999). However the Sp1-

knockout embryos exhibit multiple abnormalities, retarded development and they all die around day 11 of embryonic development. Despite the developmental abnormalities Sp1 null embryos were shown to express many putative Sp1 target genes at normal levels, including housekeeping and cell-cycle regulated genes (Marin et al., 1997). Sp4 is primarily expressed in the brain and approximately two thirds of the *Sp4*^{-/-} mice die within a few days of birth. The surviving animals are reduced in size and the male mice are infertile and unable to copulate (Gollner et al., 2001; Supp et al., 1996). In addition Sp4 null mutant mice have been shown to suffer from cardiac arrhythmia and sudden death (Nguyen-Tran et al., 2000).

Sp3 knockout mice are retarded in growth and die invariably at birth of respiratory failure (Bouwman et al., 2000). In addition, Sp3^{-/-} mice show impaired hematopoiesis, tooth and bone development (Bouwman et al., 2000; Gollner et al., 2001; Van Loo et al., 2003). Bone formation is blocked in Sp7 knockout mice (Nakashima et al., 2002) and Sp5 knockout mice showed no overt phenotype (Harrison et al., 2000). Mice with a targeted deletion of *Sp8* gene gave a dramatic phenotype including the absence of tails and severely truncated forelimbs and hindlimbs. In addition Sp8^{-/-} mice showed central nervous system defects which included a failure to close both the anterior and posterior neuropores, leading to exencephaly and spina bifida (Bell et al., 2003). Thus, it is evident from the results of knockout mice that Sp proteins play critical and specific roles in cellular functions of growth and development.

1.5 Nuclear Receptor Superfamily

1.5.1 Introduction

Nuclear receptors (NRs) are a superfamily of transcription factors that play an important role in embryonic development, reproduction, homeostasis, metabolism and other biological processes (Aranda and Pascual, 2001). Dysfunction of nuclear receptor signaling is implicated in several human diseases including diabetes, infertility, obesity, neurological dysfunctions, and leukemia, breast and prostate cancers. This makes nuclear receptors potential targets for drug discovery. Indeed, ligands for several nuclear receptors are currently used clinically to treat many diseases including cancer. For example tamoxifen targets the ER and is used to treat breast cancer. Ligands for the glucocorticoid receptor (GR) such as dexamethasone are used as anti-inflammatory drugs and chemotherapeutic agents; the mineralocorticoid receptor ligand, spironolactone, is used for prevention of cardiac fibrosis; and PPAR gamma ligands such as thiazolidinediones are used for treating type II diabetes (Pearce et al., 2004).

There have been 21 NRs identified in the complete genome of the fly *Drosophila melanogaster* (Adams et al., 2000), 270 in nematode *Caenorhabditis elegans* (Sluder et al., 1999) and 48 NRs in the human genome (Maglich et al., 2003). Although many NRs have known ligands, the majority of nuclear receptors are called orphan receptors for which ligands have not yet been discovered. Out of 48 human NRs, 24 are orphan receptors, however, those that will remain as true orphan receptors have not been established. The mammalian NR superfamily can be divided in to six classes based on nuclear receptor sequences using molecular phylogeny (Aranda and Pascual, 2001;

Gronemeyer et al., 2004). Table 7 outlines different receptors within each class along with their ligands and response elements. Ligands that bind NRs include steroid hormones such as estrogens (binds ER), progesterone (binds PR), mineralocorticoids [binds mineralocorticoid receptor (MR)], glucocorticoids (binds GR) and androgens [binds androgen receptor (AR)]; in addition other steroidal molecules such as vitamin D, oxysterols and bile acids that bind vitamin D receptors (VDR), LXR and FXR. Other ligands for NRs include retinoic acids (all-trans and 9-cis isoforms) (binds RAR and RXR respectively), thyroid hormones (bind TR), xenobiotics (bind CAR and PXR), fatty acids, leukotrienes, prostaglandins (PPARs), various metabolites and growing numbers of lipophilic molecules (Escriva et al., 2000; Chawla et al., 2001). Many known natural ligands of NRs including corticoids, vitamin D, estrogen, progesterone, androgen and retinoic acid derivatives that arise from isoprenoid-derived biochemicals that have been further metabolized (Steinmetz et al., 2001).

1.5.2 Domain structure of NRs

Nuclear receptors share a common modular structure with autonomous functional domains that can be interchanged between related receptors without loss of function (Wrange and Gustafsson, 1978; Kumar et al., 1987). A typical nuclear receptor consists of a variable amino-terminal region (A/B domain), a conserved DNA-binding domain (DBD) or region C, a linker region D, and a conserved E region that contains the ligand-binding domain (LBD). Some receptors contain an additional F domain in the C-terminal region which exhibits a highly variable sequence and whose structure and function are not well defined.

Table 7. Subfamilies of mammalian nuclear receptors (modified from Aranda and Pascual, 2001; Gronemeyer et al., 2004).

Class	Receptor	Subtype	Denomination	Ligand	Response Element	Mono mer, Homo dimer, or Hetero dimer
Class I	TR	α, β	Thyroid hormone receptor	Thyroid hormone (T3)	Pal, DR-4, IP	H
	RAR	α, β, γ	Retinoic acid receptor	Retinoic acid	DR-2, DR-5, Pal, IP	H
	VDR		Vitamin D receptor	1-25-dihydroxy vitamin D3	DR-3, IP-9	H
	PPAR	α, β, γ	Peroxisome proliferator activated receptor	Benzotriene B4; Wy 14.643 Eicosanoids; thiazolidinediones (TZDS); 15-deoxy-12,41-prostaglandin J2; polyunsaturated fatty acids	DR-1	H
	PXR		Pregnane X receptor	Pregnanes; xenobiotics	DR-3	H
	CAR/MB 67	α, β	Constitutive androstane receptor	Xenobiotics, Androstanes; 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene	DR-5	H
	LXR	α, β	Liver X receptor	Oxysterols	DR-4	H
	FXR		Farnesoid X receptor	Bile acids, fexaramine, lanosterol	DR-4, IR-1	H
	RevErb	α, β	Reverse ErbA	Orphan	DR-2, Hemisite	M, D
	RZR/RO R	α, β, γ	Retinoid Z receptor/retinoic acid-related orphan receptor	Retinoic acid, cholesterol and cholesteryl sulphate	Hemisite	M
UR		Ubiquitous receptor	Orphan	DR-4	H	
Class II	RXR	α, β, γ	Retinoid X receptor	9-Cis-retinoic acid	Pal, DR-1	D

Table 7 continued

Class	Receptor	Subtype	Denomination	Ligand	Response Element	Monomer, Homodimer, or Heterodimer
Class II	COUP-TF	α, β, γ	Chicken ovalbumin upstream promoter transcription factor	Orphan	Pal, DR-5	D, H
	HNF-4	α, β, γ	Hepatocyte nuclear factor 4	Fatty acyl-CoA thioesters	DR-1, DR-2	D
	TLX		Tailles-related receptor	Orphan	DR-1, Hemisite	M, D
	PNR		Photoreceptor-specific nuclear receptor	Orphan	DR-1, Hemisite	M, D
	TR2	α, β	Testis receptor	Orphan	DR-1 to DR5	D, H
	EAR2		ErbA2-related gene-2	Orphan		
Class III	GR		Glucocorticoid receptor	Glucocorticoids	Pal	D
	AR		Androgen receptor	Androgens	Pal	D
	PR		Progesterone receptor	Progestins	Pal	D
	ER	α, β	Estrogen receptor	Estradiol	Pal	D
	MR		Mineralcorticoid receptor	Aldosterone, spiro lactone		
	ERR	α, β, γ	Estrogen-related receptor	Orphan	Pal, Hemisite	M, D
Class IV	NGFI-B	α, β, γ	NGF-induced factor B	Orphan	Pal, DR-5	M, D, H
	NURR1		Nur related factor 1	Orphan		
	NOR1		Neuron-derived orphan receptor 1	Orphan		
Class V	SF-1/FTZ-F1	α, β	Steroidogenic factor 1/Fushi Tarazu factor 1	Oxysterols	Hemisite	M
	LRH1		Liver receptor homologous protein 1	Orphan		
Class VI	GCNF		Germ cell nuclear factor	Orphan	DR-0	D
	SHP		Small heterodimeric partner	Orphan		H
	DAX-1		Dosage-sensitive sex reversal	Orphan		

M, monomer; D, homodimer; H, heterodimer; NGF, nerve growth factor; DR, direct repeat; Pal, palindrome; IP, inverted palindrome

Figure 15 is a schematic representation of a typical nuclear receptor. The A/B region is variable in both size and sequence and interacts with coactivators and/or other transcription factors in cell- and promoter specific manner (Webster et al., 1988; Berry et al., 1990; Kumar and Thompson, 2003). The A/B region in many receptors contains one constitutively active transcriptional activation function, referred to as AF-1 which contributes to ligand-independent activation of the receptor (Tora et al., 1988). The A/B domain is also a target for phosphorylation in many receptors including ER (Kato et al., 1995) and PPAR γ and this may result in activation or repression of its transcriptional activity (Shao et al., 1998).

The DBD or the C-region has the most conserved amino acid sequence among the members of the NR superfamily and is required for the recognition and binding of specific target sequences on DNA. The DBD has two highly conserved zinc-finger motifs spanning ~60-70 amino acids: C-X₂-C-X₁₃-C-X₂-C and C-X₅-C-X₉-C-X₂-C that are common to the entire family with the exception of two divergent members: (DAX-1) (Zanaria et al., 1994) and SHP (Hard et al., 1990; Schwabe et al., 1990; Seol et al., 1996).

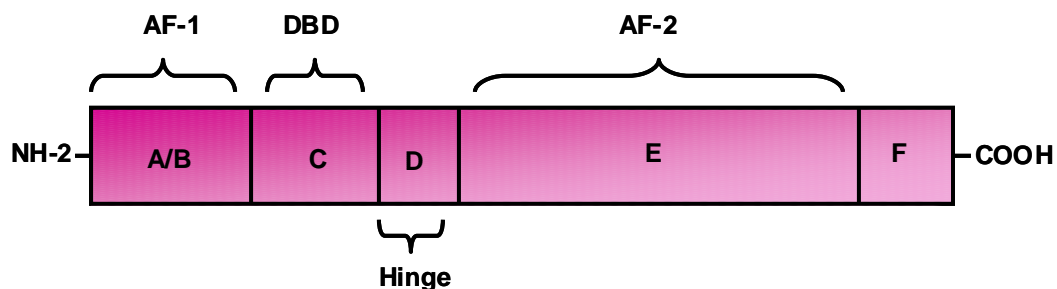


Figure 15. Schematic representation of the functional domains of nuclear receptors.

In addition, the DBD has a COOH-terminal extension (CTE) that contains the so-called T and A boxes critical for specificity and polarity of NRs in DNA binding (Hsu et al., 1998; Melvin et al., 2004). Each zinc-finger contains four highly conserved cysteine molecules that coordinate the binding of a zinc atom. Amino acids required for sequence specificity in DNA binding are present at the base of the N-terminal finger in a region termed the "P box," (Nelson et al., 1995) and residues of the second zinc finger that form the so-called "D box" are involved in dimerization (Figure 16) (Zechel et al., 1994). The core DBD contains two α -helices: the first one is known as the recognition helix and binds the major groove of DNA making contacts with specific bases, the second helix spans the COOH terminus of the second zinc finger and forms a right angle with the recognition helix (Aranda and Pascual, 2001). The DBD may also contain a nuclear localization signal (Hsieh et al., 1998) and nuclear export signals (Black et al., 2001b).

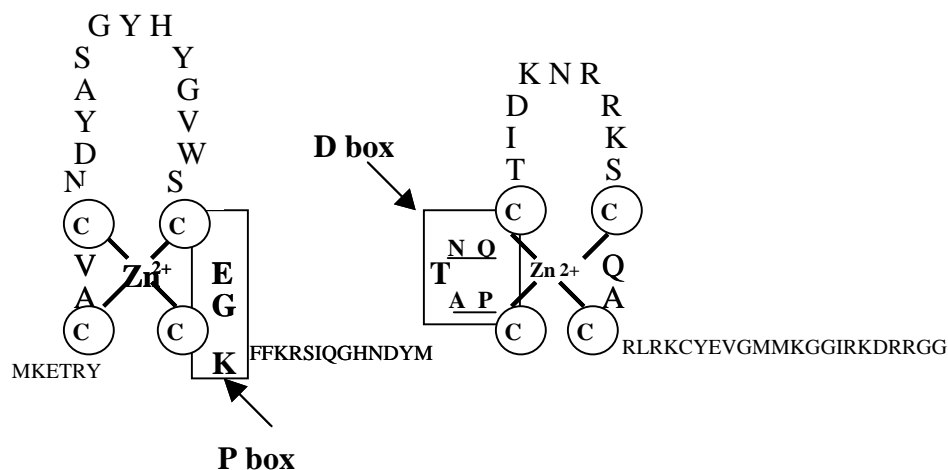


Figure 16. Schematic representation of the DBD of nuclear receptors (Ruff et al., 2000).

The D domain is not well conserved and behaves as a flexible hinge between the DBD and the LBD, allowing rotation of the DBD. The D domain in many cases may also harbor nuclear localization signals and may be important for hormone binding (Lin et al., 1991). The LBD or the E domain is moderately conserved among members of the nuclear receptor superfamily. It contains an additional transactivation domain, AF-2, which is strictly ligand dependent and is a target for interaction with several coactivator and corepressor complexes (Barettino et al., 1994; Nolte et al., 1998). The crystal structure of the LBD has been determined for several nuclear receptors and has provided insights regarding the mechanisms involved in ligand binding and transactivation (Bourguet et al., 2000).

The overall structure of the LBD is similar for several NRs and is composed of 12 helices, H1-H12, arranged together in an antiparallel, three-layered sandwich which may include two to four β -strands (Wurtz et al., 1996). Helices H1-H11 form the hydrophobic ligand-binding pocket whose entrance is guarded by H12 (Bourguet et al., 1995; Uppenberg et al., 1998). Agonist ligand binding induces a conformational change in many NRs resulting in alternate positioning of H12. This promotes recruitment of coactivators that interact with their short LXXLL-like motifs (where L is leucine and X is any amino acid) called NR-boxes. LXXLL-like motifs are present in many co-activators and are common motifs required for interacting with the LBD of NRs. The residues of the ligand-dependent activation function 2 (AF-2) are located in H12 (Danielian et al., 1992). The structural data, together with transcriptional activation data, imply that the positioning of helix 12 is crucial for receptor activation (Warnmark et al.,

2003). In addition the LBD also contains nuclear localization signals (Kanno et al., 2005), a dimerization domain (Ribeiro et al., 2001; Hentschke et al., 2002), and in some cases repression domains (Marimuthu et al., 2002).

1.5.3. Overview of mechanism underlying NR-mediated transactivation

An essential step in transcriptional activation by NRs involves binding of ligand-activated NRs to response elements, termed as hormone response elements (HREs), within the regulatory region of target genes. HREs are derivatives of the canonical sequence of 6 bp RGGTCA where R is a purine. The consensus sequence of AGAACA has been characterized for class III steroid hormone receptors, however many HREs show significant variations from the consensus sequence (Aranda and Pascual, 2001).

Although some NRs such as SF-1 can bind DNA as a monomer, most NRs bind as homodimers or heterodimers to HREs. For example class III NRs such as ER and AR forms homodimers whereas class I NRs such as TR, VDR and RAR heterodimerize with RXR. The dimeric HREs are typically composed of two hexameric core half-site motifs which are present as palindromes (Pal), inverted palindromes (IPs) or direct repeats (DRs). Figure 17 illustrates various types of DNA response elements for some NRs. The steroid hormone receptors such as GR, PR, AR and MR, each of which is a homodimer, typically bind to palindrome (symmetric repeats) of 5'-AGAACA-3' separated by 3 bp with the exception of the ER which binds similar symmetric sites but with consensus 5'-AGGTCA-3' half-sites. As shown in Figure 17B, a '1-5 rule' specifies the use of direct-repeats with variable spacing by RXR and its many partners. Some receptors, such as

the VDR or RevErb, can form homodimers as alternative to heterodimers. The size of the inter-half-site spacing (n) can vary from one to five base pairs. Sites containing just one copy of 5'-AGGTCA-3' flanked with specific 5' sequences (xxx) are used by the nerve growth factor-induced B (NGFI-B) receptors, RevErb and other orphan receptors (Figure 17C) (Khorasanizadeh and Rastinejad, 2001). The identity of different response elements resides in three features: the nucleotide sequence of the two core half-sites and their flanking sequences, the number of base pairs separating them (usually 1-5 bp), and the relative orientation of the motifs (Claessens and Gewirth, 2004). The specificity of DNA recognition by NRs resides in the first zinc finger of the DBD (Green et al., 1988).

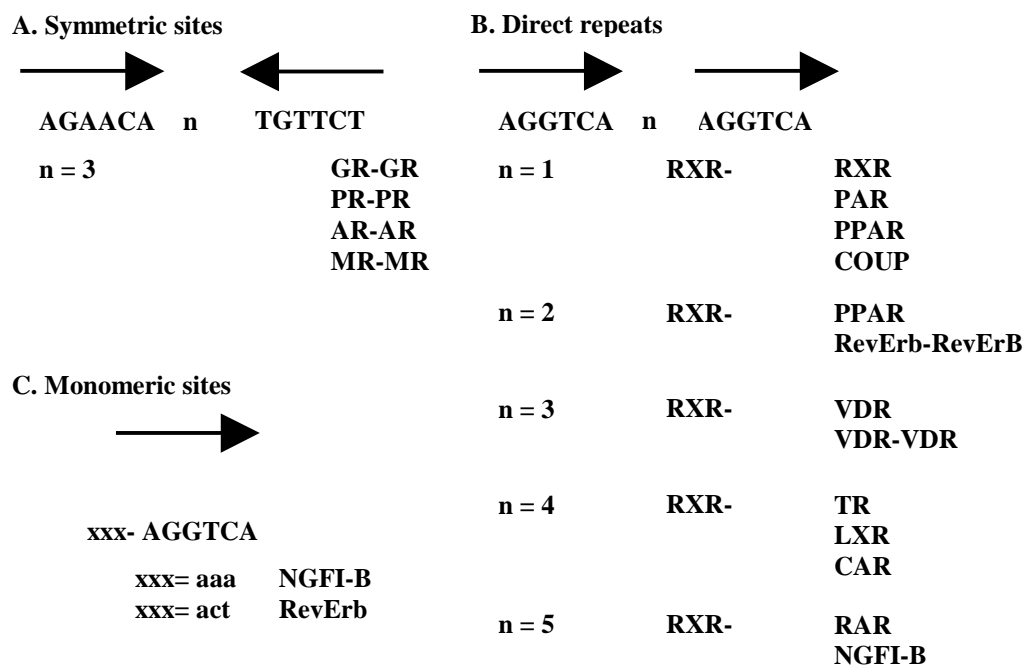


Figure 17. The types of DNA response elements used by nuclear receptors (Adapted from Khorasanizadeh and Rastinejad, 2001).

In the absence of a ligand several NRs bind HREs of target genes as a corepressor complexed with HDACs. Ligand binding releases the HDAC complex and recruits HATs and chromatin remodelling (CRM) complexes such as steroid receptor coactivator SRC/p160s and CBP [CREB (cAMP response element-binding protein) binding protein]. This binding results in disruption of local nucleosomal structure. Finally RNAP II along with GTFs and mediator complexes such as TRAP/DRIP are recruited, thereby facilitating transcription initiation. The temporal order and requirement of these complexes can occur in a receptor-, target-gene- and cell-specific manner. The ligand can also interact with its cognate receptor and exert a non-genomic action or extranuclear by interacting directly for example with kinases.

1.6 Estrogen Receptors

1.6.1 Introduction

The biological effects of estrogens are mediated by binding to one of two ERs, namely ER α and ER β . Both are ligand-dependent transcription factors that belong to the nuclear receptor family. ER α was the first ER sub-type identified in the 1960s by Jensen and his colleagues (Jensen, 1962; Toft and Gorski, 1966; Jensen et al., 1967; Jensen et al., 1968) and shown by O'Malley and coworkers to be a ligand-activated transcription factor (O'Malley and McGuire, 1968; Means et al., 1972). ER α was cloned in 1986 (Green et al., 1986; Greene et al., 1986) and it was generally accepted that this was the only receptor mediating the effects of estrogens. However, in 1996, a second genetically

distinct receptor (ER β), was identified and cloned from rat prostate and ovary (Kuiper et al., 1996), closely followed by cloning of human and mouse homologues (Mosselman et al., 1996; Enmark et al., 1997; Tremblay et al., 1997). The discovery of ER β posed many questions and possibilities on ER function and has resulted in an important paradigm shift in biology.

ER α and ER β are products of two different genes located on different chromosomes and have distinct physiological functions as demonstrated from studies on ER α (α ERKO) (Lubahn et al., 1993), ER β (β BERKO) and both ER α and ER β ($\alpha\beta$ BERKO) knockout mice (Krege et al., 1998). Although the classical 66 kDa ER α has been well-characterized, other ER α splice variants have also been identified and their biological functions are unknown (Pearce and Jordan, 2004). A variety of ER β mRNA isoforms have been identified in humans, primates, rats and mice, and the 530 amino acid subtype is considered to be the wild type, full length human ER β (Enmark et al., 1997; Lewandowski et al., 2002). ER α and ER β exhibit different expression patterns in various tissues, tumors and cancer cell lines (Kuiper et al., 1997; Hiroi et al., 1999). In humans, ER α and ER β are localized in the breast, brain, cardiovascular system, urogenital tract and bone. ER α is the main ER subtype in the liver and uterus, whereas ER β is predominant in the colon, brain and prostate (Taylor and Al-Azzawi, 2000).

1.6.2 Domains of ER

The structure of ERs and most other members of the NR family can be divided into six functional domains designated A to F (Kumar et al., 1987) (Figure 18). The transactivation function of ER is mediated by two separate but not mutually exclusive

transactivation domains namely, ligand-independent AF-1 (within the A/B) and ligand-dependent AF-2 (within the LBD)(Tora et al., 1989). The A/B domain is the least conserved region between ER α and ER β with only 30% similarity at the amino acid level. The activity of AF-1 in ER β is negligible on ERE-reporter constructs compared to the AF-1 of ER α in several different cell lines (Cowley and Parker, 1999).

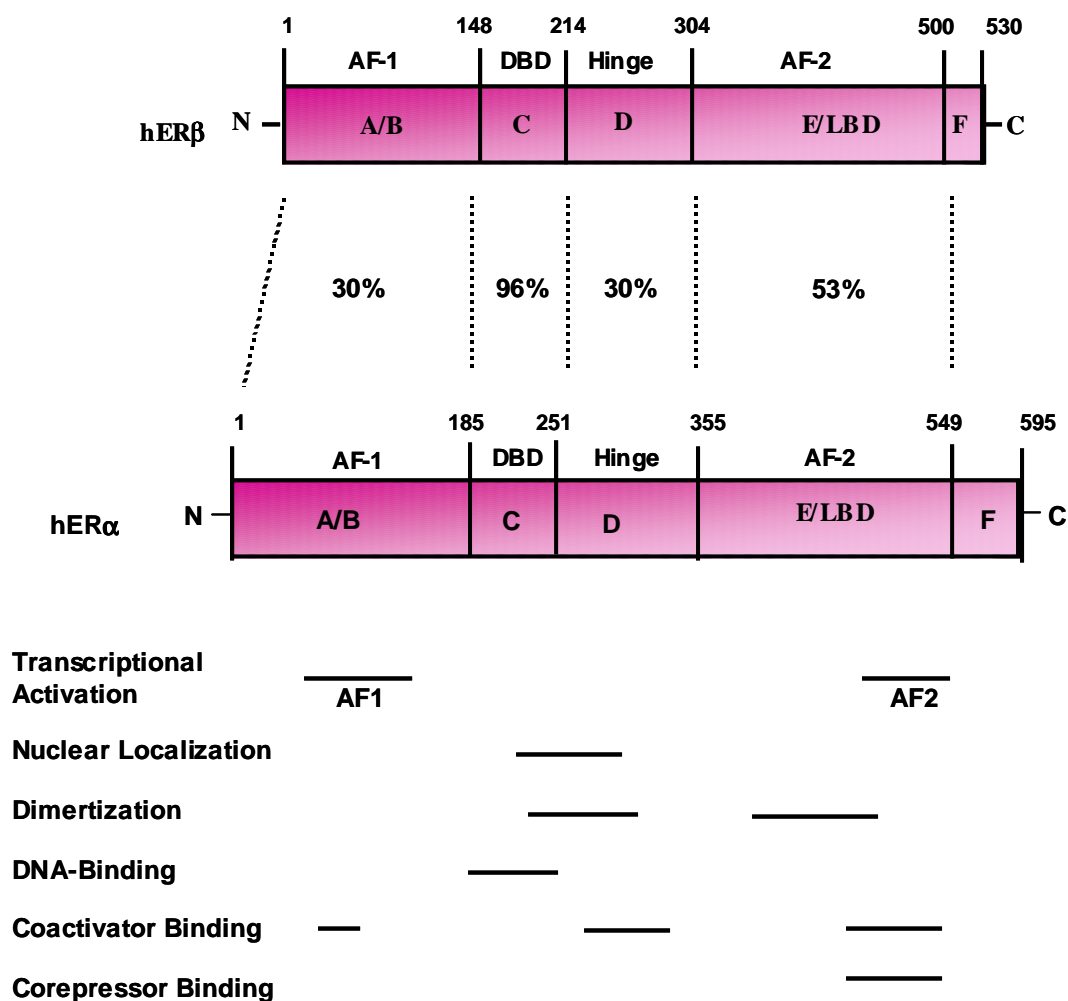


Figure 18. Structural domains of human ER α and ER β . The percent identity between the individual domains at the amino acid level is indicated (modified from Pearce and Jordan, 2004; Koehler et al., 2005).

ER α and ER β also exhibit distinctive responses to the synthetic antiestrogens such as tamoxifen and raloxifene which act as partial ER agonists for ER α and as pure ER antagonists for ER β (Barkhem et al., 1998). The functional differences between the respective A/B regions of ER α and ER β may explain their differences in ligand-dependent activation (Delaunay et al., 2000; Matthews and Gustafsson, 2003). The AF-1 region of ER α interacts with different transcriptional regulators and coactivators that affect ligand-independent transactivation. The activity of AF-1 is also regulated through kinase-dependent phosphorylation and the individual pathways involved vary with cell and promoter context (Tzukerman et al., 1994). In most cell lines, both AFs act synergistically to attain maximum transcriptional activity while in other cells only one AF may be activated (Benecke et al., 2000).

The DBD (region C) is highly conserved between ER α and ER β and exhibits 96% identity. This domain contains two zinc fingers (CI and CII) as described in section 1.5.2 for other NRs. The DBDs of both ER α and ER β bind with high affinity to EREs (Mosselman et al., 1996; Pettersson et al., 1997). The minimal consensus ERE sequence is a palindromic inverted repeat (IR): 5'-GGTCAnnnTGACC-3'. Extension of the length of the ERE palindrome, e.g. 5'-CAGGTCAnnnTGACCTG-3', forming a 17bp palindromic IR, and the sequences immediately flanking the ERE are important for determining ER binding affinities for this motif (Klinge, 2000). Three specific amino acids within the 'P box' of zinc finger CI interact in the major groove in a sequence-specific manner. The amino acid sequence of the P-box is identical between the two receptors (Mader et al., 1989). Thus both receptors bind estrogen responsive elements

(EREs) with similar specificity and affinity (Matthews and Gustafsson, 2003). The hinge region or D domain is a 40–50 aa sequence separating the DBD and LBD and is not well conserved between the two receptors. It contains sequences for receptor dimerization and nuclear localization (Picard et al., 1990; Ylikomi et al., 1992). The C-terminal E/F region encompasses the LBD, a coregulator binding surface, a dimerization domain, another nuclear localization signal, and AF-2 (Nilsson et al., 2001). Significant homology between the two receptors exists in the E/F region and both proteins display essentially the same binding affinity for E2 and many other estrogenic compounds (Kuiper et al., 1997). However, the two receptors differ in their binding affinities for some ligands including antiestrogens and phytoestrogens. For example, the phytoestrogen genistein binds with about a 30-fold higher affinity for ER β than ER α (Barkhem et al., 1998).

Crystallographic studies with the LBDs of ER α and ER β revealed that both ER α and ER β share a similar overall architecture. The AF-2 interaction surface is composed of amino acids in helix 3, 4, 5, and 12 and the position of helix 12 is altered upon ligand binding. Amino acids within helices 3, 5 and 11 are important for ligand binding since mutation of these residues significantly decrease the binding affinity for E2 (Wurtz et al., 1998). Deletion and mutation analysis have revealed that ER dimerization is mediated through helices 7-10 (Fawell et al., 1990; Lees et al., 1990). Crystal structures of the ER-LBD complexed with E2, DES, the SERMs raloxifene and tamoxifen, and the pure antiestrogen ICI 164,384, an analogue of fulvestrant (ICI 182,780), have been determined (Brzozowski et al., 1997; Shiau et al., 1998; Pike et al., 1999; Pike et al.,

2001). These structures reveal critical information regarding the agonist and antagonist activity of various ligands and showed that the structure of helix 12 within AF-2 is sensitive to ligand binding. For example when the ER α LBD is complexed with agonists such as E2, helix 12 is re-positioned over the ligand-binding pocket and it generates a functional AF-2 that interacts with LXXLL motifs of coactivators. In contrast, binding of antagonists such as raloxifene or tamoxifen with ER α -LBD results in displacement of helix 12 from its agonist position and this helix is re-positioned into the hydrophobic groove formed by helices 3, 4, and 5. This disrupts formation of the coactivator interaction surface (Shiau et al., 1998; Pike et al., 1999) and the ligand-dependent effects on helix 12 positioning is dependent on the agonistic or antagonistic activity of various ER ligands (Brzozowski et al., 1997; Pike et al., 2001). This forms the molecular basis for the action of SERMs.

1.6.3. Interaction of ERs with coregulators

Transcriptional activation by ERs is a complex process and requires recruitment of transcriptional regulators, such as GTFs, coactivators, corepressors, mediator complexes, HATs, and HDACs. Coregulators provide an additional layer of specificity and regulation of the transcriptional activity of the ER (Pearce and Jordan, 2004). ER α interacts directly with components of the basal transcription machinery such as TBP (AF-1 and AF-2 dependent) (Sadovsky et al., 1995), TAFII28 and hTAFII30 and these interactions are required for ER α -mediated transactivation (Jacq et al., 1994; Klinge, 2000). In addition to GTFs, ER α interacts with cofactors that specifically bind AF-1 or AF-2 domains and modulate transcriptional activity. The p160 steroid receptor

coactivators (SRC) were first discovered as nuclear proteins that interact with ligand-bound hormone receptors and subsequently a large number of coactivators have been identified and characterized. The AF-2 domain of ER α interacts with several coactivators including the SRC family (SRC1, SRC2/TIF2/GRIP1, and SRC3/AIB1/RAC3) and components of the mammalian mediator complex such as vitamin-D receptor interacting protein (DRIP) complexes, DRIP205 and DRIP 150 (Burakov et al., 2000; Warnmark et al., 2001; Wu et al., 2004; Lee et al., 2005). Mediators possess chromatin-remodeling ability and tether activated receptors to the basal transcriptional machinery. One of the primary functions of the p160 coactivator is to recruit other transcriptional coregulators, and HATs such as p300, CBP, and pCAF (p300/CBP-associated factor), to ER-dependent enhancers in target genes (Kamei et al., 1996; Chen et al., 2000; Matthews and Gustafsson, 2003).

The AF-2 also binds other coactivators such as PGC-1, a tissue- and promoter-specific PPAR γ -coactivator-1 (Tcherepanova et al., 2000), SNURF (Saville et al., 2002), PELP1, a proline-, glutamic acid-, leucine-rich protein 1 (Vadlamudi et al., 2001), and NCoA-7 (ERAP140) (Shao et al., 2002). With the exception of NCoA-7, most of these coactivators possess the canonical LXXLL motif that mediates their ligand-dependent interactions with the LBD of ER α (Shao and Brown, 2004). In addition a tissue-specific and kinase-regulated coactivator, GT198, interacts with the DBD of ER α (Ko et al., 2002). Coactivators that interact with AF-1 of ER α include RNA helicases p68 and p72 (Endoh et al., 1999), RNA coactivator SRA (Watanabe et al., 2001), CBP and p160 family of coactivators (Webb et al., 1998; Kobayashi et al., 2000). In addition to

coactivators, corepressors such as N-CoR and SMRT inhibit transcription of ER α -regulated genes via their recruitment of HDACs (Dobrzycka et al., 2003). For example HDACs recruited by corepressor complexes such as N-CoR/SAP31/SIN3/HDAC2 is required for the repression by tamoxifen-bound ER α (Shang et al., 2000; Huang et al., 2002a; Webb et al., 2003). In addition other proteins also repress ER-mediated transcription by distinct mechanisms. For example the ER-specific corepressor REA (Montano et al., 1999) and the orphan receptors such as SHP and DAX-1, act by competing with the p160 coactivators for binding agonist-bound ER (Johansson et al., 1999; Zhang et al., 2000a; Shao and Brown, 2004).

1.6.4 Phosphorylation of ER

ER α is phosphorylated on multiple amino acid residues and phosphorylation is enhanced upon binding E2. For example, in response to hormone binding, human ER α is predominately phosphorylated on Ser-118 (Le Goff et al., 1994; Joel et al., 1995; 1998) and to a lesser extent on Ser-104 and Ser-106 (Rogatsky et al., 1999). In response to activation of the MAPK pathway, phosphorylation occurs on Ser-118 and Ser-167. These serine residues are all located within the AF-1 region of ER α . In contrast, activation of protein kinase A (PKA) increases phosphorylation of Ser-236, which is located in the DBD (Chen et al., 1999). Although several groups have reported that ER α can be phosphorylated at tyrosine residues, Tyr-537 located in the LBD (Migliaccio et al., 1986; Arnold et al., 1995a; Arnold et al., 1995b), there are conflicting reports regarding the in vivo phosphorylation status of Tyr-537 (Lannigan, 2003). Phosphorylation of ER β has not been well examined.

1.6.5 Mechanism of transcriptional activation by ER

There are multiple mechanisms underlying transcriptional activation by ERs and these involve genomic and non-genomic pathways. The genomic pathways include the classical mechanism where ER directly binds to EREs and non-classical mechanisms that include interactions of ER with other DNA-bound transcription activators such as Sp1, nuclear factor κ B (NF- κ B), GATA-1 and AP-1. In addition ER function can be modulated in the absence of a ligand by growth factors and kinase signaling cascades. Moreover estrogen also activates non-genomic kinase signaling via a putative membrane receptor.

1.6.5.1 Classical mechanism of ligand-dependent ER action

A variety of proteins and processes affect ER function and the molecular mechanisms of ligand-induced gene expression or repression. ER α is sequestered in the nuclei of cells as part of a large inhibitory heat shock protein (hsp) complex. Upon binding E2, the ER undergoes a conformational change that results in displacement of hsps and formation of an ER homodimer which binds EREs located within the regulatory regions of target genes (Figure 19)(Klein-Hitpass et al., 1989; McDonnell and Norris, 2002). Once bound to an ERE, the ER interacts with basal transcription machinery and cofactor proteins to modulate transcription of target genes. Depending on the cell and promoter context, the DNA-bound receptor exerts either a positive or negative effect on expression of the downstream target genes. EREs were first identified in the 5'-flanking region of the *Xenopus vitellogenin A2* gene (Klein-Hitpass et al., 1986).

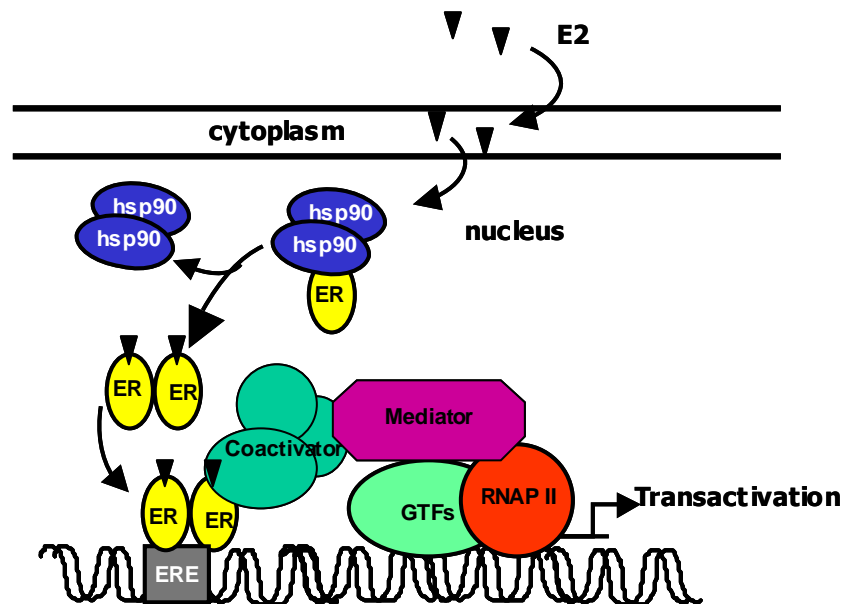


Figure 19. Classical mechanism of ER action (Grandien et al., 1997).

The minimal consensus ERE sequence is a palindromic inverted repeat (IR): 5'-GGTCAnnnTGACC-3'. Subsequently EREs have been identified in several genes including the human pS2, human complement C3 and chicken ovalbumin genes and ERE sequences vary considerably from the consensus ERE as indicated in Table 8. The ER can bind EREs as homodimers or heterodimers and these complexes activate some of the same genes although ER β tends to be less active than ER α (Cowley et al., 1997; Pettersson et al., 1997; Watanabe et al., 1997). For example overexpression of ER β decreases ER α -mediated transcriptional activation of ERE promoter construct (Hall and McDonnell, 1999). The antagonistic effects of ER β on ER α may arise from differences in their respective transactivation regions.

Table 8. Sequences of consensus and nonconsensus EREs from estrogen-responsive genes (modified from Klinge, 2001).

<i>Name</i>	<i>Sequence</i>
Xenopus vitellogenin A2 (vitERE)	5'-GTCAGGTCACAGTGACCTGATCAAAGTTAATGTAACCTCA-3' (19 bp ERE)
Chicken ovalbumin	-47/-43: 5'-TGGGTCA-3' which is half ERE and an AP-1 binding site
Human complement C3	-236: 5'-GTGTTACACCAGGTGGCCCTGACCCTGGGAG-AGTCCA-3'; +25: 5'-TGTCCCTCTGTCCCTCTGACCCTGCACTGTCC-CAGCAACCATG(<i>start</i>)-3'
Human progesterone receptor (hPR)	Form B is initiated at +744: +540: 5'-ATGGAGGCCAAGGGCAGGAGCTGACCAGCGCCGCCCT-3' Form A is initiated at +1236: +1148: 5'-TCCTGCGAGGTCACCAGCTCTTGGT-3'
Human pS2	5'-CTTCCCCTGCAAGGTCAGCGTGGCCACCCCGTGAGCCACT-3'
Rat creatine kinase B	-569: 5'-GGGCCCCGCCAAGGTCAGAA CACCCT GGGTGCTTCCGGGCGG GACC-3'
Rat <i>c-jun</i> (JUN5)	5'-GATCCTGAAGCAGAGCATGACCTTGAA-3'
Human BRCA1	+2023: 5'-TGGTCAGGCTGGTCTGGA ACTCCTGACCTG -3'
Human VEGF	-1560: 5'- AATCAGACTGACT GGCCTCAGAGCC-3'
Mouse <i>c-fos</i>	-278: 5'-GCGGAAGG CTTAGGAGACCC CCTAG-3'

The ER α /ER β heterodimer exists both in vitro and in vivo and binds ERE with affinity similar to that of the ER α homodimer (Cowley et al., 1997; Pettersson et al., 1997; Tremblay et al., 1999).

1.6.5.2 Non-classical genomic mechanisms of ER-mediated transactivation

There are several genes regulated by E2 that do not contain EREs however both ER α and ER β can modulate the expression of these genes without directly binding promoter DNA. These include binding of ER to other DNA-bound transcription factors such as AP-1, Sp1, NF- κ B, GATA-1 and other proteins. For example repression of the

IL-6 gene by E2 is mediated through the interaction of ER α with the *c-rel* subunit of NF- κ B (Ray et al., 1994; Stein and Yang, 1995) and repression of erythropoiesis gene by E2 involves interaction of ERs with GATA-1 (Blobel et al., 1995).

1.6.5.3 ER/AP1

One of the non-classical genomic mechanisms of E2 action involves interactions of ER with members of the jun/fos family of transcription factors bound to AP-1 sites. Genes that are activated by ER/AP-1 include the ovalbumin, collagenase and IGF-1 genes (Tora et al., 1988; Gaub et al., 1990; Tzukerman et al., 1991; Umayahara et al., 1994). Webb et al. (1995) showed that the ER α -DBD is not required for hormonal activation through AP-1, however AP-1 proteins are required. ER α interacts with c-Jun in vitro and the domain required for this interaction has been mapped to amino acids 259-302, which are located in the hinge domain of ER α (Jakacka et al., 2001; Teyssier et al., 2001). E2 activates AP-1 regulated genes in ER-positive MCF-7, ZR-75, and T47D breast cancer cells, but represses AP-1 target genes in other cells including ER-negative MDA-MB231 and BT20 cells (Philips et al., 1998). Moreover, estrogens and antiestrogens differentially activate ER α /AP-1 and ER β /AP-1 in HeLa, Ishikawa, MDA-453 and MCF-7 cells transfected with AP-1 reporter constructs. Agonists such as E2 and DES, and antagonists including tamoxifen and ICI 164384 activate transcription at AP-1 sites in the presence of ER α where as E2 and DES inhibited transcription in the presence of ER β . Raloxifene is only a partial agonist for the ER α /AP-1 pathway and the antiestrogens tamoxifen, raloxifene, and ICI 164384 acted as potent transcriptional activators of ER β /AP-1 in all the cell lines (Paech et al., 1997). This revealed a potential

control mechanism for differential regulation of estrogen-responsive genes by estrogens and antiestrogens and molecular analysis of these responses showed that the regions of ER required for AP-1 mediated transcription varies with cell type and ligand (Weatherman and Scanlan, 2001).

Webb and co-workers (1999) have summarized two AF dependent and independent pathways for activation of ER/AP-1 (Figure 20). The estrogen-activated ER α /AP-1 pathway requires both AF-1 and AF-2 and their interactions with the p160 family of coactivators; tamoxifen activates ER α /AP-1 through AF-1, and partly through an AF-independent pathway. The antiestrogens ICI 164384 and raloxifene activate ER β /AP-1 or AF-1-deleted (Δ AF-1) ER α /AP-1 through an AF-independent mechanism that involves interaction with the corepressor N-CoR since a mutation that eliminates ER binding to N-CoR eliminates antiestrogen activation (Webb et al., 2003). It has also been suggested that the DBD of ERs may play an important role in both AF-dependent and AF-independent pathways, even though DNA binding is not required (Bjornstrom and Sjoberg, 2002). For example mutations of a highly conserved lysine residue within the DBD (ER α .K206A/G) lead to super-activation of AP-1 through AF-dependent pathways (Uht et al., 2004).

1.6.5.4 ER/Sp1

There are two mechanisms of E2-mediated transactivation that involve ER and Sp1 and these include a DNA-dependent mechanism in which an ERE half (1/2) site and a GC-rich site are required for transactivation and a DNA-independent mechanism that requires only a GC-rich site.

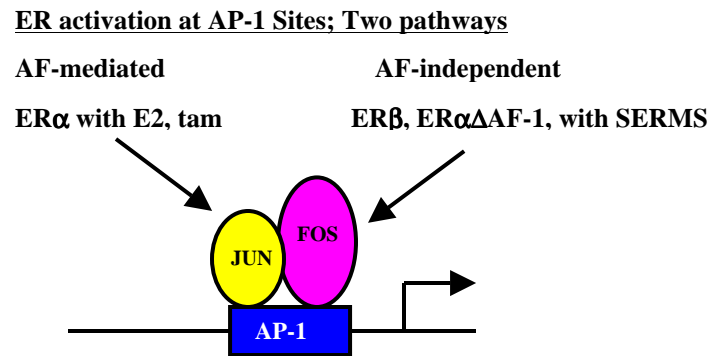


Figure 20. ERs use two separate pathways for activation of ER/AP-1 (Kushner et al., 2000).

Dubik and Shiu (1992) initially identified an E2-responsive region of the c-myc promoter that did not contain consensus EREs but an ERE 1/2 and a GC-rich site [GGGCA(N)₁₆GGCGG]. E2-mediated transactivation of this promoter construct was shown to require the DBD of ER α and involved both ER α and Sp1 bound to different sites (DNA-dependent) (Figure 21). Subsequent research in our laboratory identified similar motifs in cathepsin D (Krishnan et al., 1995), hsp27 (Porter et al., 1996) and TGF α (Vyhlidal et al., 2000) gene promoters. For example the E2-responsive region (–199 to –165) of cathepsin D gene promoter contains a GGGCGC(N)₂₃ACGGG motif and extensive mutational analysis of this promoter revealed that mutation of either the ERE 1/2 or GC-rich sites resulted in loss of hormone-induced transactivation. Similar motifs have been identified in rabbit uteroglobin (Dennig et al., 1995; Scholz et al., 1998), creatine kinase B (Wu-Peng et al., 1992), metastasis-associated protein 3 (MTA3) (Fujita et al., 2004) and progesterone receptor genes (Petz and Nardulli, 2000).

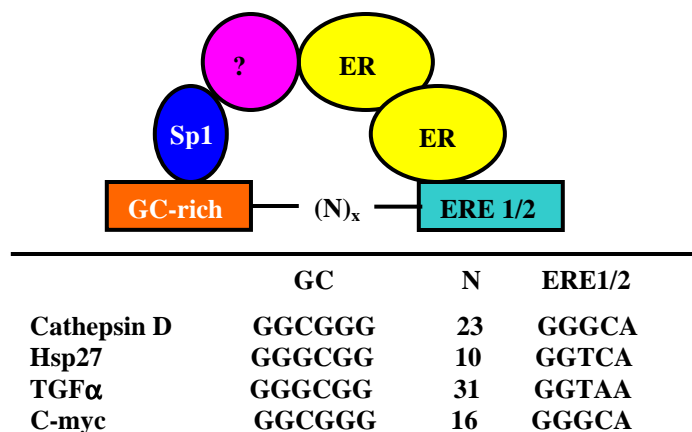


Figure 21. DNA-dependent ER α /Sp1 (Safe and Kim, 2004).

Surprisingly mutation of the ERE 1/2 site in the hsp27 gene promoter retained hormone-responsiveness in transient transfection studies suggesting that GC- rich site alone was sufficient for hormone-responsiveness (Porter et al., 1997). This led to further studies on the role of GC-rich sites in mediating ER α /Sp1 action. A construct containing consensus GC-rich Sp1 binding site linked to a chloramphenicol acetyl transferase (CAT) reporter gene (pSp1) was used in transient transfection assays in ER-positive MCF-7 and ER-negative MDA-MB-231 cells. E2 induced luciferase activity in both cell lines after transfection with pSp1 and an ER α expression plasmid, and E2 responsiveness was also observed in cells transfected with a mutant ER α lacking the DBD (HE11) suggesting that DNA binding was not required for ER α /Sp1 activation of GC-rich sites (Porter et al., 1997). In gel mobility shift assays Sp1, but not ER α , directly bound GC-rich oligonucleotides and supershifted ternary ER α / Sp1/DNA complexes were not detected. However kinetic analysis showed that ER enhanced the on-rate of Sp1-DNA binding,

but did not affect dissociation (off-rate) of the Sp1-DNA complex (Porter et al., 1997). ER α and Sp1 can be coimmunoprecipitated and in vitro studies with chimeric glutathione-S-transferase (GST)-Sp1 demonstrated that both ER α and ER β interacts with the C-terminal DBD of Sp1 while Sp1 interacts with multiple regions on ER α (Porter et al., 1997; Saville et al., 2000).

In a recent study Kim et al. (2005) confirmed ligand-dependent interactions of ER α and Sp1 in living MCF-7 cells after treatment with E2, 4'-hydroxytamoxifen (4-OHT), or ICI 182,780 using fluorescence resonance energy transfer (FRET). This led to a proposed model in which ER α interacts with GC-rich-bound Sp1 and mediates E2-dependent transactivation of genes containing GC-rich promoters (Figure 22). Research in this laboratory has shown that in MCF-7, T47D and ZR-75 breast cancer cells several hormone-induced genes associated with cell proliferation, cell cycle progression, and nucleotide metabolism are regulated by ER α /Sp1 mechanism. RNA interference using small inhibitory RNA for Sp1 (iSp1) decreased hormone-induced activation of ER α /Sp1 and cell cycle progression, demonstrating an important role for ER α /Sp1-dependent genes in the growth of ER-positive breast cancer cells (Abdelrahim et al., 2002).

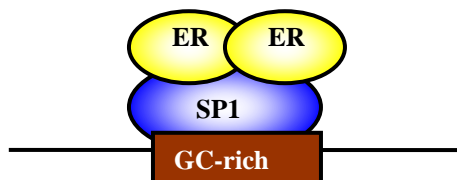


Figure 22. DNA-independent activation of GC-rich promoter by ER α /Sp1 (Porter et al., 1997).

Genes activated by ER α /Sp1 in breast cancer cells include VEGF (Stoner et al., 2004), bcl-2 (Dong et al., 1999), cyclin D1 (Castro-Rivera et al., 2001), adenosine deaminase (ADA), thymidylate synthase (TS) (Xie et al., 2000), DNA polymerase α (Samudio et al., 2001), retinoic acid receptor α (RAR α) (Sun et al., 1998), IGF binding protein 4 (IGFBP4) (Qin et al., 1999) and E2F1 (Wang et al., 1999; Ngwenya and Safe, 2003). Recently the human vitamin D3 receptor (Wietzke et al., 2005) and pS2 gene promoters (Sun et al., 2005) were also shown to be regulated by ER/Sp1 in MCF-7 cells. ER α /Sp1 regulated genes have also been identified in non-breast cancer cell lines and these include low density lipoprotein receptor (LDLR)(Sanchez et al., 1995; Li et al., 2001), epidermal growth factor receptor (EGFR) (Salvatori et al., 2000), rat SK3 (rSK3- a small conductance Ca⁺²-activated potassium channel)(Jacobson et al., 2003), and receptor for advanced glycation end products (RAGE) (Tanaka et al., 2000).

Saville and co-workers investigated the ligand- and cell context-dependent activation of ER α /Sp1 and ER β /Sp1 action in cells transfected with pSp1 containing a single GC-rich element. E2, 4-OHT and ICI 182,780 activated ER α /Sp1 in MCF-7, MDA-MB-231, and LNCaP cells, but not in HeLa cells, whereas hormone-dependent activation of ER β /Sp1 was not observed in any of the cell lines and decreased activity was observed in HeLa cells. Studies with chimeric ER proteins showed that the A/B domain of ER α were required for ER α /Sp1-mediated transactivation, and the A/B domain of ER β lacked the required transactivation function. Amino acids 79–177 region in the A/B domain of ER α were sufficient for ligand-induced ER α /Sp1 transactivation

(Saville et al., 2000). Kim et al. (2003) showed that estrogen or antiestrogen activation of ER α /Sp1 also required other regions of ER α .

1.6.6 Activation of ER by kinases

The ER can be modulated by a variety of different agents/pathways in the absence of E2 and these include peptide growth factors (Pietras et al., 1995), interleukin-2 (IL-2), dopamine (Power et al., 1991; Olesen et al., 2005), 8-bromo-cyclic adenosine monophosphate (cAMP) (Ince et al., 1994), insulin (Patrone et al., 1996), caveolin (Schlegel et al., 1999), cyclins A and D (Neuman et al., 1997; Trowbridge et al., 1997; Zwijsen et al., 1997; Zwijsen et al., 1998), and activators of the PKA and PKC pathways (Cho and Katzenellenbogen, 1993). Most growth factors activate cell proliferation, differentiation, or survival programs through binding their tyrosine kinase receptors, expressed in the plasma membrane and thereby activating various downstream kinase pathways. Growth factors that stimulate ER α include EGF, transforming growth factor (TGF- α) (Ignar-Trowbridge et al., 1996), IGF-I and heregulin-2 (Pietras et al., 1995). The mechanisms by which TGF- α and heregulin-2 activate the ER are unclear, however activation of ER α by EGF and IGF-1 has been extensively investigated.

EGF mimics several estrogen-like effects such as promotion of cell growth and induction of E2-responsive genes in the mouse reproductive tract (Nelson et al., 1991). Administration of the antiestrogen ICI 164,384 reduces the observed uterine responses to EGF (Ignar-Trowbridge et al., 1992). Moreover Curtis et al. (1996) showed that EGF did not induce DNA synthesis and transcription in uteri of α ERKO mice suggesting that EGF may act through ER α in absence of estrogen. IGF-1 activates the tyrosine kinase

receptor IGFR-I and also induces proliferation of breast cancer cells (Figure 23) (Hamelers and Steenbergh, 2003). Like EGF, IGF-I activates uterine cell proliferation *in vivo* and this is also dependent on ER α (Klotz et al., 2002). Both EGF and IGF-I activate ER α by inducing MAPK-dependent phosphorylation of ER α at Ser-118 (Kato et al., 1995; Bunone et al., 1996). EGF and IGF-1 also stimulate the phosphoinositol kinase 3 (PI3-K)/AKT pathway which in turn activates E2 responsive genes such as progesterone receptor in MCF-7 cells and these effects can be blocked by PI3-K inhibitors and ICI 182, 780 (Martin et al., 2000). IGF-I activates PI3-K- dependent phosphorylation of ER α at Ser-167 and enhances ER α -dependent transcriptional activity and cell proliferation. AKT overexpression also upregulates expression of estrogen-regulated pS2, Bcl-2, and macrophage inhibitory cytokine 1 genes in MCF-7 cells (Campbell et al., 2001).

The tyrosine kinase Src-dependent phosphorylation of ERK also stimulates AF-1 of ER α by phosphorylation of Ser-118. In addition SRC-dependent activation of JNK enhances phosphorylation of ER α at Ser-118 (Feng et al., 2001; Ho and Liao, 2002). In contrast to ER α , there is only one study showing that growth factors such as EGF activate ER β in the absence of estrogen via the MAPK pathway and this leads to recruitment of SRC1 by ER β (Tremblay and Giguere, 2001).

Activation of HER-2 by heregulin leads to direct and rapid phosphorylation of ER α on tyrosine residues and induction of progesterone receptor gene expression in breast cancer cells. It was also shown that overexpression of HER-2 in MCF-7 cells results in rapidly growing cells that are insensitive to both E2 and tamoxifen (Pietras et

al., 1995). This may explain, in part, tamoxifen resistance in breast tumors that overexpress HER-2. Shou et al. (2004) recently showed that tamoxifen behaves as an estrogen agonist in breast cancer cells that express high levels of the coactivator SRC3 and HER2, resulting in de novo resistance.

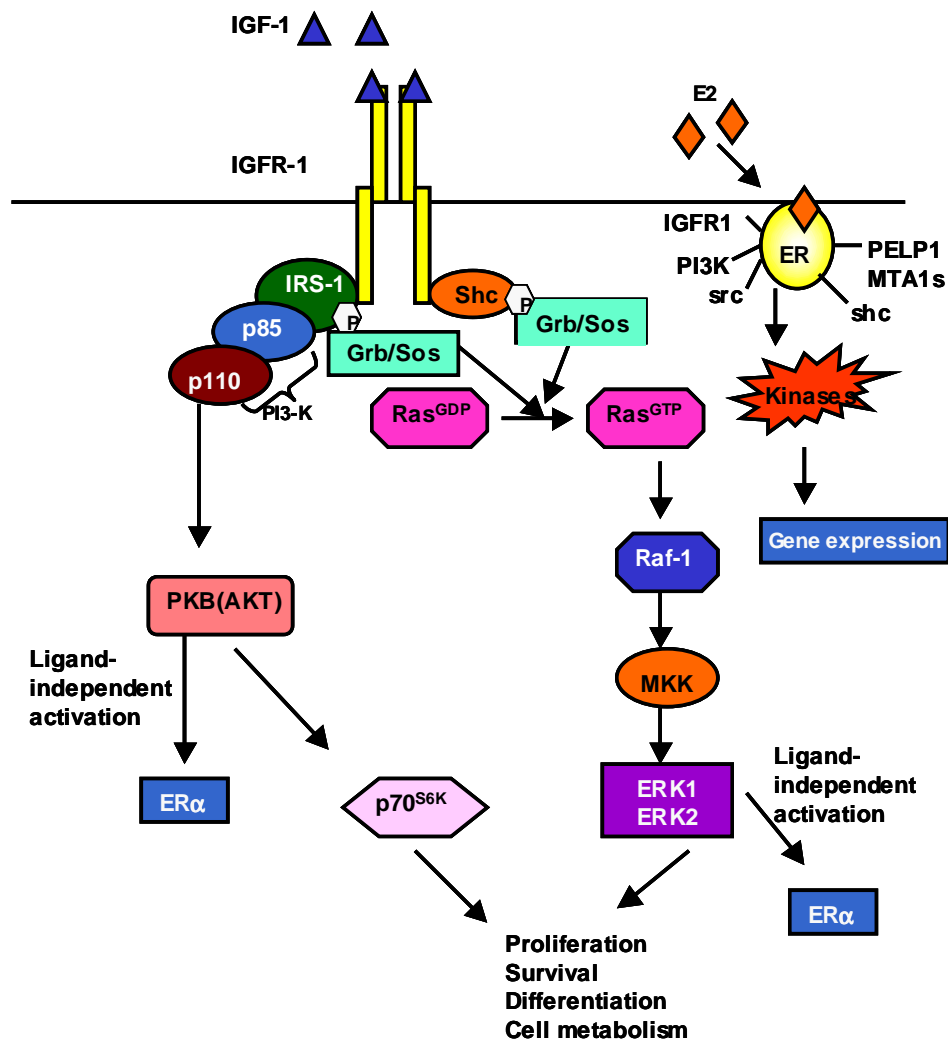


Figure 23. Activation of ERα by kinases (modified from Hamelers and Steenbergh, 2003).

Growth factors also modulate the transcriptional activity of ER by phosphorylation of NR coregulators such as SRC3 and SRC1 (Font de Mora and Brown, 2000; Rowan et al., 2000). It has been suggested that phosphorylation of coregulators may serve as point of convergence between E2 and growth factor signaling pathways and regulate the transcriptional activity of downstream target genes.

1.6.7 Non-genomic actions of estrogen

There is now considerable evidence that ER α can induce rapid non-genomic (extranuclear) responses that occur within seconds to minutes after the addition of estrogens in multiple tissues/cells including bone, breast, digestive and reproductive tract, vasculature and the nervous system (Bjornstrom and Sjoberg, 2005; Levin, 2005). These effects are rapid and induced by membrane-impermeable E2 such as E2-BSA (Taguchi et al., 2004) and are not blocked by inhibitors of protein or RNA synthesis (Losel et al., 2003) implying that transcription and translation are not induced (Simoncini and Genazzani, 2003; Acconcia and Kumar, 2005). These are called non-genomic effects of estrogens and are cell-type specific.

E2 rapidly activates several pathways including the elevation of intracellular calcium levels (Stefano et al., 2000a), stimulation of adenylate cyclase activity and cAMP production (Aronica et al., 1994; Razandi et al., 1999), secretion of prolactin (Watson et al., 1999) and generation of nitric oxide (Stefano et al., 2000b). In addition, E2 activates MAPKs in several cell types, including breast cancer, endothelial, bone, and neuroblastoma cells (Migliaccio et al., 1996; Bjornstrom and Sjoberg, 2005). Migliaccio et al. showed that the liganded ER α binds and activates c-Src in MCF-7 cells. Activated

c-Src phosphorylates Shc, which then associates with the Grb2/mSos complex and subsequently results in activation of MAPK (Migliaccio et al., 1996, 2000). E2 also activates the PI3-K pathway in MCF-7 cells and activated ER α forms a ternary complex with src and p85 α (Castoria et al., 2001; Sun et al., 2001). Activation of kinase pathways by estrogen affects cell growth, cell cycle progression, and survival in several cell lines including breast cancer cells. Table 9 depicts non-genomic activities of ER and proposed physiological relevance. In cultured endothelial cells (ECs), estrogen enhances NO release within minutes without altering expression of endothelial NO synthase (Adams et al., 2000) and this is due to activation of MAPK and PI3-K pathways. These effects may play a significant role in the cardioprotective properties of E2. The protective effects of estrogen in the bone is also attributed due, in part, to rapid activation of MAPK by E2 (Endoh et al., 1997; Jessop et al., 2001).

E2 also activates growth factor receptors such as IGF-1R (Kahlert et al., 2000) and EGFR (Brandeis et al., 1994; Filardo et al., 2000; Yang et al., 2004b). E2 activates EGFR by a mechanism that involves activation of G proteins, Src kinase, and matrix metalloproteinases, leading to increased MAPK and Akt (protein kinase B) activities (Razandi et al., 2003). Activation of growth factor signaling pathways has been implicated in breast cancer progression (Shou et al., 2004). Tamoxifen also activates membrane ER and this may explain acquired tamoxifen resistance in breast tumors (Kurokawa et al., 2000). The crosstalk between estrogen and growth factor signaling pathways may play critical role in resistance to endocrine therapies and breast cancer progression. The non-genomic effects of estrogen are mediated in part by cell-

surface/membrane ER forms that are linked to intracellular signal transduction proteins. A putative membrane ER was first proposed in 1977 (Pietras and Szego, 1977; Pietras and Szego, 1980), however no such membrane ER has been cloned and the precise nature of this receptor remains elusive (Ho and Liao, 2002). Razandi et al. (1999) demonstrated that membrane and nuclear ERs can arise from a single transcript by transfecting cDNAs for ER α and ER β into Chinese hamster ovary (CHO) cells, which lack endogenous ER.

Table 9. Summary of tissue-specific extranuclear activities of ER α and their proposed physiological relevance (Adapted from Ho and Liao, 2002).

Cell type	Signalling cascades	Proposed physiological effect
Neuroblastoma	MAPK	LHRH Secretion
Primary cortical neuron	PI3-K/Akt	Neuroprotection
	Src/Ras/MAPK	Neuroprotection
Cortical explant	Src/ERK/B-Raf	Neuronal Differentiation
Hippocampus	cAMP	Excitatory neurotransmission
Lactotroph	ERK ½	Prolactin production
Mammary cancer	Src/Ras/ERK	
	Src/PI3-K	Cell cycle progression
	P38	Apoptosis
	JNK	Anti-apoptosis
Cardiac myocyte	ERK ½	Cardioprotection
	JNK	
	P38	Cardioprotection
Lung myofibroblast	ERK ½	Anti-proliferation
Vascular smooth muscle	Calcium influx	Vasorelaxation
	ERK	Antiproliferation
Endothelial	P38	EC protection
	PI3-K/Akt/eNOS	No release decreased leukocyte accumulation
	MAPK/eNOS	NO release
	G proteins/eNOS	NO release
Colon cancer	Src/ERK ½	Cell growth
Uterus	cAMP	
Prostate cancer	Src/Ras/ERK	Cell proliferation
Osteoblast	ERK ½	
Macrophage Overexpression system	Src/Shc/ERK	
	Calcium influx	NO release, cell activation
	G proteins	
	IGFR	

Several other groups have also identified membrane ER using antibodies against the classical nuclear ER α and ER β (Pappas et al., 1995; Kim et al., 1999; Norfleet et al., 2000; Chambliss et al., 2002). For example Western blot analysis of purified membrane proteins with ER α antibodies revealed multiple high molecular weight membrane ERs (92 k, 110 k, and 130 k) as well as a 67 k ER α . In addition fluorescence, confocal and electron microscopy studies of MCF-7 cells have identified specific membrane ER α sites (Powell et al., 2001). The association of wild type and truncated ER α isoforms with the plasma membrane may be due to palmitoylation of the receptor (Li et al., 2003; Acconcia et al., 2004; Rai et al., 2005). Recent studies have shown that the E domain of ER α is critical for membrane localization (Razandi et al., 2003; Chambliss et al., 2005).

Alternatively ERs have been found in caveolae of endothelial cells where they activate eNOS through protein kinase-mediated phosphorylation (Kim et al., 1999; Chambliss et al., 2002; Razandi et al., 2002). It has also been shown that cytosolic ER α physically associates with signaling proteins including modulator of nongenomic activity of estrogen receptor (MNAR) (Wong et al., 2002), the adapter proteins Shc, p85 α of PI3-K, caveolin (Zschocke et al., 2002), IGF-1R (Song et al., 2004), and striatin (Lu et al., 2004) which leads to ER α membrane translocation and the activation of cell membrane-initiated kinases. Furthermore two nonclassical ERs, ER-X (Li et al., 2003), and GPR30 (Qiu et al., 2003; Revankar et al., 2005) have been identified in endothelial cell membranes and neurons respectively suggesting that the nongenomic effects of E2 are mediated by a distinct ER different from wild type ER α and ER β . The exact nature of

membrane/cytosolic ER mediating rapid effects of estrogen is controversial and this area of study is under intense investigation.

1.6.8 ER α and ER β knockout mice

The role of ER α and ER β in development and homeostasis has been analyzed using three knockout models that include α ERKO (Lubahn et al., 1993), β BERKO (Krege et al., 1998) and $\alpha\beta$ ERKO mice (Couse and Korach, 1999; Dupont et al., 2000). Loss of either ER α or/and ER β is not lethal and all three knockout mice survive to adulthood (Walker and Korach, 2004). Both male and female α ERKO mice are infertile but external phenotypes of both sexes are normal (Lubahn et al., 1993). However, α ERKO females showed noticeable internal gross differences, with hypoplastic uteri and E2 did not induce increases in uterine wet weight, hyperemia, or the alteration of vaginal epithelial cell morphology. The ovaries lacked corpora lutea and contained cystic and hemorrhagic follicles with few granulosa cells. α ERKO females exhibit decreased patterns of sexual behavior and do not show any lordosis posture or receptiveness to wildtype males even after treatment with E2. This lack of estrogen responsiveness is due to abrogation of ER α in the central nervous system.

The α ERKO mammary gland exhibits normal prenatal and prepubertal development, but remains rudimentary after puberty, lacking the epithelial branching and lobuloalveolar development as evident in wild-type glands (Bocchinfuso and Korach, 1997). Male α ERKO mice have decreased fertility, and low sperm count, low testis weight and decreased sperm motility (Lubahn et al., 1993; Eddy et al., 1996). They exhibit normal motivation to mount females but achieved less intromissions, have greatly

reduced aggressive behavior and rare ejaculations (Ogawa et al., 1997; Pearce and Jordan, 2004). β ERKO females exhibit normal sexual behavior and normal mammary gland development but have reduced fertility with fewer and smaller litters. The reduction in fertility is the result of reduced ovarian efficiency since the ovaries have fewer corpora lutea. β ERKO males show no abnormalities in sexual behavior (Krege et al., 1998; Ogawa et al., 1999; Dupont et al., 2000).

$\alpha\beta$ ERKO males and females are infertile. $\alpha\beta$ ERKO males exhibit a complete disruption of sexual behavior, from both consummatory and motivational aspects in addition to an 80% reduction in sperm count (Couse et al., 1999; Dupont et al., 2000; Ogawa et al., 2000). The uterine phenotype observed in $\alpha\beta$ ERKO mice is quite similar to that observed in α ERKO mice, however the $\alpha\beta$ ERKO mice exhibit a distinct ovarian phenotype where structures are similar to those observed in male seminiferous tubules of the testis. The granulosa cells of these "sex-reversed" follicles have undergone redifferentiation to a Sertoli cell phenotype, as indicated by both morphological and biochemical markers. These structures are not present in the prepubertal ovaries. The α/β ERKO mammary gland phenotype resembles that of α ERKO adult females (Korach et al., 2003).

Bone phenotypes in ERKO mice show shorter femurs in α ERKO and $\alpha\beta$ ERKO mice but not in β ERKO mice (Vidal et al., 1999). Female α ERKO mice have smaller bone diameters and males have lower bone density (Vidal et al., 1999). β ERKO female mice had higher bone density whereas β ERKO male mice showed normal bone density (Windahl et al., 1999; 2001). These results emphasize the importance of ER α in

maintenance of bone. Studies with knockout mice also suggest that ER α and ER β may play redundant roles in E2-mediated cardioprotection (Iafrati et al., 1997; Karas et al., 1999, 2001). Evidence for an obligatory role of ER α in mediating the detrimental actions of neonatal DES exposure in the murine reproductive tract was provided by Couse and Korach (2004) using knockout mice. The results in the α ERKO uterus and prostate showed that DES-effects on gene expression and tissue differentiation were not observed. Although ER β is highly expressed in the prostate epithelium, neonatal DES treatment induced similar responses in wild type and β ERKO male mice.

1.7 Aryl Hydrocarbon Receptor

1.7.1 Introduction

The aryl hydrocarbon receptor (AhR) is a ligand-dependent transcription factor that regulates expression of a battery of genes in a wide range of species and tissues. The AhR was first identified by Poland et al. (1976) who showed specific binding of radiolabeled 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), to an intracellular component in hepatic cytosol in C57BL/6J mice (Poland et al., 1976). TCDD is a persistent environmental contaminant that is a by-product of industrial processes and combustion of organic materials including municipal garbage and the most toxic among the halogenated aromatic hydrocarbon (HAH) environmental pollutants. TCDD binds the AhR with an extremely low K_d value and has been used extensively as a prototypical AhR ligand (Poland and Knutson, 1982). TCDD and other related halogenated aromatic hydrocarbons (HAHs) elicit a diverse spectrum of sex-, strain-, age-, and species-

specific responses. These effects include a severe wasting syndrome, tumor promotion, immunotoxicity, hepatotoxicity, teratogenesis, cardiac dysfunction, enzyme induction, and the modulation of endocrine systems (Kociba et al., 1978; Pitot et al., 1980; Poland and Knutson, 1982; Safe, 1986). In rodents, TCDD increases the incidence of hepatic carcinoma and pulmonary and skin tumors (Flodstrom et al., 1991; Kociba et al., 1978). In skin of hairless mice, TCDD promotes tumor formation at 1/100th the dose rate of phorbol-12-myristate-13-acetate (TPA), which is a known tumor promoter, in skin of hairless mice (Knutson and Poland, 1982; Poland et al., 1982). However TCDD was found to be non-mutagenic in Ames test and appears to be non-genotoxic (Geiger and Neal, 1981). Moreover treatment of female Sprague-Dawley rats with TCDD or PCDD for 2 and 6 months showed no covalent DNA adduct formation in kidney or liver of these animals (Randerath et al., 1988). Therefore it is likely that the carcinogenic responses are associated with the action of TCDD as a tumor promoter.

The most established effect of exposure to TCDD and other HAHs in humans is chloracne. The carcinogenicity of TCDD in humans had been controversial but TCDD was designated as a human carcinogen (Group1) in 1997 by International Agency for Research on Cancer (IARC). This classification was based on sufficient evidence in animals as a multisite carcinogen and limited evidence from humans where increased overall cancer mortality in industrial cohorts was observed (Steenland et al., 2004).

1.7.2 AhR ligands

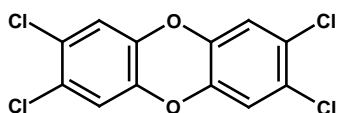
There are several synthetic and naturally occurring ligands that bind AhR but to date no high affinity endogenous ligand has been identified. The most extensively

characterized classes of AhR ligands are environmental contaminants that include HAHs such as the polyhalogenated dibenzo-p-dioxins (PCDDs), dibenzofurans (PCDFs), and biphenyls (PCBs) and polycyclic aromatic hydrocarbons (PAHs) such as 3-methylcholanthrene, benzo(a)pyrene, benzanthracenes, benzoflavones, and related compounds (Figure 24) (Kafafi et al., 1993; Safe, 1990; Waller and McKinney, 1995). PCDDs and PCDFs are formed as byproducts during synthesis of various organochlorine products (such as the herbicide 2,4,5-T), as a result of chlorine bleaching of wood pulp, during municipal, hospital and industrial waste incineration, metal production and fossil fuel or wood burning and other sources (Denison and Heath-Pagliuso, 1998). PCBs were produced commercially for use in transformers and capacitors, heat transfer and hydraulic fluids and for other applications.

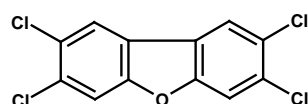
During the past fifteen years relatively large number of AhR ligands whose structure and physiochemical characteristics are dramatically different from that of the “classical” HAH and PAH ligands have been identified suggesting that AhR has a promiscuous ligand binding site (Cheung et al., 1996; Lee et al., 1996; Nagy et al., 2002; Quattrochi and Tukey, 1993). In addition there are several naturally occurring non-classical ligands that have been identified and characterized that can activate or inhibit AhR signaling pathways. Earlier studies showed that indole-3-carbinol (I3C), a secondary plant metabolite produced in vegetables of the Brassica genus, including cabbage, cauliflower and brussels sprouts, induced CYP1A1 activity. Subsequently it was found that Indol-[3,2]-carbazole and diindolylmethane (DIM) which are acidic

condensation products produced from I3C in vitro and in vivo exhibit more potent AhR agonists activities (Figure 25) (Bjeldanes et al., 1991).

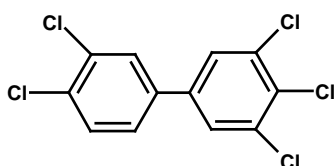
A. Classical AhR ligands



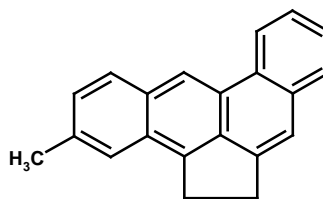
2,3,7,8-Tetrachlorodibenzo-p-dioxin



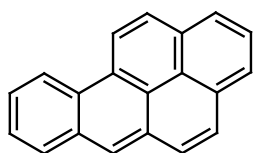
2,3,7,8-Tetrachlorodibenzofuran



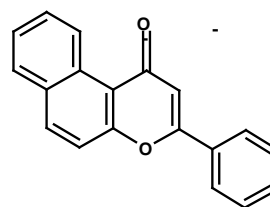
3,3',4,4',5-Pentachlorobiphenyl



3-Methylcholanthrene

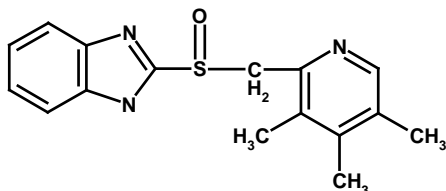


Benzo(a)pyrene

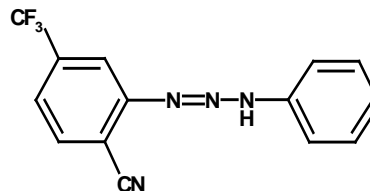


β -Naphthoflavone

B. Non-classical synthetic AhR ligands



Omeprazole



SKF71739

Figure 24. Structures of selected classical (A) and non-classical (B) synthetic ligands of AhR (Denison and Nagy, 2003).

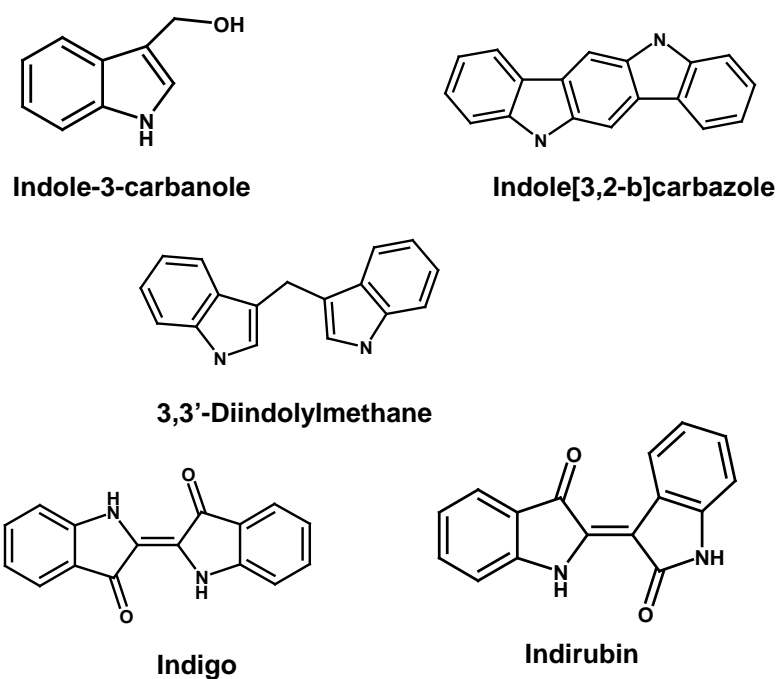


Figure 25. Structures of selected naturally occurring AhR ligands (Denison and Nagy, 2003).

Several other phytochemicals such as dibenzoylmethanes, 7,8-dihydrorutacarpine, curcumin, carotenoids [e.g., canthaxanthin, astaxanthin] and flavonoids have been shown to competitively bind the AhR and/or induce AhR-dependent gene expression (Gillner et al., 1993; Gradelet et al., 1996; Perdew and Babbs, 1991; Washburn et al., 1997). The existence of an endogenous ligand for the AhR has been inferred from studies reporting identification of the active nuclear AhR in unexposed cells/ tissues and from the phenotypes observed in AhR knockout mice which suggest a role for the AhR in normal development and physiology (Singh et al., 1996). Numerous laboratories have reported the existence of endogenous AhR ligands and these include indoles, tetrapyroles, arachidonic acid metabolites and others (Heath-Pagliuso et al., 1998; Miller,

1997; Seidel et al., 2001). However, these compounds are relatively weak AhR agonists compared to TCDD and are rapidly degraded by detoxification enzymes. Adachi et al. (2001) reported two tryptophan metabolites, indigo and indirubin, present in human urine as extremely potent AhR agonists (Figure 25). Indigo and indirubin activated AhR in an AhR/Arnt containing yeast cell bioassay system and were recently shown to act as inducers for cytochrome P450 1A1/2 mediated by AhR in mammals in vivo suggesting the possibility of indigoids as physiological ligands of the AhR (Sugihara et al., 2004).

1.7.3 Characterization of AhR and Arnt proteins

AhR cDNAs have been cloned and characterized from several mammalian species and strains and the rat (91-96 kDa), mouse (90-96 kDa) and human (96 kDa) AhRs have been studied in great detail. Mammals have only one gene for AhR (AhR1) but at least two AhR genes (AhR1 and AhR2) have been found in fish (Hahn, 2002; Hahn et al., 1997; Karchner et al., 1999). The human AHR locus encoding the structural gene for the Ah receptor has been localized to Chromosome 7p21-->p15 (Le Beau et al., 1994). AhR and its heterodimerization partner, the AhR nuclear translocator (Arnt) are members of the basic helix-loop-helix-per/Arnt/sim (bHLH-PAS) family of transcription factors (Burbach et al., 1992). Arnt was first identified as a factor required to translocate ligand bound AhR from the cytosol to the nucleus to form a transcriptionally active nuclear AhR complex in mouse hepatoma Hepa c4 cells (Hoffman et al., 1991). ARNT exists as an alternatively spliced form in both mouse and human, containing an additional 15 amino acids just N-terminal of the basic region encoded by exon 5 (Reisz-Porszasz et al., 1994). Other BHLH/PAS proteins include hypoxia inducible factors

(HIF-1 α , HIF-2 α /HLF, HIF-3 α), which mediate hypoxic responses, single minded proteins (SIM1 and SIM2), which are involved in control of neural development and circadian rhythm proteins (BMAL1 and BMAL2) (Kewley et al., 2004). The AhR is incapable of forming a homodimer and partners only with Arnt. However, on the other hand Arnt or hypoxia inducible factor-1 β is capable of homodimerizing (Antonsson et al., 1995; Sogawa et al., 1995) and is a heterodimeric partner for other bHLH/PAS proteins including SIM1, SIM2 (Probst et al., 1997) and HIF1 α (Wang et al., 1995). In addition Arnt has been shown to heterodimerize with CHF1 (Cardiovascular basic helix loop helix factor 1) (Chin et al., 2000) and EPAS1 (Endothelial PAS domain protein 1) (Tian et al., 1997) which are putative regulators of cardiovascular development and pathological angiogenesis respectively.

Several functionally important domains have been identified in the AhR and ARNT proteins (Figure 26). The amino-terminal region of AhR and ARNT contains a bHLH motif, which is involved in heterodimerization and is shared by other transcription factors such as Myc, Max and MyoD. AhR/Arnt binds to the dioxin response elements (DRE) rather than typical E-box recognized by other bHLH proteins. This binding to DNA occurs primarily through their basic domains in the N-terminal region. The sequence adjacent to the bHLH region constitutes the PAS domain, which contains two imperfect repeats of 50 amino acids, PAS A and PAS B. The PAS domain was initially identified as a conserved sequence among *Drosophila* PER, human ARNT and *Drosophila* SIM (Citri et al., 1987; Hoffman et al., 1991; Nambu et al., 1991; Reddy et al., 1986). PAS domains act as regulated protein interaction surfaces and are involved

in a wide variety of sensory/signaling processes in both eukaryotes and prokaryotes (Gu et al., 2000). Ligand binding to AhR occurs over the PAS B domain (Fukunaga et al., 1995; Whitelaw et al., 1993). In its latent (non-DNA binding) state, the AhR is found in the cytoplasm of many cell types, stably associated with two molecules of the 90 kDa molecular chaperone hsp90, p23 and hepatitis B virus X-associated protein (XAP2/AIP/Ara9) (Meyer et al., 1998).

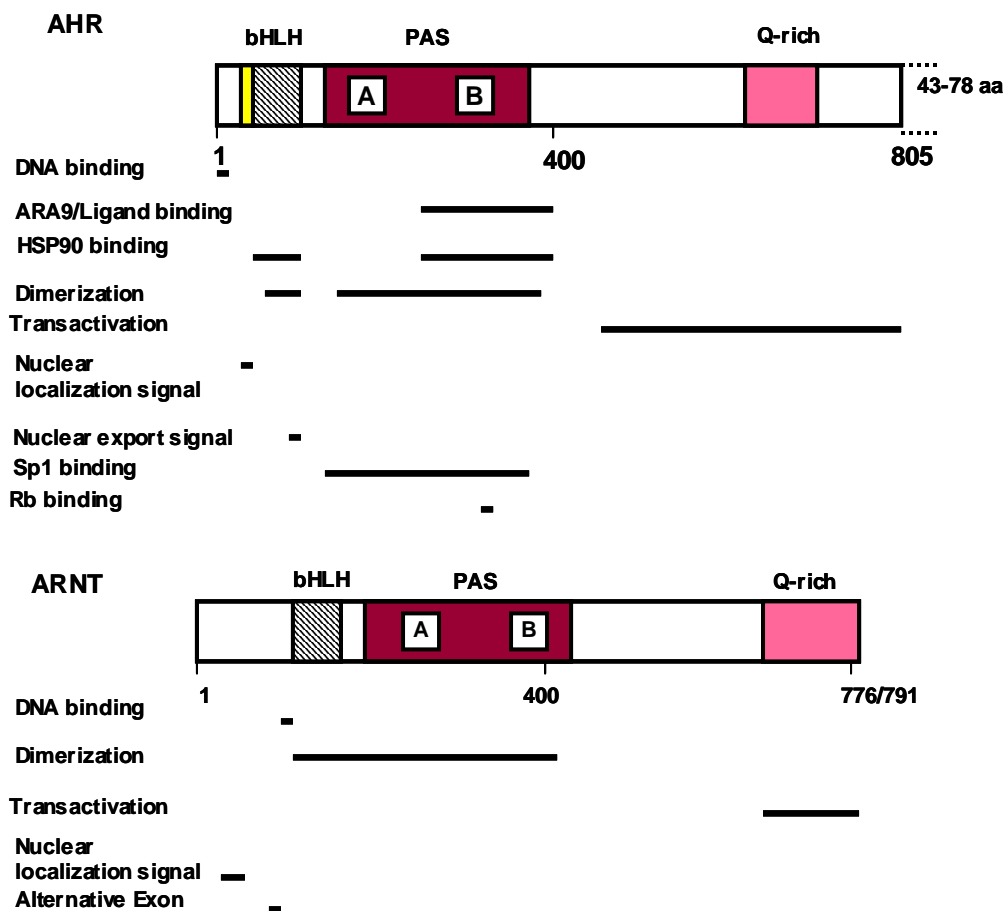


Figure 26. Schematic representation of functional domains of AhR and Arnt proteins (modified from Gu et al., 2000).

Hsp90 interacts with the AhR via both the bHLH region and PAS B domain, and this association is essential for AhR signaling (Perdew and Bradfield, 1996). ARA9/AIP interacts with the PAS-B/ligand-binding domains (Carver et al., 1998). Also both nuclear localization signal (NLS) and nuclear export signal (NES) sequences have been identified in the NH₂-terminal region of the AhR protein (Ikuta et al., 1998). AhR and ARNT also contain carboxyl-terminal trans-activation domains (TAD). The TAD of AhR is complex and composed of multiple segments that function independently and exhibit varying levels of activation whereas the TAD of ARNT is constitutive and limited to 43 amino acids. One of the TAD domains of the AhR that is rich in glutamine (Q-rich) is critical for transcriptional activation of dioxin-responsive genes (Jain et al., 1994; Kumar et al., 2001; Reisz-Porszasz et al., 1994; Rowlands et al., 1996).

The interactions between the AHR/ARNT with basal transcription factors such as TFIIB (Swanson and Yang, 1998), TFIIE (Rowlands et al., 1996), and TBP and, with the coactivator proteins such as SRC1, RIP140 and, p300, GRIP1 and BRG-1 (Kobayashi et al., 1997; Kumar and Perdew, 1999; Kumar et al., 1999; Rushing and Denison, 2002; Wang and Hankinson, 2002) facilitate gene activation by the AHR/ARNT heterodimer. Potential binding sites for NF κ B (Tian et al., 1999), Rb (Ge and Elferink., 1998), COUP-TF1 and ERR α 1 (Klinge et al., 2000) have been identified in the AhR protein (Swanson, 2002). In addition both bHLH and PAS domains of AhR and ARNT cooperatively interact with the zinc finger of Sp1 protein (Kobayashi et al., 1996).

1.7.4 AhR and Arnt null mice

The AhR is ubiquitously expressed in most tissues (Carver et al., 1994). Three independent groups have generated AhR-null mice and these mice are fertile and viable. There are considerable phenotypic differences observed among the three different knockout mice, but all three groups consistently reported hepatic defects with reduced liver weight and loss of inducibility of drug metabolizing enzymes (Fernandez-Salguero et al., 1995; Mimura et al., 1997; Schmidt et al., 1996).

It has been reported that reproductive success is adversely affected in Ahr-null females which have difficulties in surviving pregnancy and lactation, and rearing pups to weaning (Abbott et al., 1999). In addition, Thackaberry et al. (2003) demonstrated that AhR is required for normal insulin regulation in pregnant and older mice and for cardiac development in embryonic mice. The hepatic defects, changes in immune function and effects in other tissues in AhR(-/-) animals, strongly support a role for the AhR in cell and tissue physiology and homeostasis and other normal developmental processes.

Extensive studies on the AhR function using AhR-deficient mice have revealed that AhR is responsible for most, if not all, of the diverse biological, toxic (Nakatsuru et al., 2004) and biochemical effects caused by TCDD (Schmidt et al., 1996; Shimizu et al., 2000). Peters et al. (1999) reported that TCCD-induced teratogenicity is mediated by AhR by evaluating teratogenicity of TCDD in AhR-null mice. AhR-deficient mice has been shown to mount normal productive immune responses to two model antigens, allogeneic P815 tumor cells and sheep red blood cells, and neither the cellular nor the humoral response were suppressed by exposure to TCDD (Vorderstrasse et al., 2001).

These results confirmed the obligatory role of AhR in TCDD-induced immune suppression. Bunger and coworkers generated mice with a mutation in the AHR nuclear localization/DRE binding domain (Bunger et al., 2003). These AhR (nls) mice were found to be resistant to all TCDD-induced toxic responses including hepatomegaly, thymic involution, and cleft palate formation. Moreover, aberrations in liver development observed in these mice were identical to that observed in mice harboring a null allele at the Ahr locus. These results further support a model where most, if not all, AHR-regulated biology requires nuclear localization. Using AhR(-/-) mice, Nishimura et al. (2005) demonstrated that disruption of thyroid hormone and retinoid homeostasis after exposure to TCDD is mediated entirely via AhR.

Arnt is found exclusively in the nucleus of a range of cultured cells from different tissues and species (Pollenz et al., 1994). Arnt has an ubiquitous tissue expression pattern (Carver et al., 1994) but has been shown to localize within the cytoplasm to some level in several tissues (Hushka et al., 1998; Sojka et al., 2000). Arnt-deficient mice are embryonically lethal and cannot survive past 10.5 days. The primary cause of lethality appeared to be failure of the embryonic component of the placenta to vascularize and may be related to ARNT's role in hypoxia, angiogenesis and other important signaling pathways (Kozak et al., 1997; Maltepe et al., 1997).

1.7.5 Mechanism of AhR-mediated transactivation

The induction of CYP1A1 gene expression has been extensively used as a model for understanding the mechanism of AhR action (Figure 27) (Whitlock, 1999). In the absence of a ligand, AhR is mainly in the cytoplasm and exists in a complex with two

molecules Hsp90, and one molecule each of the co-chaperones, XAP2/AIP/Ara9 and a 38 kDa p23 protein (Carver et al., 1998; Kazlauskas et al., 1999; Ma and Whitlock, 1997; Meyer et al., 1998; Perdew, 1988; Pollenz et al., 1994). Ara9 is a tetratricopeptide repeat protein of the immunophilin family and binds to both Hsp90 and AhR. Several groups have implicated the role of ARA9 and p23 in stabilizing the cytoplasmic AhR (Bell and Poland, 2000; Kazlauskas et al., 1999; Kazlauskas et al., 2000; Ramadoss et al., 2004).

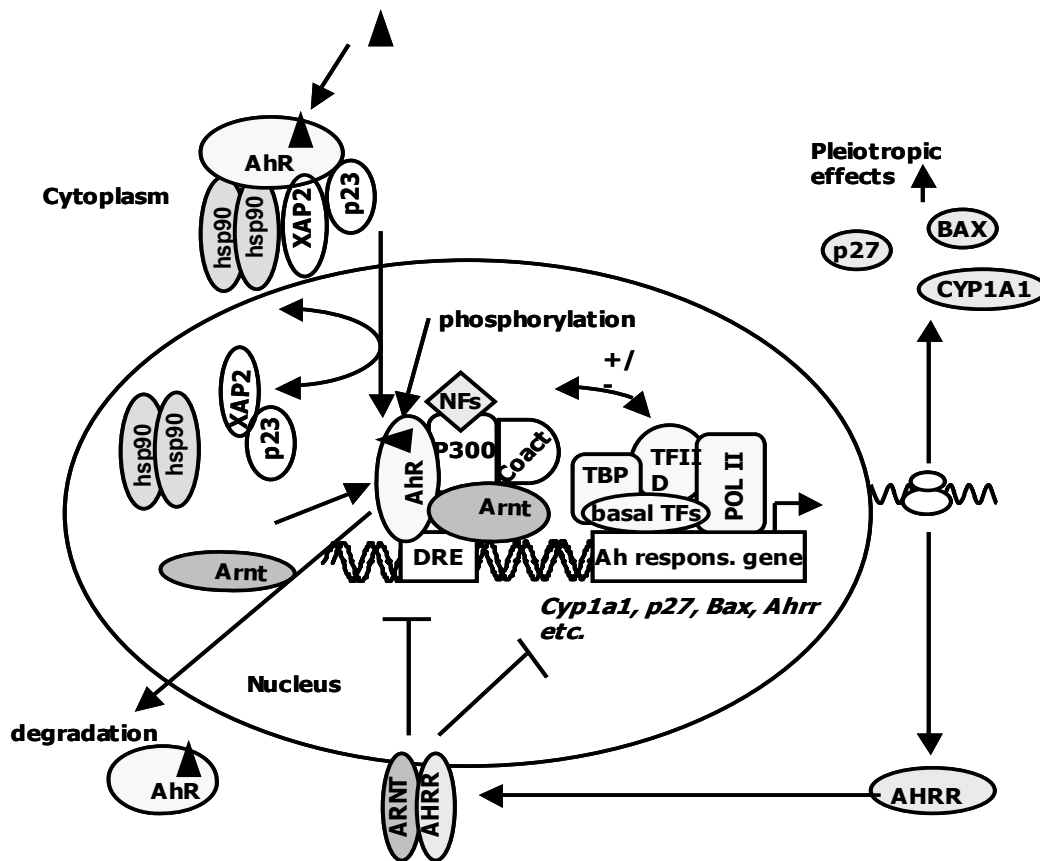


Figure 27. Mechanism of transcriptional activation by AhR (Mimura and Fujikuriyama 2003).

Hsp90 appears to be required for proper folding of AhR into a high affinity ligand binding conformation and is also involved in the retention of the AhR in the cytoplasm, perhaps by masking its nuclear localization sequence (NLS) (Ikuta et al., 1998; Pongratz et al., 1992). Ligand binding causes a conformational change in AhR protein, exposing its nuclear localization signal that triggers nuclear translocation of AhR and in the nucleus AhR exchanges hsp90 with its partner molecule Arnt (Lees and Whitelaw, 1999). The stage at which the release of p23 and XAP2 takes place from the AhR complex is not known. The AhR/Arnt heterodimer then binds to the dioxin or xenobiotic response elements (DRE/XRE) in the CYP1A1 gene promoter and promoters of other Ah- responsive target genes (Dolwick et al., 1993; McLane and Whitlock, 1994; Shen and Whitlock, 1992). It then recruits coactivators (CBP/300 for Arnt and RIP140, SRC1 for AhR) and general transcription machinery, to initiate the transcription of the target gene (Beischlag et al., 2002; Hankinson, 2005; Kim and Stallcup, 2004; Kobayashi et al., 1997; Kumar and Perdew, 1999; Kumar et al., 1999).

DRE motifs were initially identified in the 5' regulatory regions of CYP1A1 gene by several groups (Fujisawa-Sehara et al., 1986; Jones et al., 1986). Based on sequence alignment of several known DREs, a consensus DRE containing an N T/G TGCGTG A/C C/G A/T A/G G/C N sequence has been derived. The pentanucleotide core GCGTG is required for AhR/Arnt binding and flanking sequences are important for transcriptional activation (Safe, 2001). The transcriptional activity of the AhR can be autoregulated. Mimura et al. (1999) reported that the liganded AhR complex activated gene expression of another bHLH/PAS protein designated as the AhR repressor (AhRR)

(Figure 28). AhRR inhibits AhR function by competing with AhR for dimerizing with Arnt and binding to the XRE sequence. This represents a novel mechanism for regulating AhR function by negative feedback inhibition. The AHRR–ARNT complex itself is capable of binding to gene promoter XREs but does not transactivate these target genes (Baba et al., 2001; Korkalainen et al., 2004; Mimura et al., 1999).

Basal expression of AHRR mRNA has not been detected in the tissues of untreated mice, but AHRR mRNA levels were induced in lung, thymus, heart, liver, kidney, and intestine after treatment with 3-methylcholanthrene. However, in humans, AHRR is constitutively expressed in various normal tissues, especially in testis (Mimura et al., 1999; Tsuchiya et al., 2003; Yamamoto et al., 2004). An additional regulation of AhR function is due to induced degradation of AhR protein. Degradation of AhR in response to TCDD has been observed in several cell lines and tissues and it is established that ligand-induced downregulation of AhR is mediated by ubiquitin and 26S proteasome pathway following nuclear export (Davarinos and Pollenz, 1999; Pollenz, 2002; Roberts and Whitelaw, 1999). It has been demonstrated that inhibition of proteasomes blocked AhR degradation resulting in strong and prolonged activation of AhR mediated genes (Ma et al., 2000). There is also evidence suggesting that phosphorylation of AhR/Arnt heterodimer is important for transactivation since the binding of AhR/Arnt to DRE was shown to be abolished by phosphatase treatment (Park et al., 2000). Recently several reports have suggested involvement of MAPK and other kinase pathways in modulating the activity of the AhR complex (Tan et al., 2002; Tan et al., 2004; Yim et al., 2004).

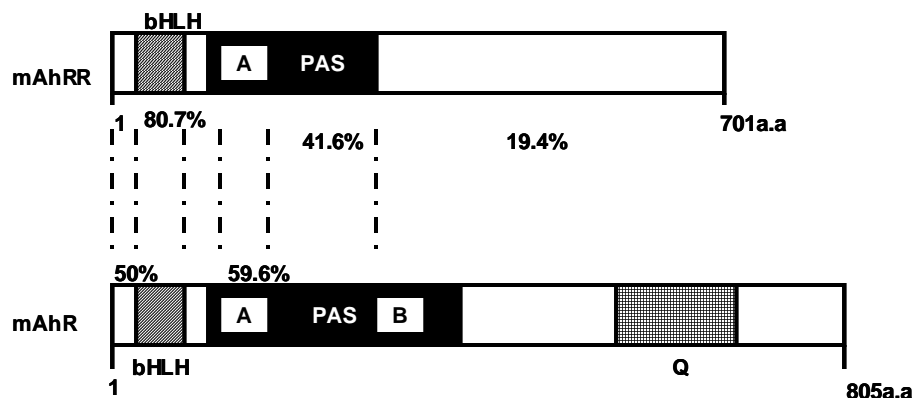


Figure 28. Domain structures of mAHR and mAHR proteins. Percentage identity between mAHR and mAHR are indicated (Mimural et al., 1999).

1.7.6 Target genes for TCDD

It has been suggested that most of the pathological effects of TCDD are due to transcriptional activation or repression of genes. TCDD induces several phase I enzymes (cytochrome P450s CYP1A1, CYP1B1, and CYP1A2) and phase II (NAD(P)H-dependent quinone oxidoreductase-1, glutathione-S-transferase GST-Ya, UDP-glucuronosyltransferase) drug metabolizing enzymes (Whitlock, 1999). In addition a number of other genes involved in cell proliferation (TGF- β , IL-1 β and PAI-2), cell cycle regulation (p27, p21 and jun-B), apoptosis (Bax)(Matikainen et al., 2001) and other pathways, are also induced by AhR ligands (Kolluri et al., 1999; Son and Rozman, 2002; Sutter et al., 1991). TCDD inhibits cell cycle progression in 5L rat hepatoma cells by direct induction of p27 mRNA and protein levels (Kolluri et al., 1999). Treatment of PC-3 and DU145 human prostate cancer cells with TCDD results in dose and time dependent increase in matrix metalloproteinases MMP-9 expression and also stimulates MMP-9 protein secretion (Haque et al., 2005). Most AhR-regulated genes contain XRE

in their regulatory regions and are regulated by direct binding of ligand-activated AhR to XRE sequences, however, there is considerable evidence that TCDD can affect gene expression through other indirect mechanisms, such as AhR complex formation with retinoblastoma protein (RB), RelA, and SP1 (Dunlap et al., 2002; Ge and Elferink, 1998; Kim et al., 2000; Wang et al., 1998) and effects on kinases such as src kinase (Dunlap et al., 2002). For example TCDD induces the transcription of *fos* and *jun* genes by both AhR- dependent and -independent mechanisms (Hoffer et al., 1996; Puga et al., 1992).

Recently, Weiss et al. (2005) showed that the induction of *c-jun* in 5L- rat hepatoma cells depends on activation of p38-mitogen-activated protein kinase (MAPK) by an AhR-dependent mechanism and differs from the classical mode of AhR-dependent regulation of gene expression by binding of the receptor to its XRE recognition sequences in promoters of regulated target genes. In addition, TCDD affects gene expression by stimulation of growth factors (Davis et al., 2000) and through interference with other nuclear receptors such as ER (Wormke et al., 2003). Several groups have used microarray technology to identify novel genes regulated by TCDD. Boverhof et al (2005) performed comprehensive temporal and dose-response microarray analyses using custom cDNA microarrays containing 13,362 cDNA clones on hepatic tissue from immature ovariectomized C57BL/6 mice treated with TCDD. The group reported changes in expression of genes involved in several physiological processes such as oxidative stress and metabolism, differentiation, apoptosis, gluconeogenesis, and fatty acid uptake and metabolism. Table 10 describes a list of several other genes regulated by TCDD.

Table 10. List of genes regulated by TCDD.

mRNA/promoter/protein upregulated or induced by TCDD	
Aldehyde dehydrogenase	(Dunn et al. 1988)
Aldehyde oxidase 1 (AOX1).	Mouse hepatoma cell line Hepa-1 (Rivera et al., 2005).
Basic leucine zipper transcription factor NRF2 (NF-E2 p45-related factor 2).	Mouse hepatoma 1c1c7 (Miao et al., 2005).
Cyp2a5.	Primary hepatocyte cultures derived from livers of DBA/2 and C57BL/6 mice (Arpiainen et al., 2005).
Insulin-like growth factor binding protein-1 gene expression.	Human hepatocytes and HepG2 human hepatoma cells (Marchand et al., 2005).
Suppressor of cytokine signaling 2 (Socs2).	Murine B cells (Boverhof et al., 2004).
Plasminogen activator inhibitor-1.	Mouse hepatoma cell lines.(Son and Rozman, 2002).
p21.	Lncap and MCF-7 cells (Barnes-Ellerbe et al., 2004).
HES-1(protein and mRNA levels).	T47D human mammary carcinoma cells (Thomsen et al., 2004).
CAAT/enhancer binding protein-alpha (C/EBPalpha).	Mouse adipose tissue and liver (Liu et al., 1998).
Nuclear factor, DIF-3.	Mouse embryonic stem (ES) cells(Ohbayashi et al., 2001).
MMP-1.	Human Keratinocytes (Murphy et al., 2004).
mRNA/promoter/protein downregulated by TCDD	
C/EBP beta.	Mouse adipose tissue and liver (Liu et al., 1998)
Gamma-catenin.	Rat liver epithelial cells (WB-F344).(Dietrich et al., 2003).
Spermatogenesis-related factor-2 (SRF-2).	Rat testis (Yamano et al., 2005).
T-cadherin mRNA.	Rat aortic smooth muscle cells (Niermann et al., 2003).

1.7.7 Inhibitory AhR/ER crosstalk

TCDD inhibits several E2-induced responses both in vitro and in vivo.

Kociba and co-workers (1978) first reported that several age-dependent spontaneous mammary and uterine tumors were decreased in female Sprague-Dawley rats exposed to TCDD in the diet for up to two years. These results suggested that TCDD exhibits antiestrogenic activity and inhibited formation and/or growth of two E2-dependent tumors. Subsequent studies showed that TCDD inhibits mammary tumor formation and

tumor growth in 7,12-dimethylbenzanthracene-induced female Sprague-Dawley rats and inhibits tumor growth in athymic nude mice bearing human breast cancer cell xenograft (Gierthy et al., 1993; Holcomb and Safe, 1994; Tritscher et al., 1995).

TCDD exhibited several antiestrogenic effects in the uterus of immature or ovariectomized rats and mice. In these animals, TCDD inhibited several E2-induced uterine responses including uterine wet weight increase (Romkes et al., 1987), DNA synthesis, progesterone receptor (PR) binding, peroxidase activity (Astroff and Safe, 1990), EGF receptor binding and increased EGFR (Astroff et al., 1990) and c-fos mRNA levels (Astroff et al., 1991). The AhR is expressed in the rodent uterus and the structure-dependent antiestrogenic activities of TCDD and related compounds as antiestrogens corresponded to other AhR binding affinities thereby supporting a role for the AhR in mediating the responses (Romkes and Safe, 1988; Safe et al., 2000).

Buchanon et al. (2000) showed that TCDD inhibits E2-induced uterine epithelial labeling index (LI) and lactoferrin (LF) mRNA expression in wild type but not in AhR knockout mice. Recently, Takemoto et al. (2004) demonstrated that cotreatment of mice with E2+TCDD resulted in significant decrease in E2-induced uterine peroxidase activity (UPA) in wild type AhR mice but not in AhR^{-/-} and cyp1b1^{-/-} knockout mice suggesting that antiestrogenic effects of AhR requires AhR as well as CYP1b1. Moreover, TCDD also inhibited E2-induced cyclin A1, cyclin B1, and cyclin D2 in the uterus of ovariectomized C57BL/6J mice. This was associated with increased expression of TGF β , which may play a role in the growth inhibitory effects of TCDD (Buchanon et al., 2002). In humans, accidental exposure of residents of Seveso, Italy (1976) to TCDD

resulted in a decrease in mammary and endometrial cancer (Bertazzi et al., 1998; Bertazzi et al., 2001), thus supporting the anti-estrogenic effects of TCDD in humans.

TCDD inhibits expression of a large number of E2-induced genes/proteins and other related activities in various cell lines including ER positive breast and endometrial cancer cells. Gierthy and coworkers initially reported that TCDD inhibited E2-induced secretion of tissue plasminogen activator activity, postconfluent focus production, and proliferation of ER-positive MCF-7 cells, whereas no effects were observed in ER-negative MDA-MB-231 cells (Gierthy et al., 1987; Gierthy and Lincoln, 1988). Subsequent studies revealed that TCDD and other AhR agonists inhibited secretion of pro-cathepsin D, cathepsin D, 160-kD protein and pS2 (Biegel and Safe, 1990) in addition to inhibition of E2-induced glucose metabolism (Narasimhan et al., 1991).

TCDD also inhibited E2-induced mRNA levels of several genes including c-fos (Duan et al., 1999), pS2 (Zacharewski et al., 1994; Gillesby et al., 1997), Hsp27 (Porter et al., 2001), prolactin receptor (Lu et al., 1996), PR (Harper et al., 1994), and cyclin D1 (Wang et al., 1998). TCDD inhibited reporter gene activity in cells transfected with constructs containing EREs (Nodland et al., 1997) and promoter fragments of cathepsin D (Krishnan et al., 1995; Wang et al., 2001b), creatine kinase B (Castro-Rivera et al., 1999), pS2 (Gillesby et al., 1997), Hsp27 (Porter et al., 2001), c-fos (Duan et al., 1999) and E2F1 genes (Ngwenya, S unpublished). Wang et al. reported that TCDD significantly inhibited E2-induced hyperphosphorylation of RB, cyclin D1 protein, and cdk2-, cdk4-, and cdk7-dependent kinase activities in MCF-7 cells and the anti-proliferative activity of TCDD may be due, in part to downregulation of several E2-

induced cell cycle proteins/activities (Wang et al., 1998). Kohle et al. (2002) investigated biological effects of a constitutively active mutant of the aryl hydrocarbon (Ah) receptor (CA-AhR), in modulating estrogen receptor function in MCF-7 cells. The conditional expression using the tet repressor of CA-AhR inhibited estrogen-dependent cathepsin D expression and growth of MCF-7 cells thereby suggesting the role of AhR in mediating growth inhibitory effects. Moreover TCDD inhibited selected estrogen-induced responses in wild-type but not benzo[*a*]pyrene resistant (BaPR) MCF-7 cells which expresses ER α and Arnt but only minimal levels of the AhR thus further confirming the requirement of AhR expression for mediating AhR/ER crosstalk (Moore et al., 1994). TCDD inhibited several E2-induced responses including cell proliferation in Ishikawa and ECC1 endometrial cancer cell lines (Castro-Rivera et al., 1999; Wormke et al., 2000a) and inhibitory AhR/ER crosstalk is also observed in ovarian cancer cells (Rowlands et al., 1993; Rogers and Denison, 2002).

1.7.8 Mechanism of Inhibitory AhR-ER α crosstalk

The interaction between AhR and ER α signaling pathway is complicated and several mechanisms have been proposed to explain the inhibitory AhR/ER α crosstalk. These include increased metabolism of E2, direct interactions of the AhR with critical promoter regions of E2 responsive genes, induction of inhibitory factors, competition for common nuclear coregulatory proteins, and proteasome-dependent degradation of ER α (Safe and Wormke, 2003).

1.7.8.1 Increased metabolism of E2

Spink and co-workers demonstrated that TCDD and other AhR agonists induce CYP1A1 and CYP1B1 expression in breast cancer cells. CYP1A1 and CYP1B1 are involved in oxidative metabolism of E2 resulting in depletion of intracellular hormone levels (Spink et al., 1990; Spink et al., 1992; Spink et al., 1994). The limiting levels of E2 may result in decreased hormone-responsiveness that may contribute to the observed inhibitory crosstalk between AhR and ER signaling pathways. However, there is evidence that alteration in E2 metabolism cannot fully account for observed antiestrogenic responses. For example, circulating E2 levels are not altered in rodents treated with TCDD (Shiverick and Muther, 1982). TCDD and other related compounds inhibit E2-induced responses at time points that precede induction of CYP1A1 protein (Krishnan et al., 1995). Several SAhRMs do not induce CYP1A1 in breast cancer cells or in rodent models in vivo but inhibit cell/tumor growth. These results suggest that CYP1A1-dependent depletion of E2 through oxidative metabolism is not necessary for inhibitory AhR-ER α cross-talk (Safe and Wormke, 2003).

1.7.8.2 Proteasome-dependent degradation of ER α

TCDD and other AhR agonists were initially reported to decrease the levels of ER α protein levels in MCF-7 cells and this correlated with their binding affinities for the AhR (Harris et al., 1990). Later, Wormke et al. (2000b) demonstrated that TCDD causes proteasome-dependent degradation of both AhR and ER α resulting in decreased levels of immunoreactive AhR and ER α proteins in T47D, MCF-7, and ZR-75 breast cancer cells. Moreover, cotreatment with TCDD plus E2 resulted in 80-90% (enhanced)

degradation of cellular ER α levels within 3 h (Wormke et al., 2003). Thus cotreatment of cells with TCDD plus E2 may result in proteasome-dependent degradation of ER α below critical levels required for hormone-induced transactivation.

1.7.8.3 Induction of Inhibitory factors

TCDD inhibited E2-induced pS2 mRNA and ERE-reporter gene activity in BG-1 ovarian cancer cells and this was blocked by cycloheximide, a protein synthesis inhibitor. These results suggest that the inhibitory effects of TCDD may be due to induction of an inhibitory factor (Rogers and Denison, 2002). Hairy and Enhancer of Split homolog-1 (HES-1) is downregulated by estrogen and is involved in mediating proliferative effects of E2 on breast cancer cells (Strom et al., 2000). Thomsen et al. (2004) recently showed that HES-1 is up-regulated by TCDD both at protein and mRNA levels in T47D cells and that AhR-mediated up-regulation of HES-1 mRNA is caused by transcriptional activation of the HES-1 gene. This upregulation of HES-1 expression was correlated with suppression of cell proliferation and this may be in part, to inhibition of E2F-1 through a HES element. Thus, the regulation of HES-1 by TCDD and E2 with opposite effects may represent a novel pathway for the repression of E2 signaling by TCDD-activated AhR.

1.7.8.4 Inhibitory DREs

One of the mechanisms of inhibitory AhR/ER α crosstalk involves direct interactions of the nuclear AhR complex with "critical" regions of E2 responsive gene promoters that results in suppression of gene expression. Functional inhibitory DREs (iDREs) with GCGTG sequence that corresponds to the pentanucleotide core of a DRE

required for AhR-mediated transactivation, have been identified in the cathepsin D, pS2, Hsp27, and c-fos gene promoters; however the mechanisms of inhibition are gene promoter specific.

1.7.8.5 Competition for shared transcription factors

AhR and ER interact with several common transcription factors and cofactors including nuclear factor 1 (Ricci et al., 1999) and steroid receptor coactivators such as estrogen receptor associating protein 140 (ERAP 140), RIP140 (Kumar et al., 1999), SRC1 (Kumar and Perdew, 1999) and CBP/p300 (Kobayashi et al., 1997). In addition the silencing domain of SMRT corepressor interacts with the PAS B domain of the AhR (Nguyen et al., 1999; Rushing and Denison, 2002; Fallone et al., 2004).

1.7.9 SAhRMs

Research in our laboratory has identified two groups of selective AhR modulators (SAhRMs) as potential therapeutic agents that target the AhR for treatment of breast cancer. This includes alternate-substituted (1,3,6,8- and 2,4,6,8-) alkyl PCDFs and ring-substituted diindolylmethanes (DIMs) (Safe et al., 1999; Safe et al., 2000). These compounds interact with the AhR and exhibit partial AhR antagonist activities for many of the AhR-mediated toxic responses while retaining antiestrogenic properties. Prototypical SAhRMs such as 6-methyl-1,3,8-trichlorodibenzofuran (6-MCDF) (Figure 29) and related alternate-substituted alkyl PCDFs inhibited E2-induced responses in vitro as well as in the rat uterus. 6-MCDF also inhibited mammary tumor growth in DMBA-induced rat mammary tumor model without any significant changes in liver/body weight ratios, liver morphology or induction of hepatic CYP1A1-dependent

activity (McDougal et al., 1997). Cotreatment with tamoxifen (TAM) and 6-MCDF resulted in synergistic and additive effects on inhibition of DMBA-induced rat mammary tumor growth. Moreover, 6-MCDF inhibited two TAM-induced markers of estrogenicity in the uterus without affecting the desirable ER agonist activity of TAM on bone growth (McDougal et al., 2001). TCDD and 6-MCDF also inhibited pancreatic cancer growth via induction of p21 and cell cycle arrest (Koliopoulos et al., 2002).

On the other hand, the ring-substituted diindolylmethanes (DIMs) are structurally related to phytochemicals such as indole-3-carbinol (I3C) that are components of cruciferous vegetables and exhibit antitumorigenic activity. DIM (Figure 29) is a major acid-catalyzed metabolite of I3C formed in the gut that binds to the AhR and exhibits anticarcinogenic activity *in vivo* in rodent models as well as inhibiting growth of cancer cell lines (Wattenberg and Loub, 1978; Stoewsand et al., 1988; Kojima et al., 1994). For example, Chen et al. (1998) showed that DIM inhibits E2-induced proliferation of MCF-7 cells, reporter gene activity in cells transiently transfected with an E2-responsive plasmid (containing a frog vitellogenin A2 gene promoter insert) and down-regulated the ER.

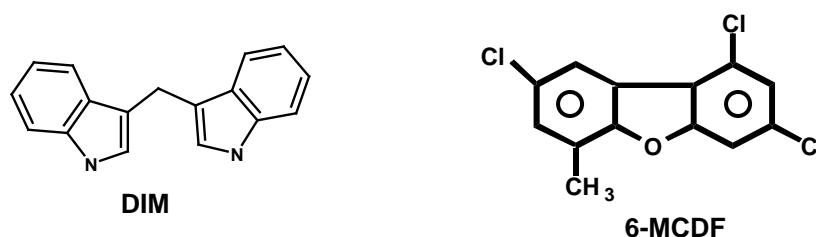


Figure 29. Structure of SAhRMs (McDougal et al., 1997, 2001).

Moreover, DIM also inhibits DMBA-induced mammary tumor growth in Sprague-Dawley rats and this was not accompanied by induction of hepatic CYP1A1-dependent activity. Several ring-substituted DIMs synthesized in this laboratory inhibited E2-induced growth of MCF-7 cells and DMBA-induced mammary tumor growth in Sprague-Dawley rats (McDougal et al., 2000). These results are consistent with the epidemiological evidence showing that cruciferous vegetable consumption is associated with prevention of several cancers including breast cancer and this could be due, in part, to indole-3-carbinol and related compounds that activate the AhR (Murillo and Mehta, 2001).

1.8 CAD

Pyrimidine nucleotides play a critical role in cellular metabolism serving as activated precursors of RNA and DNA; and CDP-diacylglycerol phosphoglyceride for the assembly of cell membranes and UDP-sugars for protein glycosylation and glycogen synthesis. Pyrimidines can either be synthesized *de novo* from small metabolites or via salvage pathways and recycling of preformed pyrimidine bases (Evans and Guy, 2004). The salvage pathway is usually sufficient for resting cells (Zaharevitz et al., 1992), however, activation of *de novo* pyrimidine biosynthesis is essential for growth of tumors and proliferating cells (Roux et al., 1973; Huisman et al., 1979; Fairbanks et al., 1995). The *de novo* biosynthesis of pyrimidines is a highly regulated multistep (six) pathway and has been studied genetically and biochemically in a variety of organisms (Figure 30). In mammalian cells, the first, second and third steps are catalyzed by a large

multifunctional protein CAD, that contains three enzymatic activities: carbamoyl phosphate synthetase II (CPSase II), aspartate transcarbamoylase (ATCase) and dihydroorotase (DHOase)(Coleman et al., 1977).

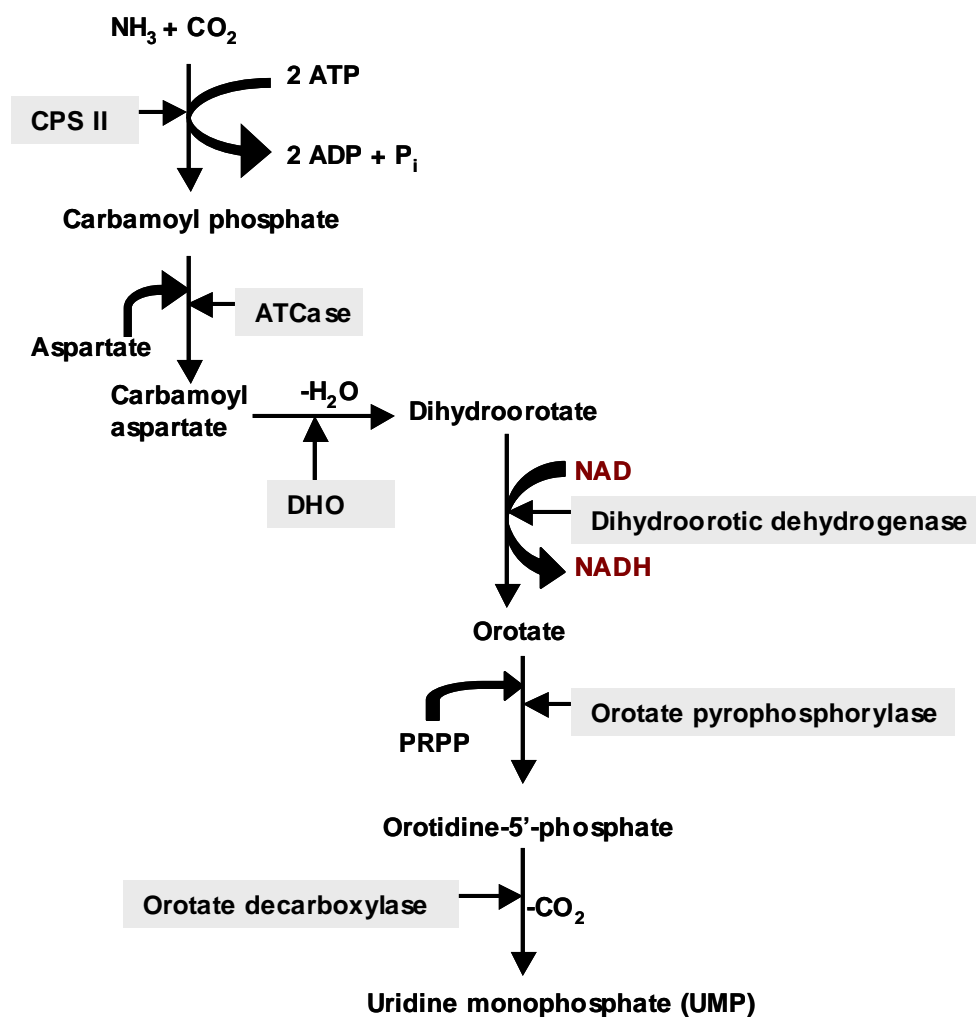


Figure 30. Schematic representation of de novo pyrimidine biosynthesis in mammalian cells (modified form Evans and Guy, 2004).

CAD is a 243kDa polypeptide that is functionally active as a hexamer and is organized into autonomously folded functional domains connected by inter-domain linkers (Figure 31) (Carrey et al., 1985; Lee et al., 1985; Kim et al., 1992; Qiu and Davidson, 2000). The rate limiting reaction of de novo pyrimidine biosynthesis is catalyzed by CPSase II, that is allosterically inhibited by UTP and activated by 5-phosphoribosyl-1-pyrophosphate (PRPP) (Levine et al., 1971; Simmons et al., 2004). The activity of CPS II is also regulated by MAPK and PKA phosphorylation; for example, PKA-mediated phosphorylation of Ser1406 results in loss of UTP inhibition and reduced activation by PRPP (Carrey et al., 1985; Carrey and Hardie, 1988; Sahay et al., 1998). CAD is also phosphorylated at Thr-456 in the CPS II domain by MAPK *in vitro* and in response to growth factors (EGF, PDGF) *in vivo*. MAPK phosphorylation both *in vivo* and *in vitro* relieves UTP inhibition and stimulates PRPP activation of CPSase II that may result in increased rate of pyrimidine biosynthesis (Graves et al., 2000). CAD protein is also a selective target for caspase-mediated inactivation and degradation during apoptosis *in vitro* and *in vivo* in a myeloid precursor cell line (Huang et al., 2002b).

Elevated activities of many of the key enzymes of nucleotide metabolism, including CAD, CTP synthetase, thymidylate synthase (TS), dihydrofolate reductase and ribonucleotide reductase have been reported in transformed and malignant tumor cells (Aoki and Weber, 1981; Weber, 2001). For example CPSase II activity of CAD was significantly higher in hepatomas compared to normal liver cells (Aoki et al., 1982; Reardon and Weber, 1985).

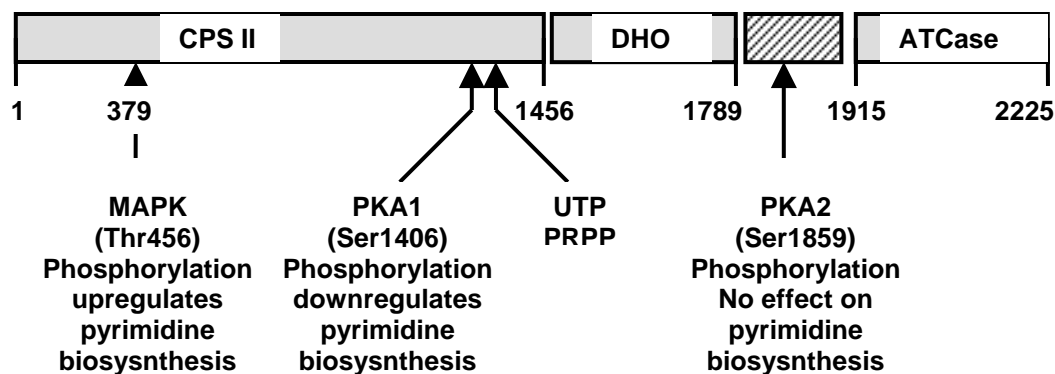


Figure 31. Structural organization and regulation of CAD (modified from Sigoillot et al., 2004).

Sigoillot et al. recently showed that the activity of the *de novo* pyrimidine biosynthetic pathway and the intracellular CAD concentration in MCF-7 cells was around 4-fold higher than in normal MCF-10A breast cells (Sigoillot et al., 2004). Thus inhibition of these enzymes is another target for developing anticancer drugs.

1.9 Research Objectives

1.9.1 Molecular mechanisms underlying hormonal regulation of CAD gene expression in MCF-7 and ZR-75 breast cancer cells

E2 stimulates proliferation of MCF-7 and other ER-positive breast cancer cell lines, and this is accompanied by cell cycle progression and transactivation of multiple genes including several that are involved in the proliferative response. Lippman and coworkers have investigated effects of E2 in MCF-7 cells on several enzymes required for DNA synthesis including those involved in nucleotide biosynthesis (Bronzert et al., 1981; Aitken and Lippman, 1982; Aitken and Lippman, 1985). They reported that E2

induced dihydrofolate reductase, thymidylate synthase, and thymidine kinase activities, and these were accompanied by increased DNA synthesis as determined by radiolabeled thymidine uptake (Aitken and Lippman, 1985; Aitken et al., 1985). In addition, activity of several enzymes required for pyrimidine biosynthesis, including carbamylphosphate synthetase, aspartate transcarbamylase, orotidine pyrophosphorylase, and orotidine decarboxylase, were also induced by E2 (Aitken and Lippman, 1983).

Research in this laboratory has demonstrated that the mechanisms of hormonal and growth factor regulation of some genes, including those associated with nucleotide biosynthesis and cell growth, are regulated by a nonclassical DNA-independent mechanism that involves ER α /Sp1 interactions at E2-responsive GC-rich promoter motifs. Genes regulated by ER α /Sp1 in ER-positive MCF-7 or ZR-75 breast cancer cells include cyclin D1, bcl-2, RAR α 1, IGF binding protein 4, adenosine deaminase, DNA polymerase α , c-fos, cathepsin D, E2F-1, and creatine kinase B (Duan et al., 1998; Sun et al., 1998; Dong et al., 1999; Qin et al., 1999; Xie et al., 2000; Castro-Rivera et al., 2001; Samudio et al., 2001; Ngwenya and Safe, 2003). The aim of this research was to investigate the mechanisms underlying hormonal regulation of CAD gene expression in MCF-7 and ZR-75 breast cancer cells.

1.9.2. Molecular mechanisms of inhibitory AhR-ER α crosstalk using the hormone-responsive CAD gene as a model

ER α and ER β are members of the NR superfamily of transcription factors, and studies in ER knockout mice and humans show the important role for this receptor in reproductive tract development, neuronal and vascular function, and bone growth (Krege

et al., 1998; Couse and Korach, 1999). ER expression and activation by estrogens also plays a pivotal role in mammary tumor development and growth (Hulka et al., 1994), and early stage ER-positive breast cancer has been successfully treated with antiestrogens such as tamoxifen and other SERMs (MacGregor and Jordan, 1998).

Although tamoxifen and other SERMs have been extensively used in clinical applications, there is evidence that prolonged use of these compounds may lead to increased risk for endometrial cancer (for tamoxifen) or development of tumors resistant to endocrine therapy (Clarke et al., 2001). An alternative approach for inhibiting estrogen-dependent mammary tumor growth using ligands for the aryl hydrocarbon receptor (AhR) has been investigated in this laboratory. Compounds such as 6-MCDF activate inhibitory AhR-ER α crosstalk in breast and endometrial cancer cells, the rodent uterus, and rodent mammary tumors *in vivo*. For example, 6-MCDF significantly inhibited DMBA-induced mammary tumor growth in female Sprague Dawley rats at doses as low as 50 $\mu\text{g}/\text{kg}/\text{d}$ (McDougal et al., 2001). Moreover, in combination with tamoxifen, 6-MCDF synergistically inhibited mammary tumor growth in the rat model and protected against tamoxifen-induced estrogenic responses in the uterus but did not affect bone lengthening induced by tamoxifen (McDougal et al., 2001).

Hormone-mediated mammary tumor growth is dependent on modulation of gene expression and in breast cancer cells, AhR agonists, such as 6-MCDF or the high affinity AhR ligand TCDD, inhibit E2-induced progesterone receptor, prolactin receptor, cathepsin D, heat shock protein 27, *c-fos*, pS2 and cyclin D1 mRNA and/or protein expression (Safe and Wormke, 2003). Based on results of promoter analysis, one

inhibitory mechanism may involve direct interaction of the AhR complex with inhibitory iDREs in E2-responsive gene promoters (Krishnan et al., 1995; Gillesby et al., 1997; Duan et al., 1999; Porter et al., 2001). Both E2 and TCDD induce proteasome-dependent degradation of ER α (Wormke et al., 2000b; Wormke et al., 2003) and in breast cancer cells cotreated with E2 plus TCDD, the resulting low levels of ER α may become limiting and thereby decrease expression of some hormone-dependent genes. The objective of this study was to investigate the mechanism of inhibitory AhR-ER α crosstalk using the hormone-responsive CAD gene as a model.

CHAPTER II

MATERIALS AND METHODS

2.1 Cells, Chemicals and Biochemicals

MCF-7, ZR-75 and COS-1 cells were purchased from American Type Culture Collection (ATCC, Manassas, VA). MCF-7 and COS-1 cells were cultured in DME/F12 (Sigma Chemical Co., St. Louis, MO) media supplemented with 5% fetal bovine serum (Intergen, Des Plains, IA; JRH Biosciences, Lenexa, KS; or Atlanta Biologicals, Inc., Norcross, GA.), 1.5 g/l sodium bicarbonate and 10 ml/l antibiotic-antimycotic solution (Sigma Chemical Co., St. Louis, MO). ZR-75 cells were maintained in RPMI 1620 medium with phenol red and supplemented with 10% fetal bovine serum, 1.5 g/l sodium bicarbonate, 2.38 g/l HEPES, 4.5 g/l dextrose and 0.11 g/l sodium pyruvate. Cells were maintained in 37°C incubators under humidified 5% CO₂: 95% air.

Dimethyl sulfoxide (DMSO), Phosphate buffered saline (PBS), E2, and antibiotic/antimycotic solution were purchased from Sigma Chemical Co. (St. Louis, MO). MG132 was purchased from Calbiochem (EMD Biosciences, Inc., CA). TCDD was prepared in this laboratory and was shown to be >99% pure by gas chromatographic analysis. ICI 182,780 was provided by Alan Wakeling (AstraZeneca Pharmaceuticals, Macclesfield, UK). [γ -³²P]ATP (3000 Ci/mmol) and [γ -³²P]CTP (3000 Ci/mmol) were purchased from Perkin-Elmer Life Sciences (Foster City, CA). Restriction enzymes, Polydeoxy- (inosinic-cytidylic) acid [poly d(I-C)], T4 DNA ligase, and other enzymes

were purchased from Promega Corp. (Madison, WI) and or Boehringer Mannheim (Indianapolis, IN). Reporter lysis buffer and Luciferase Assay Reagent were purchased from Promega. β -Galactosidase activity was measured using Tropix Galacto-Light Plus Assay System (Tropix, Bedford, MA, USA). Instant Imager and Lumicount micro-well plate reader were purchased from Packard Instrument Co. (Downers Grove, IL). Anti-FLAG M2 antibody was purchased from Stratagene (La Jolla, CA) and all other antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

2.2 Plasmids, Oligonucleotides and Cloning

Human ER α expression plasmid was originally provided by Dr. Ming-Jer Tsai (Baylor College of Medicine, Houston, TX) and was recloned into pcDNA3 and human ER β expression plasmid was kindly provided by Drs. E. Enmark and J.A. Gustafsson from the Center for Biotechnology, Novum (Huddinge, Sweden). ER α deletion constructs HE11, HE15, and HE19 were obtained from Dr. Pierre Chambon (Institut de Genetique et de Biologie Moleculaire et Cellulaire, Illkirch, France) and cDNAs for these constructs were then subcloned into the EcoRI site of pcDNA3.1 in this laboratory. ER α -null and TAF1 plasmids were derived from HE19 and ER α , respectively in this laboratory. Dominant negative Sp1 (pBGENSp1) and the corresponding empty vector (pBGENO) were provided by Drs. Yoshihiro Sowa and Toshiyuki Sakai (Kyoto Prefectural University of Medicine, Kyoto, Japan). Basic pGL2 luciferase plasmid was purchased from Promega. DRE-luciferase (DRE-luc) reporter construct was constructed in this laboratory and contains three tandem consensus dioxin response elements (DRE).

PCI/hAhR-FLAG and pcDNA3/hArnt-FLAG were kindly provided by Dr. Gary Perdew (Pennsylvania State University, PA). Plasmid pSp1₃ contains three consensus Sp1 binding sites linked to a luciferase reporter gene cloned into pXP2 plasmid. All primers and oligonucleotides were prepared either by Genosys/Sigma (The Woodlands, TX, USA) or Integrated DNA Technologies (IDT) (Coralville, IA) or Promega Corp (Madison, WI). CAD promoter luciferase constructs pCAD1 (-90/+115) and pCAD2 (-90/+25) were kindly provided by Dr. Peggy Farnham (University of Wisconsin, Madison, WI). CAD promoter fragments were synthesized or amplified by PCR as double-stranded DNA and inserted into the pGL2 basic luciferase reporter plasmid (Promega Corp.) vector between Nhe I and Hind III polylinker sites. pCAD3 (-67/+115), pCAD4 (-67/+25), pCAD5 (-47/+115), pCAD7 (-20/+115), pCAD8 (1/+115), and pCAD9 (-47/+60) were made by PCR amplification using pCAD1 as the template and forward (fwd) and reverse (rev) primers as listed in Table 11.

Plasmids pCAD6 (-47/+25) and pCAD10 (-30/+25) were constructed by inserting the double-stranded oligonucleotides into pGL2 basic vector. PCAD constructs containing point mutations were constructed by PCR mutagenesis using **pCADm-rev: 5'-CCA ACA GTA CCG GAA TGC CAA GCT TAC TTA GAT-3'** as the reverse primer. PCAD1m1, pCAD1m2 and pCADm4 were made using pCAD1 as the template; pCADm5 was made using pCADm1 as the template, and pCADm6 was made using pCADm2 as the template. The fwd primers used for PCR mutagenesis are listed in Table 12. All ligation products were transformed into competent Escherichia coli cells and clones were confirmed by DNA sequencing (Gene Technologies Laboratory, Texas

A&M University). Plasmid preparation kits were purchased from Qiagen (Valencia, CA) or Biorad Lab. (Hercules, CA). pECFP-C1 and pEYFP-C1 mammalian expression vectors were obtained from BD Biosciences CLONTECH Laboratories, Inc. (Palo Alto, CA). CFP-hER α , CFP-YFP chimera, CFP-Sp1 and YFP-hER α were constructed by Dr. Kyoungyun Kim in this laboratory (Kim et al., 2005). Full length YFP-AhR was constructed using forward primer 5' CAG TAG ATC TAT GAA CAG CAG CAG CGC CAA CAT 3' and reverse primer 5' CAG TGT CGA CTT ACA GGA ATC CAC TGG ATG TCA A 3' and cloned in between bgl II and SalI sites of pEYFP-C1 plasmid.

Table 11. Primers for pCAD deletion constructs.

pCAD deletion constructs	Primers used
pCAD3 (-67/+115)	pCAD3-fwd: 5'- CAG TGC TAG CCT TAC GTG CCC GGC CCC-3' pCAD3-rev: 5'-CAG TAA GCT TTG CAA ACT CCA CTG GAA CCA C-3'
pCAD4 (-67/+25)	pCAD3-fwd: 5'- CAG TGC TAG CCT TAC GTG CCC GGC CCC-3' pCAD4-rev: 5'-CAG TAA GCT TGC AGC GTA GGA GCC TCG C -3'
pCAD5 (-47/+115)	pCAD5-fwd: 5'-CAG TGC TAG CCT CAC GCC GCC TGT GTC-3' pCAD3-rev: 5'-CAG TAA GCT TTG CAA ACT CCA CTG GAA CCA C-3'
pCAD7 (-20/+115)	pCAD7-fwd: 5'-CAG TGC TAG CCA GTC TCT GCT GCT GCC-3' PCAD3-rev: 5'-CAG TAA GCT TTG CAA ACT CCA CTG GAA CCA C-3'
pCAD8 (1/+115)	pCAD8-fwd: 5'-CAG TGC TAG CCA AGC GCG CCC GAG GCT CCT AC-3' PCAD3-rev: 5'-CAG TAA GCT TTG CAA ACT CCA CTG GAA CCA C-3'
pCAD9 (-47/+60)	pCAD5-fwd: 5'-CAG TGC TAG CCT CAC GCC GCC TGT GTC-3' PCAD9-rev: 5'-CAG TAA GCT TGC TAA CGG CGC GGG GCG CTG-3'

fwd= forward and rev= reverse

Table 12. Primers for pCAD constructs containing point mutations.

Plasmids	Primers used
pCAD1m1	pCADm1-fwd: 5'-CAG TGC TAG CCC GTG GCT CCG CGG ACC CGC CCC TTA CGT GCC CGG CCC CCA ACC TCA C-3'
pCAD1m2	pCADm2-fwd: 5'-CAG TGC TAG CCC GTG GCT CCG CGG ACC CCA ACC TTA CGT GCC CGG CCC CCA ACC TCA C-3'
pCAD1m4	pCADm3-fwd: 5'-CAG TGC AGC CCG TGG CTC CGC GGA CCC GCC CCT TAC GTG CCC GGC CCC GCC CCT AAA ACC GCC TG-3'
pCAD1m5	pCADm5-fwd: 5'-CAG TGC TAG CCC GTG GCT CCG CGG ACC CGC CCC TTA CGT GCC CGG CCC CCA ACC TAA AAC CGC CTG-3'
pCAD1m6	pCADm6-fwd: 5'- CAG TGC TAG CCC GTG GCT CCG CGG ACC CCA ACC TTA CGT GCC CGG CCC CCA ACC TAA AAC CGC CTG-3'

fwd= forward and rev= reverse

2.3 Semiquantitative Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

MCF-7 cells were seeded in DME/F12 medium supplemented with 2.5% charcoal-stripped serum. RNA was extracted using RNazol B (Tel-Test, Friendswood, TX) or RNeasy mini kit (Qiagen, Valencia, CA) following manufacturer's protocol. RNA was quantitated by measuring the 260/280-nM absorption ratio, and concentration was adjusted to 100–200 ng/μl RNA for use in RT-PCR. RNA was reverse-transcribed at 42°C for 25 min using oligo-deoxythymidine primer, followed by PCR amplification of RT product using 2 mM MgCl₂, 1 μM each gene-specific primer, 1 mM deoxynucleotide triphosphate (dNTP), and 2.5 U AmpliTaq DNA polymerase (Perkin-Elmer, Boston, MA). Primer sets for CAD were added to the mixture, and gene products were amplified (30 cycles) in a PTC-200 thermal cycler (MJ Research, Inc., Watertown, MA). The

resulting 837-bp CAD probe was ligated into pcDNA3. The CAD probe was PCR amplified, gel purified and then sequenced by the Gene Technologies Laboratory, Texas A&M University. Primers used for CAD amplification were as follows:

CAD-fwd: 5'-CTAAGCTTAC-TGTGGCCTCAAGTATAAT-3'

CAD-rev: 5'-CTGGATCCTATGGGAAGA AAATAGACCT-3'

2.4 Northern Blot Analysis

Cells were seeded in DME/F12 medium supplemented with 2.5% charcoal-stripped serum and then synchronized in serum-free media for 3 days. Cells were treated with various compounds for different time periods and RNA was extracted using RNazol B (Tel-Test), following the manufacturer's protocol; 15-20 μ g of RNA were separated on a 1.2% agarose/1 M formaldehyde gel and transferred onto nylon membrane for 48 h. RNA was cross-linked by exposing the membrane to UV light for 10 min, and the membrane was baked at 80°C for 2 h. The membrane was then prehybridized for 18 h at 60°C using ULTRAhyb-Hybridization Buffer (Ambion, Austin, TX) and hybridized in the same buffer for 24 h with the [γ ³²P]-labeled cDNA probe. The membrane was then washed in 2X SSC (0.3 M sodium chloride, 0.03 M sodium Citrate, pH 7) and 0.5% SDS for 1 h and then washed in 2X SSC for 6-8 h. The resulting blots were quantitated using a Phosphor Imager (Molecular Dynamics, Sunnyvale, CA). β -Actin or β -tubulin mRNA were used as an internal control to normalize CAD mRNA levels.

2.5 Transient Transfection Assays

Cells were seeded in DME/F12 medium supplemented with 2.5% charcoal-stripped serum overnight in 12-well plates. Transfection was carried out either using calcium phosphate-DNA co-precipitation method or Fugene 6 transfection reagent (Roche, IN) or lipofectamine 2000 (Invitrogen, CA). The reporter plasmids were cotransfected with ER α , ER β , or other ER variant expression vectors using the calcium phosphate method for 5–6 h. After 5–6 h cells were shocked with 0.5 ml of 25 % glycerol for 1 min in a 12-well plate. Cells were then dosed for 36–48 h with different treatments. Transfection using Fugene 6 or lipofectamine 2000 was carried out according to the manufacturer's protocol in absence of antibiotics in serum-free media. After 4 h, serum was then either added directly on to the wells or serum-free media was changed with 5 % charcoal-stripped media. Cells were then treated with dimethylsulfoxide (DMSO) or other treatments. After 24–36 h cells were washed with PBS and then harvested in 100 μ l of cell lysis buffer (Promega Corp., Madison, WI). Cell lysate was frozen in liquid nitrogen for 30 sec and then thawed at room temp in a water bath. Freeze-thaw cycle was repeated three times and then sample was vortexed, centrifuged at 12000 rpm for 1 min and supernates were transferred to fresh tubes. Luciferase activities in the various treatment groups were performed on 20 μ l of cell extract using the luciferase assay system (Promega Corp., Madison, WI) in a luminometer (Packard Instrument Co., Meriden, CT), and results were normalized to β -galactosidase enzyme activity which was also performed on 20 μ l of cell extract.

2.6 Electrophoretic Mobility Shift Assay (EMSA)

Cells were seeded in DME/F12 medium supplemented with 2.5% charcoal-stripped serum overnight and treated with 10 nM E2 for 30 min. Nuclear extracts were obtained using NE-PER nuclear and cytoplasmic extraction reagents (Pierce Chemical Co., Rockford, IL). Oligonucleotides were synthesized, purified, and annealed, and 5 pmol of specific oligonucleotides were labeled at the 5'-end using T₄ polynucleotide kinase and [³²γ] ATP. 2 μg of nuclear extract was incubated in 0.75 M HEG (2 mM HEPES, 1.5 mM EDTA, and 10% glycerol (vol/vol), pH 7.6) buffer with 1 μl of 0.1 M ZnCl₂ and 1 μl of poly [d(I-C)] for 5 min. 100-150 fold excess of unlabeled wild-type or mutant oligonucleotides were added for competition experiments and incubated for 10 min. The mixture was incubated with 2 μl labeled oligonucleotides (40,000 cpm) for 10 min, and antibodies were then added for 30 min on ice for supershift experiments. The reaction mixture was loaded onto a 5% polyacrylamide gel and ran at 150 V for 2 h. The gel was dried for 45 min and protein/DNA complexes were visualized by either autoradiography or exposed to phosphoscreen and visualized on STORMTM 860 (Molecular Dynamics, Sunnyvale, CA). The oligonucleotides (mutations are underlined and substituted bases are indicated in bold) used in EMSAs are listed in Table 13.

2.7 Chromatin Immunoprecipitation Assay

ZR-75 or MCF-7 cells were grown in 150-mm tissue culture plates and treated with 20 nM E2 for various times. Formaldehyde was then added to the medium to a final concentration of 1% and incubated with shaking for 10 min at room temperature

followed by the addition of glycine (0.125 M) and incubation for 10 min. Cells were washed with PBS and 1 mM phenylmethylsulfonyl fluoride (PMSF), scraped, and collected by centrifugation. Cells were then resuspended in swell buffer (85 mM KCl, 0.5% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 5 µg/ml leupeptin and aprotinin at pH 8.0) and homogenized. Nuclei was isolated by centrifugation at 1500 x g for 3 min, then resuspended in sonication buffer [1% sodium dodecyl sulfate (SDS); 10 mM EDTA; 50 mM Tris-HCl, pH 8.1], and sonicated for 45 sec. This extract was then centrifuged at 15,000 x g for 10 min at 4 C, aliquoted, and stored at -80° C until used. The cross-linked chromatin preparations were diluted in buffer (1% Triton X-100; 100 mM NaCl; 0.5% SDS; 5 mM EDTA; and Tris-HCl, pH 8.1), and 20 µl of Ultralink protein A or G or A/G beads (Pierce Chemical Co., Rockford, IL) were added per 100 µl of chromatin and incubated for 4 h at 4 C. A 100-µl aliquot was saved and used as the 100% input control. Salmon sperm DNA, specific antibodies, and 20 µl of Ultralink beads were added, and the mixture was incubated overnight at 4 C.

Table 13. Oligonucleotides used for EMSA.

Oligos	Sequences
CADa1 (-75/-48)	5'-CCC CGC CCC TTA CGT GCC CGG CCC CGC TCA CGC CC- 3'
CADE (+54/+78)	5'-GCC GTT AGC CAC GTG GAC CGA CTC-3'
CADE (mutant)	5'-GCC GTT AGC CTG CAG GAC GAC CGA CTC-3'
Sp1 (consensus)	5'-AGC TTA TTC GAT CGG GGC GGG GCG AGC G-3'
Sp1 (mutant)	5'-AGC TTA TTC CG AA GC GGG GCG AGC G-3'

Samples were then centrifuged; beads were resuspended in dialysis buffer, vortexed for 5 min and centrifuged at 15,000 x g for 3 min. Beads were resuspended in immunoprecipitation buffer (11 mM Tris-HCl; 500 mM LiCl; 1% Nonidet P-40; and 1% deoxycholic acid, pH 8.0) and vortexed for 5 min at 20 C. The procedures with the dialysis and immunoprecipitation buffers were repeated (3–4x), and beads were resuspended in elution buffer (50 mM sodium bicarbonate, 1% SDS, 1.5 µg/ml sonicated salmon sperm DNA), vortexed, and incubated at 65° C for 15 min. Supernatants were isolated by centrifugation and incubated at 65° C for 6 h to reverse cross-links. Wizard PCR kits (Promega Corp.) were used to purify DNA, and PCR was used to detect the presence of promoter regions immunoprecipitated with ER α , Sp1, Brahma-related gene 1 (Brg-1) or acetylated histone 4 (Ac. H4) antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). The primers -176 5'-CTT GGG GTG GGA GGG ACT-3' and -19 5'-GCG GCA GCA GCA GAG ACT-3' (CAD gene promoter) and +2465 5'-TGT AGT TCT TGA GCA CCT CG-3' and +2605 5'-TGC ACA AGT TCA CGT CCA TC-3' (cathepsin D, exon II) were synthesized and used for PCR analysis of immunoprecipitated DNA. PCR reactions were carried out in the presence of 1 M betaine and/or varying amounts of DMSO 3-6 % in a stringent two-step PCR program (95-60° or 95-65°) to increase the specificity.

2.8 Coimmunoprecipitation

MCF-7 cells were seeded in 10 mm plates in DME/F12 medium supplemented with 2.5% charcoal-stripped serum and were grown for two days. Cells were transfected

with 3 μg of AhR-FLAG using Fugene 6 as per the manufacturer's protocol for 18 h and then treated with different compounds for 20 min. Cells were washed with ice-cold PBS (2x) and cell lysates were prepared in 800 μl of lysis buffer [50 mM Tris HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, 1 % Igepal CA-630 and protease inhibitor mixture (Sigma)] on ice for 45 min with intermittent vortexing at medium speed and then centrifuged at 13000 x g for 15min. Five hundred μg of protein was used for immunoprecipitation for each sample. 20 μl of EZview™ Red ANTI-FLAG M2 agarose affinity gel (Sigma Chemical Co.) was added to the protein samples and immunoprecipitation was carried out for 6 h at 4°C on a rocking platform. In control samples, 1 μg of mouse IgG was added for 5 h followed by addition of 15 μl protein- L- agarose beads (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) for 1 h. The beads were washed with the lysis buffer (5x) for 15 min each at 4°C followed by centrifugation at 8,000 x g for 30 sec. After the final wash, beads were resuspended in 30 μl of 1X sample buffer (50 mM Tris-HCl, 2% sodium dodecyl sulfate [SDS], 0.1% bromphenol blue, 175 mM β -mercaptoethanol), boiled for 5 min, separated on 7.5-10% SDS-PAGE gel for 5 h at 130V and Western blot analysis was performed.

2.9 Western Blot Analysis

Protein samples were boiled in 1X sample buffer for 5 min, separated on 7.5-10% gel and then transferred to polyvinylidene difluoride (PVDF) membrane (BioRad) overnight at 30V. Membranes were blocked in Blotto (5% milk, Tris-buffered saline [10 mM Tris-HCl, pH 8.0, 150 mM NaCl], and 0.05% Tween 20) for 30 min and probed

with primary antibodies ER α (H184), Sp1 (PEP2) or FLAG (M2) for 2-4 h. Membranes were washed for 30 min in 1X TBS-Tween, probed with peroxidase-conjugated secondary antibody for 1-2 h and then washed in 1X TBS-Tween for 30 min. Ten ml of HRP-substrate (Dupont-NEN, Boston, MA) was added and incubated for 1 min and visualized by autoradiography. Quantitation was performed using a Sharp JX-330 scanner (Sharp Electronics, Mahwah, NJ, USA) and Zero-D Scanalytics software (Scanalytics Corp., Sunnyvale, CA, USA).

2.10. Fluorescence Resonance Energy Transfer (FRET)

To perform FRET, cells were seeded in 2-well Labtek chamber slides w/cvr (Nalge Nunc. International, Rochester, New York) in DME/F12 medium supplemented with 5% charcoal-stripped serum and grown for 36 h. Cells were then transfected with 750 ng of CFP-Sp1, 500 ng CFP-ER, 500 ng YFP-AhR or 500 ng of YFP-ER alone or in combination using Fugene 6 in absence of serum. After 4 h, 5 % charcoal-stripped serum was directly added on to the cells. After 14-18 h, cells were washed with PBS and then put on the stage of the BioRad 2000MP system equipped with a Nikon T#300 inverted microscope with a 60X (NA1.2) water immersion objective lens and a Titanium:Sapphire pulsed laser tuned to 820 nm wavelength and a continuous wavelength Argon/Krypton laser tuned to 488 nm excitation (Table 14). Cells were pre-treated with TCDD for 15 min and images were acquired between 8 and 18 min after addition of DMSO or E2. Images of cells transfected with CFP and YFP fusion construct alone or in combination, were collected using 2 photon-820 nM excitation wavelength.

Table 14. Excitation and emission of fluorescent proteins.

Channel	Excitation (nm)	Emission (nm)	Laser
CFP channel	820	488	Titanium: Sapphire
FRET channel	820	525	Titanium:Sapphire
YFP channel	488	525	Argon/Krypton

Both CFP and YFP were excited at 820 nm to effectively generate 410 nm for excitation of the CFP and FRET channels. Emission of CFP (CFP channel; donor signal) was collected using a 500 DCLP dichroic and 450 nm /80 nm filter while emission of YFP (FRET channel; acceptor 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 the YFP fusion construct alone. For determination of YFP-hER α localization in the YFP channel, YFP-hER α was excited at 488 nm with Argon/Krypton laser and emission of YFP-hER α was collected at 525/50 nm. 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).

2.11 Small Inhibitory RNA

Small inhibitory RNAs (siRNAs) were prepared by IDT or Dharmacon Research (Lafayette, CO) and targeted the coding region 153–173, 672–694, and 1811–1833 relative to the start codon of GL2, lamin B1, and Sp1 genes, respectively. Single-stranded RNAs were annealed by incubating 20 μ M of each strand in annealing buffer (100 mM potassium acetate; 30 mM HEPES-KOH, pH 7.4; and 2 mM magnesium acetate) for 1 min at 90° C followed by 1 h at 37° C. Cells were cultured in six-well

plates in 2 ml of DME/F12 medium supplemented with 5% fetal bovine serum. After 16–20 h when cells were 50–60% confluent, iRNA duplexes and reporter gene constructs (pCAD1) were transfected using LipofectAMINE Plus Reagent (Invitrogen Life Technologies, Carlsbad, CA). The effects of iSp1 on hormone-induced transactivation of CAD gene was investigated in ZR-75 cells treated with 15 nM E2 and transfected with pCAD1 (500 ng) and ER α expression plasmid (200 ng). iRNA duplex (0.75 μ g) was transfected in each well to give a final concentration of 50 nM. Cells were harvested 48 h after transfection by manual scraping in 1x lysis buffer (Promega Corp. Madison, WI). The sequences for the siRNAs used in this study are listed in Table 15.

2.12 Real-Time PCR

Cells were seeded in DME/F12 medium supplemented with 2.5% charcoal-stripped serum and then synchronized in serum-free media for 2 days. Cells were pretreated with DMSO or 10 μ M MG132 for 30 min before treating with various compounds for different time periods.

Table 15. Sequences for siRNA duplexes.

Target gene	siRNA duplex
GL2	5'-CGU ACG CGG AAU ACU UCG ATT TT GCA UGC GCC UUA UGA AGC U-5'
LMN	5'-CUG GAC UUC CAG AAG AAC ATT TT GAC CUG AAG GUC UUC UUG U-5'
Sp1	5'-AUC ACU CCA UGG AUG AAA UGA TT TT UAG UGA GGU ACC UAC UUU ACU-5'

RNA was extracted using Qiagen RNeasy minikit (Qiagen, Valencia, CA, USA) following the manufacturer's protocol. Four μg of RNA was reverse transcribed for cDNA synthesis using Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Two μl of cDNA reaction mixture was then used to carry out PCR using SYBR Green PCR Master Mix from PE Applied Biosystems (Warrington, UK) on an ABI Prism 7700 Sequence Detection System (PE Applied Biosystems). PCR was performed as follows: Step 1: 95° for 10 min (1 cycle) and step 2: 95° for 15 sec and 60° for 1 min (40 cycles). The relative quantitation of samples was carried out using comparative C_T method. TATA binding protein (TBP) was used for normalization. Primers used to perform PCR were purchased from Integrated DNA technologies (Coralville, IA) and are as follows:

CAD (Fwd): 5'- TCC TCT GAT CGG CAA CTA TGG -3'

CAD (Rev): 5'- AGG ATT CAA ACC ACT TGC AGA GA -3'

TBP (Fwd): 5'- TGC ACA GGA GCC AAG AGT GAA - 3'

TBP (Rev): 5'- CAC ATC ACA GCT CCC CAC CA - 3'

2.13 Statistical Analysis

Experiments were repeated two or more times, and data are expressed as either the mean \pm SD or mean \pm SE for at least three replicates for each treatment group. Statistical differences between treatment groups were determined using SuperANOVA and Scheffé's test. Treatments were considered significantly different from controls if $P < 0.05$.

CHAPTER III

RESULTS

3.1 Mechanism of Hormonal Regulation of CAD Gene Expression in MCF-7 and ZR-75 Breast Cancer Cells*

3.1.1 Hormonal regulation of CAD gene expression in MCF-7 and ZR-75 cells

In order to determine if regulation of CAD gene expression is critical for E2-induced breast cancer growth we first investigated the effects of E2 on CAD mRNA levels. E2-responsive MCF-7 and ZR-75 cells were maintained in serum-free media before treatment with 10 nM E2 for 1, 3, 6, 12, and 24 h. CAD mRNA levels were determined at all time points in both cell lines and significant induction was observed in MCF-7 cells after 3 and 6 h (5- and 3-fold increase) (Figure 32) and after 12 h (>4.5-fold) in ZR-75 cells (Figure 33). Although the time course induction of CAD mRNA levels by E2 was different in MCF-7 and ZR-75 cells, the induced mRNA appeared to be transient in both cell lines. *

* Reprinted with permission from “Estrogen receptor/Sp1 complexes are required for induction of cad gene expression by 17beta-estradiol in breast cancer cells” by Shahen Khan, 2003. *Endocrinology*, 144, 2325-2335. Copyright 2003, *The Endocrine Society*.

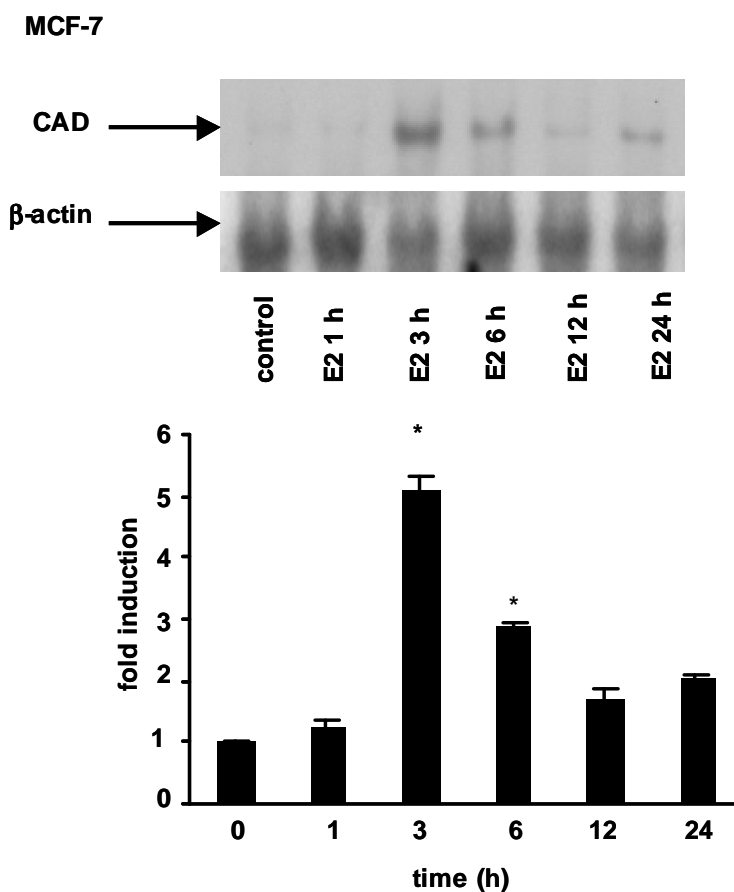


Figure 32. Induction of CAD mRNA levels by E2 in MCF-7 cells. MCF-7 cells were treated with DMSO for 24 h (lane 1) and 10 nM E2 for 1, 3, 6, 12, and 24 h (lanes 2–5, respectively). Cell extracts were obtained, and total RNA was isolated and subjected to Northern blot analysis as described in Materials and Methods. The intensity values were quantified using a Phosphor Imager and were normalized to the values of β -actin mRNA. Significant induction ($P < 0.05$) of CAD mRNA was observed at 3 h and 6 h and is indicated with an *asterisk*.

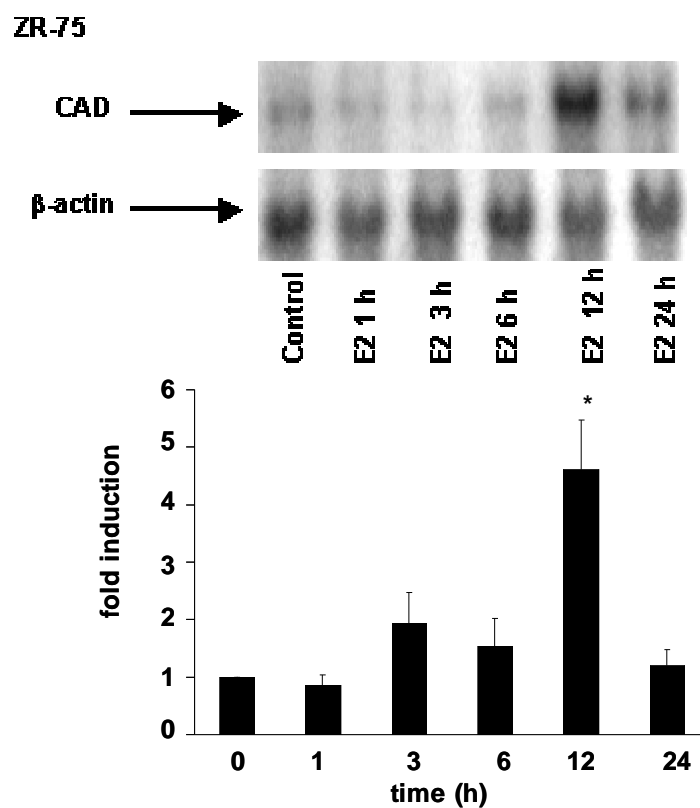


Figure 33. Induction of CAD mRNA levels by E2 in ZR-75. ZR-75 cells were treated with DMSO for 24 h (lane 1) and 10 nM E2 for 1, 3, 6, 12, and 24 h (lanes 2–5, respectively). Cell extracts were obtained, and total RNA was isolated and subjected to Northern blot analysis as described in Materials and Methods. The intensity values were quantified using a Phosphor Imager and were normalized to the values of β -actin mRNA. Significant induction ($P < 0.05$) of CAD mRNA was observed at 12 h and is indicated with an *asterisk*.

After determining that E2 induced CAD mRNA levels we then investigated effects of E2 on CAD gene promoter constructs in transient transfection assays in both cell lines. The proximal growth-responsive region of the human and hamster CAD gene promoter are similar and contain three upstream GC-rich binding sites and 1 (hamster) or 2 (human) downstream E box motifs. pCAD1 contains the -90 to +115 region of the human CAD gene promoter linked to the firefly luciferase reporter gene (Mac and Farnham, 2000) and was initially used to determine the effects of E2. Treatment of MCF-7 or ZR-75 cells with E2 alone (10–100 nM) did not induce reporter gene activity suggesting that nongenomic hormonal activation of kinases may not be required for transactivation of the growth-responsive CAD gene promoter (Figure 34).

Studies in several laboratories have demonstrated that hormonal activation of E2-responsive promoters containing EREs, AP-1, and GC-rich motifs are minimal in ER-positive breast cancer cells without cotransfection with ER α (Berry et al., 1989; Weisz and Rosales, 1990; Porter et al., 1996; Sathya et al., 1997; Castro-Rivera et al., 2001). This has been attributed to overexpression of the construct in the transfected cells where ER becomes limiting and E2-responsiveness is restored by cotransfection with ER α or ER β expression plasmids. The results in Figure 34 show that E2 induced luciferase activity only in MCF-7 or ZR-75 cells cotransfected with ER α and pCAD1, whereas hormone-induced transactivation was not observed in cells cotransfected with ER β . These results suggest that ER α but not ER β play a critical role in E2-mediated transactivation of CAD gene promoter.

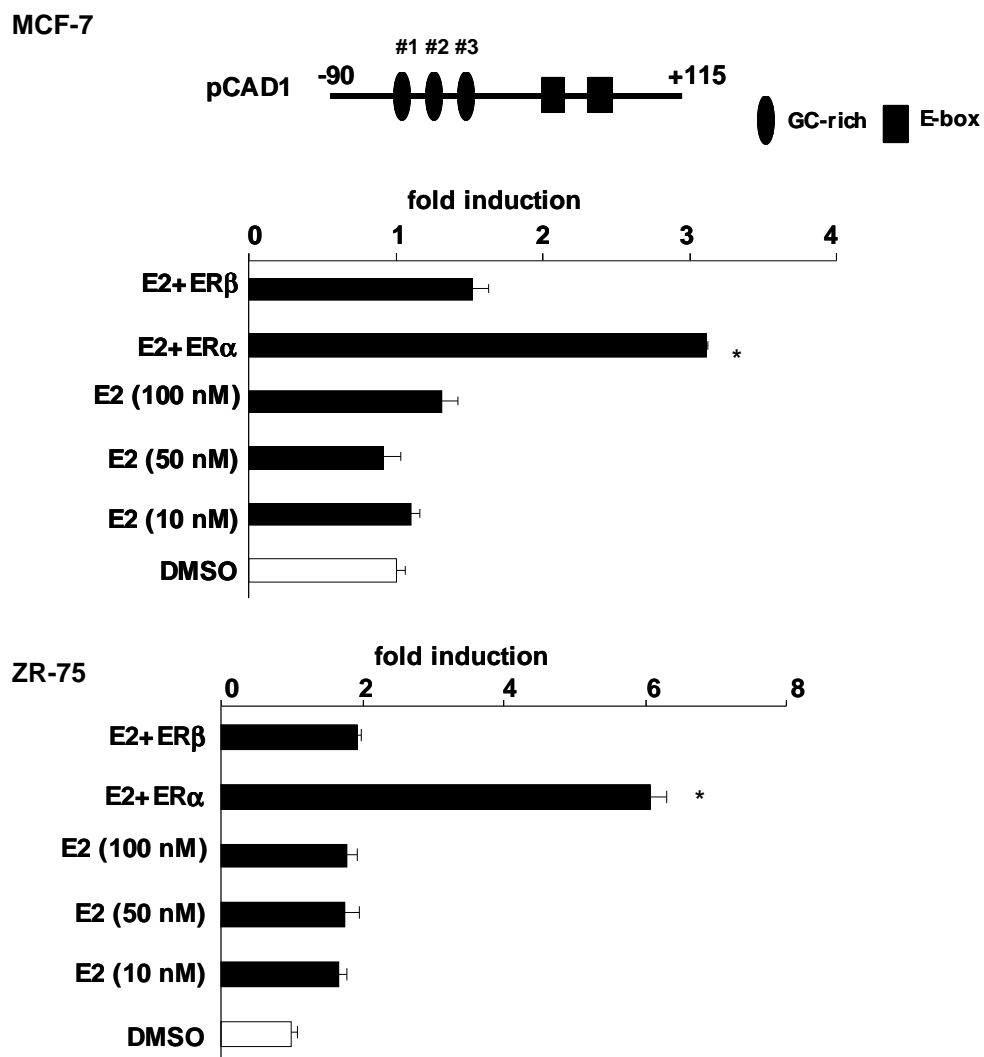


Figure 34. Activation of pCAD1 by E2 in MCF-7 (A) and ZR-75 (B) cells. Cells were transfected with 1 μg of pCAD1 and 250 ng of ER α or ER β expression plasmids, and the effects of 10 nM E2 on luciferase activity were determined as described in Materials and Methods. Results are expressed as means \pm SD for at least three replicate determinations or each treatment group and significant induction ($P < 0.05$) is indicated with an *asterisk*.

3.1.2 Analysis of domains of ER α required for E2-mediated activation of CAD gene promoter in MCF-7 and ZR-75 cells

The importance of different domains of ER α in E2-mediated transactivation of CAD gene promoter was investigated by transfecting MCF-7 and ZR-75 cells with pCAD1 plus wild type or variant ER α expression plasmids. Figure 35 is a schematic representation for each of the ER α expression plasmids used in these studies. These include ER α variants containing mutations in helix 12 of AF-2 (TAF1) and deletions of AF-2 (HE15), AF-1 (HE19), and the DNA-binding domain (HE11) regions. ER α -null contains both mutations in helix 12 and deletion of AF-1. Mutations in helix 12 within the AF-2 domain disrupt the interaction of coactivators with transcription factors without affecting ligand binding or dimerization. In MCF-7 cells, hormone inducibility was observed in cells cotransfected with pCAD1 and ER α or HE11 and decreased hormone-responsiveness but significant induction was also observed in cells transfected with TAF1 and HE19. In ZR-75 cells hormone inducibility was observed in cells cotransfected with pCAD1 and ER α and decreased hormone-responsiveness but significant induction was also observed in cells transfected with HE11 and TAF1 (Figure 36). Analysis of domains of ER α required for activation of pCAD2 was also investigated. The pCAD2 construct has three upstream GC-rich sites but lacks the two downstream E-boxes. E2-mediated transactivation of pCAD2 was observed with ER α , HE11 and TAF1 in MCF-7 cells whereas in ZR-75 cells, E2-mediated transactivation of pCAD2 was observed with ER α , HE11 and HE15 (Figure 37). In summary hormone inducibility was

observed in n MCF-7 and ZR-75 cells cotransfected with ER α and HE11; however, transactivation by HE19 and TAF1 was dependent on promoter and cell context. Moreover the fold-inducibility of pCAD constructs was higher in ZR-75 cells (>8-fold) transfected with ER α compared with MCF-7 cells (<3.5-fold); inducibility in MCF-7 and ZR-75 cells transfected with HE11 or TAF1 was 2- to 4-fold. These data are consistent with results of previous studies on other genes regulated by ER α /Sp1 where transactivation is observed for ER α and HE11, demonstrating that DNA binding by ER α is not required for hormone-dependent transactivation (Duan et al., 1998; Sun et al., 1998; Porter et al., 2001; Saville et al., 2002).

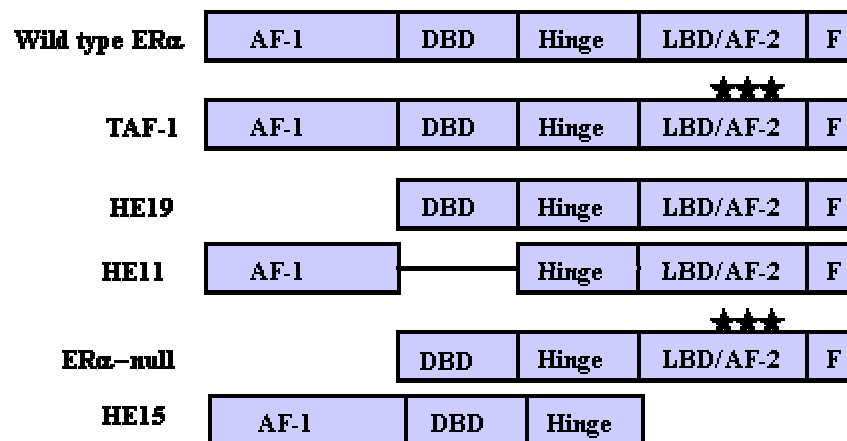


Figure 35. Schematic representation for ER α variants containing deletions and point mutants. HE11 contains deletion of DBD of ER α , HE15 contains a deletion of the AF-2 domain of ER α , TAF-1 contains mutations in helix 12 of the AF-2 domain of ER α (D538N, E542Q and D545N), HE19 contains a deletion of the AF-1 domain of ER α and ER α -null is similar to HE19 but has three point mutations in AF-2.

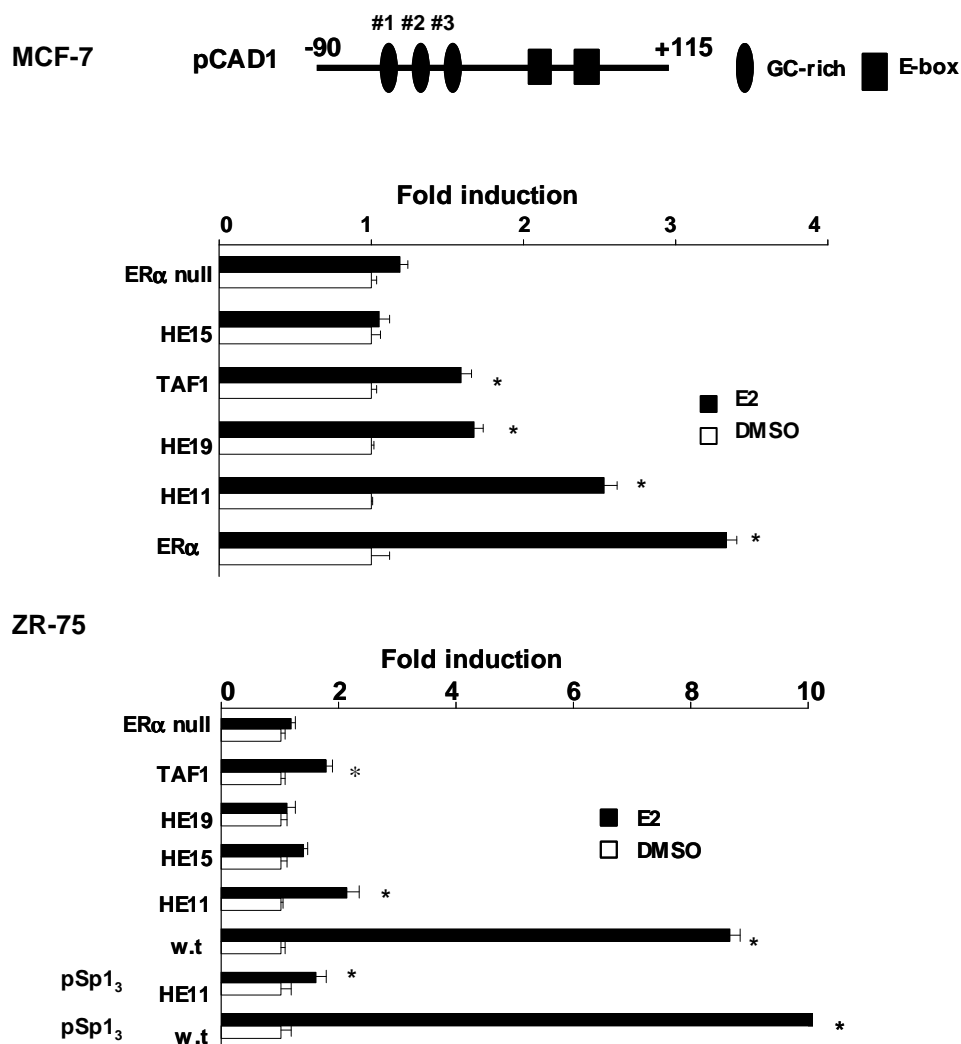


Figure 36. Effect of wild type and variant ER α on pCAD1 in MCF-7 and ZR-75 cells. Cells were transfected with 1 μ g of pCAD1 and 250 ng of ER α or HE11, HE15, HE19, ER α -null, and TAF1. pSp1₃ was used as a positive control for HE11 in ZR-75 cells. Luciferase activity was determined as described in Materials and Methods. Results are expressed as means \pm SD for at least three replicate determinations for each treatment group and significant ($P < 0.05$) induction is indicated with an *asterisk*.

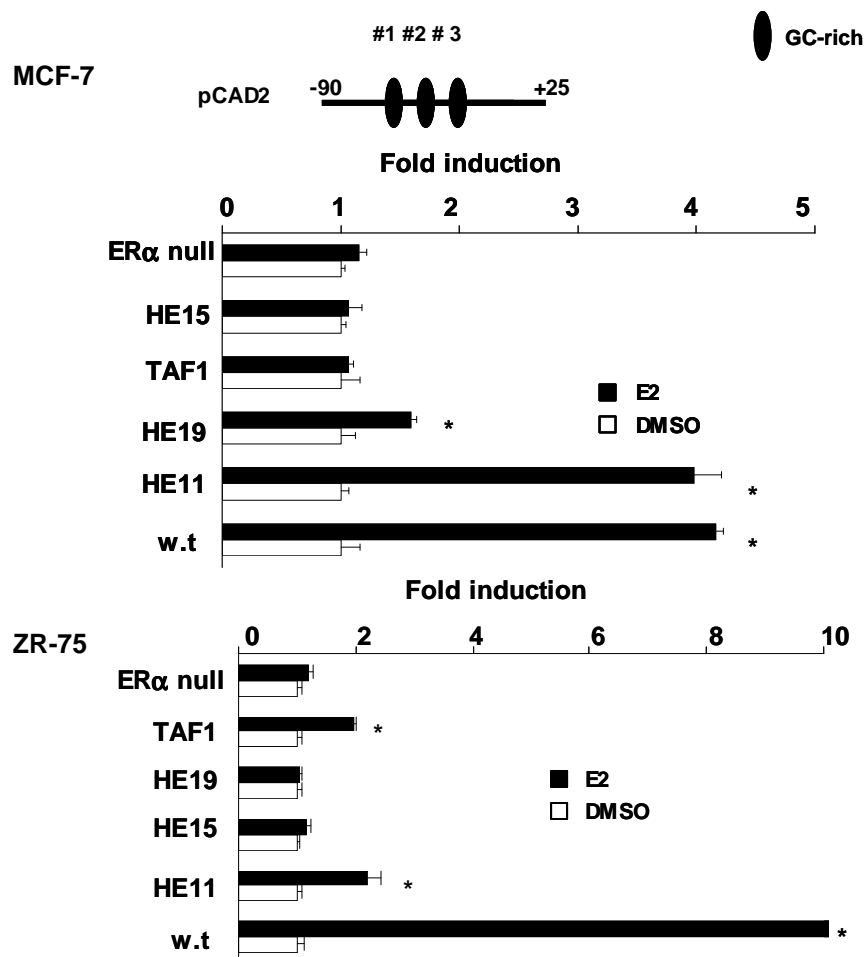


Figure 37. Effects of wild type and ER α variants on pCAD2 in MCF-7 and ZR-75 cells. Cells were transfected with 1 μ g of pCAD2 and 250 ng of ER α or HE11, HE15, HE19, ER α -null, and TAF1. pSp1₃ is used as a positive control for HE11 in ZR-75 cells. Luciferase activity was determined as described in Materials and Methods. Results are expressed as means \pm SD for at least three replicate determinations for each treatment group and significant ($P < 0.05$) induction is indicated with an *asterisk*.

3.1.3 Deletion and mutation analysis of the CAD gene promoter

The E2-responsive -90 to +115 region of the human CAD gene promoter contains three GC-rich Sp1 binding sites and two E-boxes. E2-induced reporter gene activity was observed in MCF-7 (Figure 38) and ZR-75 (Figure 39) cells transfected with pCAD1 which contains both GC-rich and E-box motifs. Deletion analysis of the growth-responsive region of the CAD gene promoter gave a unique pattern of responses in both cell lines. The effects of successive 5'-deletions of the upstream GC-rich sites on basal activity and E2-responsiveness was determined in transient transfection studies using pCAD1, pCAD3, pCAD5, pCAD7, and pCAD8. As shown in Figure 38, basal activity observed with these constructs in MCF-7 cells decreased with increasing deletion of GC-boxes 1–3; however, GC-box 2 appeared to be the most essential element for high basal activity. Moreover, E2 responsiveness of pCAD1, pCAD3, pCAD5, pCAD7, and pCAD8 in MCF-7 cells was dependent on GC-boxes 1 and 2 but not 3, and GC-box 2 was primarily responsible for E2-mediated transactivation. Constructs containing GC-box 3 alone (pCAD9, pCAD5, pCAD6, or pCAD10) or in combination with the two E-box motifs (pCAD5) were E2 non-responsive.

Transfection of constructs containing the E-box motifs alone (pCAD7 and pCAD8) or comparison of activities associated with deletion of the E-boxes (*e.g.* pCAD1 vs. pCAD2; pCAD3 vs. pCAD4) indicated that in MCF-7 cells these motifs were not E2-responsive and inhibited basal activity in MCF-7 cells. These results are in contrast to previous studies with both the human and hamster CAD gene promoter constructs in other cell lines where the E-box regions were primarily responsible for high basal

activity (Boyd and Farnham, 1997; Boyd et al., 1998; Boyd and Farnham, 1999; Mac and Farnham, 2000).

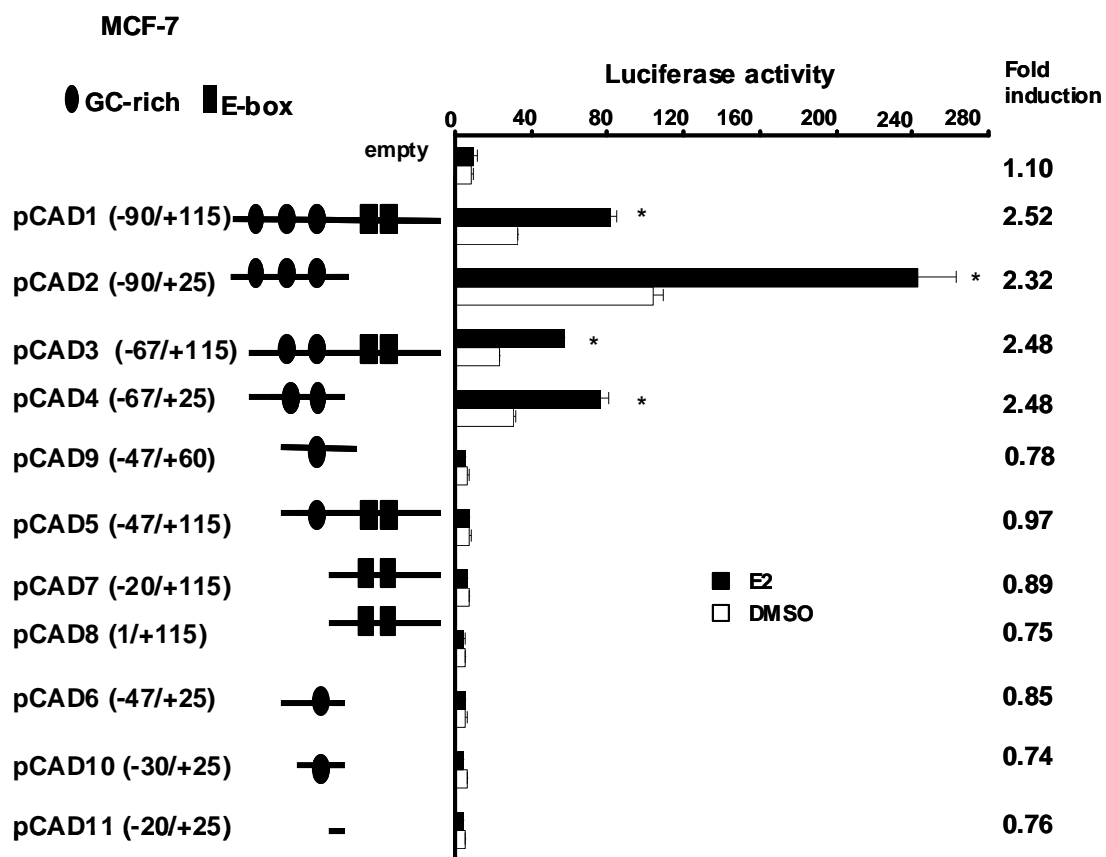


Figure 38. Deletion analysis of pCAD constructs in MCF-7 cells. Cells were transiently transfected with various pCAD deletion constructs, ER α expression plasmid and treated with DMSO or 10 nM E2; luciferase activity was determined as described in Materials and Methods. Results are expressed as means \pm SD for at least three replicate determinations for each treatment and significant ($P < 0.05$) induction is indicated with an *asterisk*.

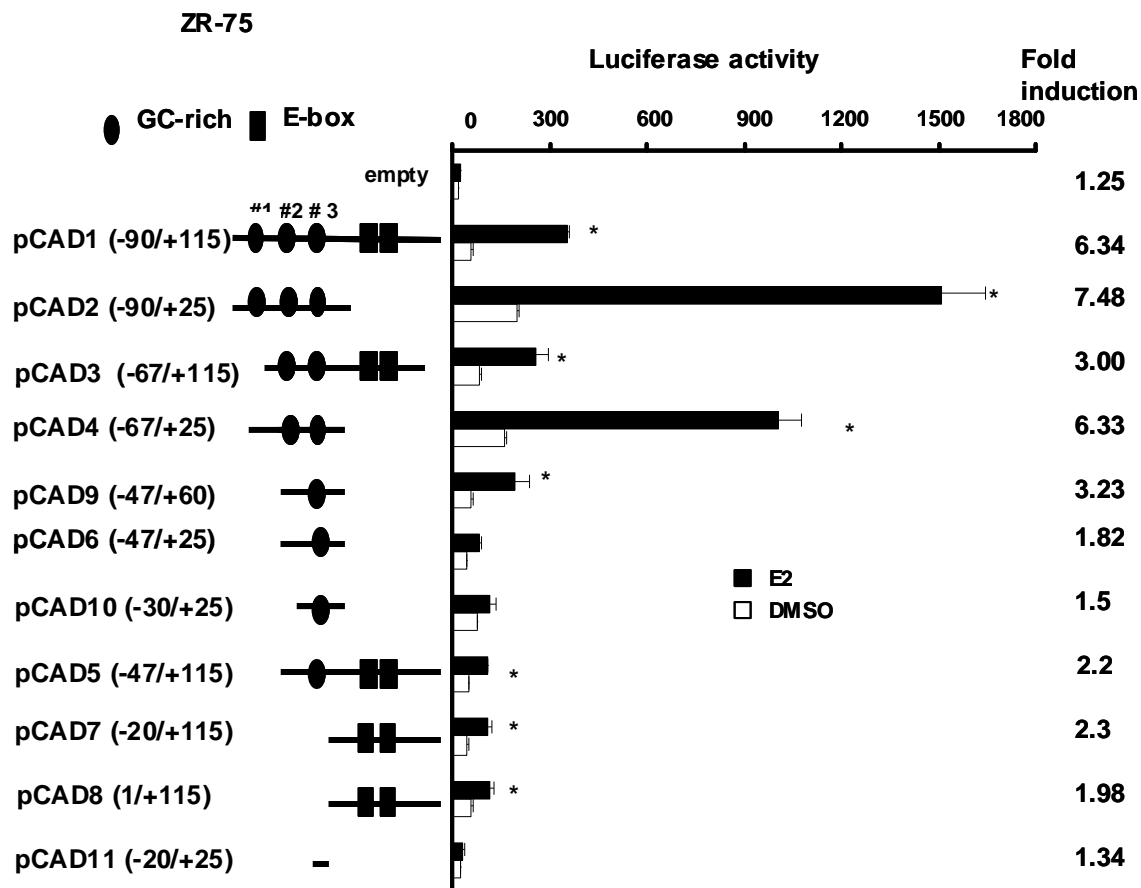


Figure 39. Deletion analysis of pCAD constructs in ZR-75 cells. Cells were transiently transfected with various pCAD deletion constructs, ER α expression plasmid and treated with DMSO or 10 nM E2; luciferase activity was determined as described in Materials and Methods. Results are expressed as means \pm SD for at least three replicate determinations for each treatment and significant ($P < 0.05$) induction is indicated with an *asterisk*.

In ZR-75 cells transfected with the same constructs (Figure 39), there was a higher level of E2-inducibility and less variability in basal activity compared with MCF-7 cells. Deletion of the E-boxes in constructs containing GC-boxes 1–3 (pCAD1 vs. pCAD2) or GC-boxes 2–3 (pCAD3 vs. pCAD4) resulted in increased basal activity and hormone-inducibility in ZR-75 cells, whereas in MCF-7 cells (Figure 38), only increased basal activity was observed. In ZR-75 cells, a comparison of a series of constructs containing successive deletion of GC-boxes 1–3 (pCAD1/pCAD3/pCAD5 and pCAD2/pCAD4/pCAD9, pCAD6 and pCAD10) indicated that E2 responsiveness was primarily associated with GC-boxes 1 and 2. The hormone inducibility observed for pCAD9 (-47 to +60), which contains GC-box 3 was not observed for pCAD6 (-47 to +25) or pCAD10 (-30 to +25), suggesting that a GC-box-independent region between +60 and +25 retained some hormone responsiveness. Constructs containing the E-boxes (pCAD7 and pCAD8) or GC-box 3 and promoter sequences up to the first E-box were also E2 responsive. These results suggest that the E-box and/or +25 to +60 region of the CAD gene promoter exhibited E2-inducibility in ZR-75 but not MCF-7 cells; however, GC-boxes 1 and 2 were the major E2-responsive sites in the human CAD gene promoter.

The effects of mutation analysis of the CAD promoter GC-boxes on E2-responsiveness were also investigated in MCF-7 and ZR-75 cells (Figure 40). Mutation of GC-box 2 (pCAD1m1) did not eliminate E2 responsiveness; however, deletion of both GC-boxes 1 and 2 (pCAD1m2) resulted in the loss of hormone responsiveness.

These results further confirm that cooperative binding to both GC-rich motifs contribute to hormone-dependent activation of the CAD gene promoter constructs.

3.1.4 Protein interactions with the CAD gene promoter

In order to determine the binding of Sp1 with GC-rich sites of CAD gene promoter we performed gel mobility shift assays using oligonucleotides from the CAD gene promoter containing wild type or mutant GC-rich sites. Interactions of nuclear extracts from MCF-7 and ZR-75 cells treated with DMSO (lanes 6 and 12) or 10 nM E2 (lanes 1–5 and 7–11) with the GC-rich [³²P]CADa1 (-75 to -35 region of the CAD promoter) and [³²P]Sp1 (containing consensus GC-rich site) oligonucleotides were investigated. Nuclear extracts from both cell lines bound [³²P]-Sp1 to give several bands which have previously been identified as Sp1 and Sp3 proteins (Dong et al., 1999; Wang et al., 1999; Xie et al., 2000; Castro-Rivera et al., 2001; Samudio et al., 2001). A major low mobility Sp1-[³²P]Sp1 complex (indicated with an *arrow*) was observed (lanes 1, 3, 5, and 6) and this band was unaffected by nonspecific IgG antibody (lane 1) or competition with unlabeled mutant Sp1 oligonucleotide (lane 3); in contrast, Sp1 antibody supershifted (SS) this complex (lane 2), and all bands were competitively decreased using unlabeled wild-type Sp1 oligonucleotide (lane 4). There was an increase in intensity of the Sp1-DNA complex band using nuclear extracts from MCF-7 cells treated with E2 (Figure 41, lane 5) compared with Me₂SO (Figure 41, lane 6); comparable differences in band intensities were not observed in ZR-75 cells (Figure 42, lanes 5 and 6).

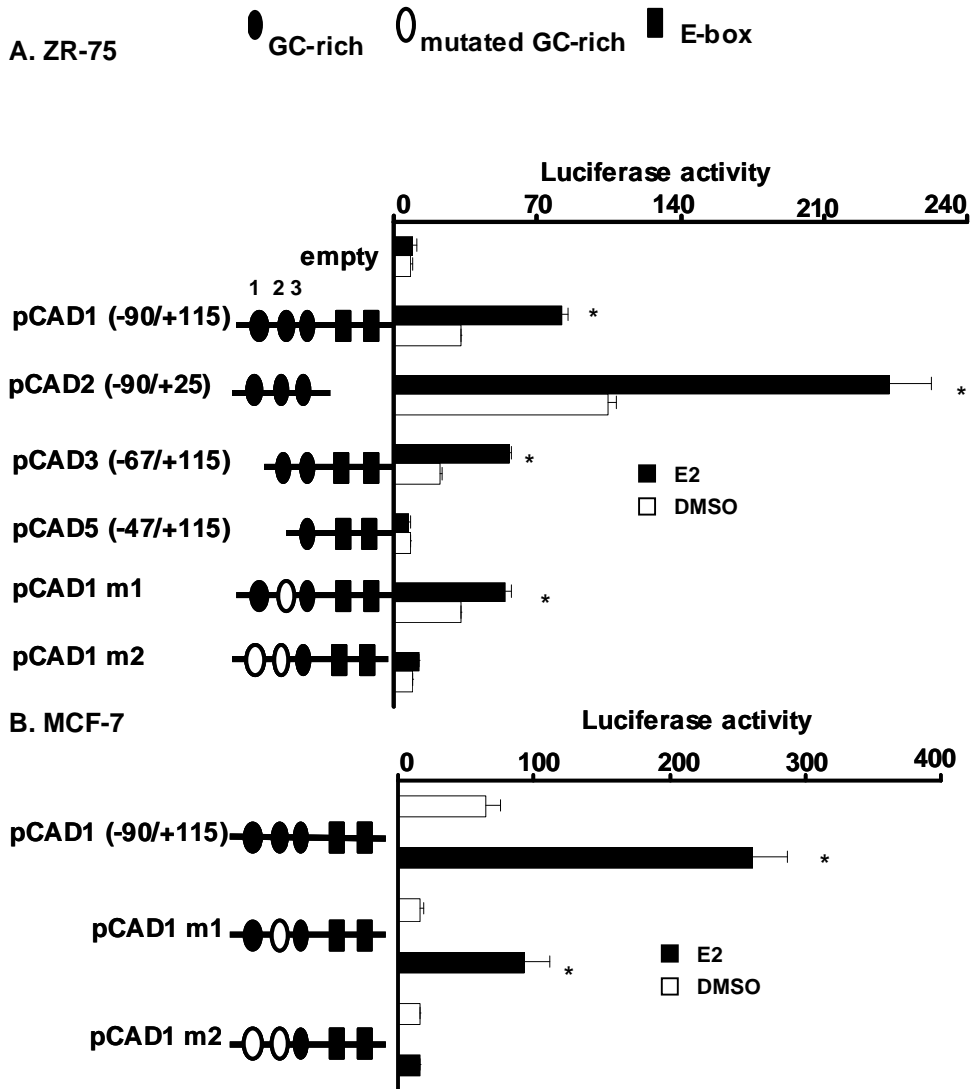


Figure 40. Mutation analysis of pCAD constructs in MCF-7 and ZR-75 cells. ZR-75 [A] or MCF-7 [B] cells were transiently transfected with various pCAD mutant constructs, ER α expression plasmid and treated with DMSO or 10 nM E2; luciferase activity was determined as described in Materials and Methods. Results are expressed as means \pm SD for at least three replicate determinations for each treatment and significant ($P < 0.05$) induction is indicated with an *asterisk*.

The patterns of low mobility nuclear extract- ^{32}P CADa1 bands were similar to those observed using ^{32}P Sp1; however, there were several additional higher mobility complexes observed using ^{32}P CAD1 (lanes 6–12). The Sp1- ^{32}P CADa1 and Sp1- ^{32}P Sp1 complexes exhibited similar mobilities (lanes 7–12 vs. 1–6; complex indicated with an *arrow*). The specifically bound Sp1- ^{32}P CADa1 complex (lanes 7–9, 11, and 12) was unaffected after coincubation with nonspecific IgG (lane 7) or mutant unlabeled Sp1 oligonucleotide (lane 9), whereas Sp1 antibody supershifted (SS) the complex to give a band (lane 8) with similar mobility to the supershifted complex observed in lane 2; and coincubation with an excess of unlabeled consensus Sp1 oligonucleotide (lane 10) decreased intensities of the higher mobility complexes. The lower mobility complexes were not affected by coincubation with Sp1 antibody or consensus oligonucleotide and were not further investigated.

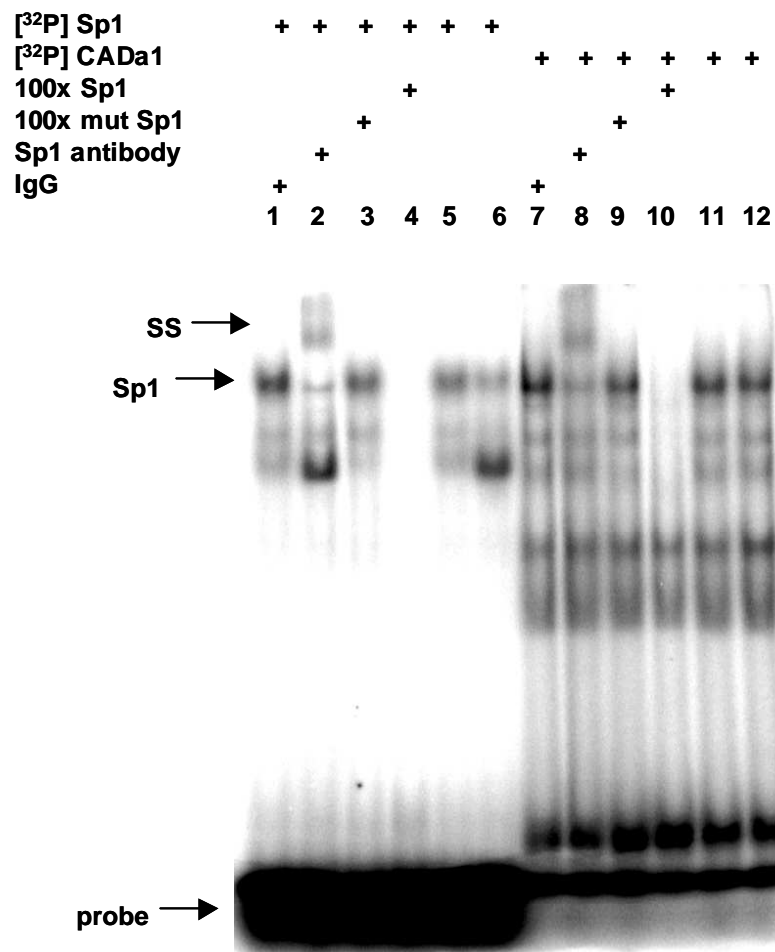


Figure 41. Binding of Sp1 to CAD promoter in MCF-7 cells. Nuclear extracts from MCF-7 cells treated with DMSO (lanes 6 and 12) or 10 nM E2 (lanes 1–5 and 7–11) were incubated with [³²P]Sp1 or [³²P]CAdA1 [containing the proximal GC-rich (-75 to -39) region of the CAD promoter] and unlabeled wild-type or mutant Sp1 oligonucleotides, Sp1 antibody, or nonspecific IgG antibody as described in Materials and Methods.

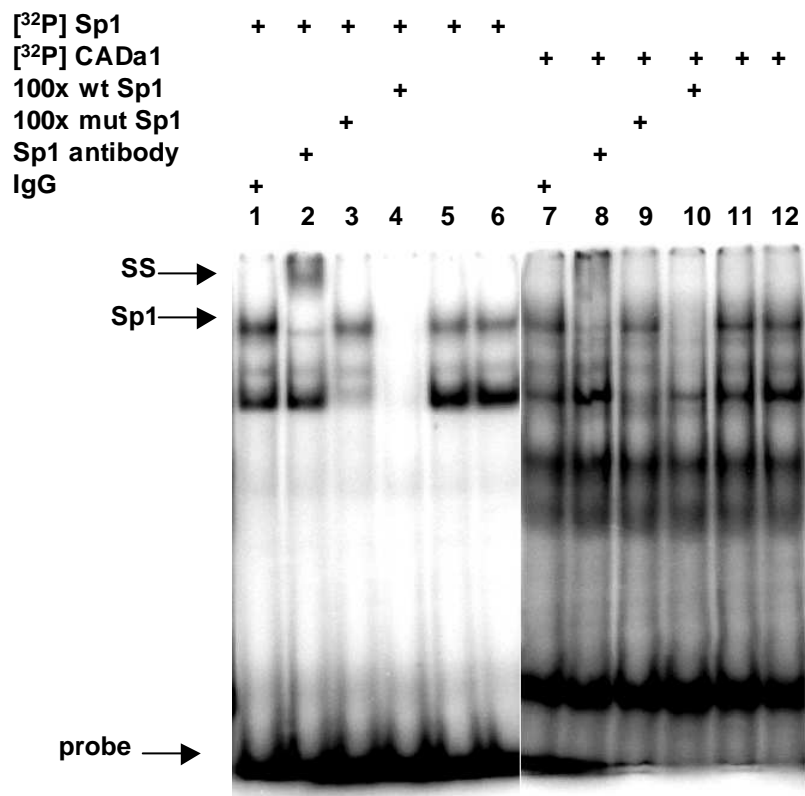


Figure 42. Binding of Sp1 to CAD promoter in ZR-75 cells. Nuclear extracts from ZR-75 cells treated with DMSO (lanes 6 and 12) or 10 nM E2 (lanes 1–5 and 7–11) were incubated with [³²P]Sp1 or [³²P]CADA1 and unlabeled wild-type or mutant Sp1 oligonucleotides, Sp1 antibody, or nonspecific IgG as described in Materials and Methods.

Nuclear extracts from MCF-7 cells were also incubated with [³²P]CADE which contained the E-box motif in the +54 to +78 region of the human CAD gene promoter as shown in Figure 43. Incubation of [³²P]CADE with nuclear extracts gave a retarded band (lane 1, indicated with an *arrow*), which was competitively decreased by a 50-fold excess of unlabeled wild-type CADE (lane 3) but not mutant CADE oligonucleotide (lane 4). This specifically bound band was supershifted by USF1 antibody (lane 5) but not by nonspecific IgG (lane 6). The specifically bound complex was not observed using radiolabeled mutCADE (lane 7); however, two more mobile bands were observed and only the more mobile band was formed with wild-type [³²P]CADE. Proteins interacting with [³²P]CADE to form the less mobile band were not identified. These results demonstrate that USF1/2 in nuclear extracts of MCF-7 cells bind the E-box motifs in the CAD gene promoter, and interactions of USF1/2 in MCF-7 cell nuclear extracts with the E-box major late promoter element in the cathepsin D gene promoter has previously been reported (Xing and Archer, 1998; Wang et al., 2001b).

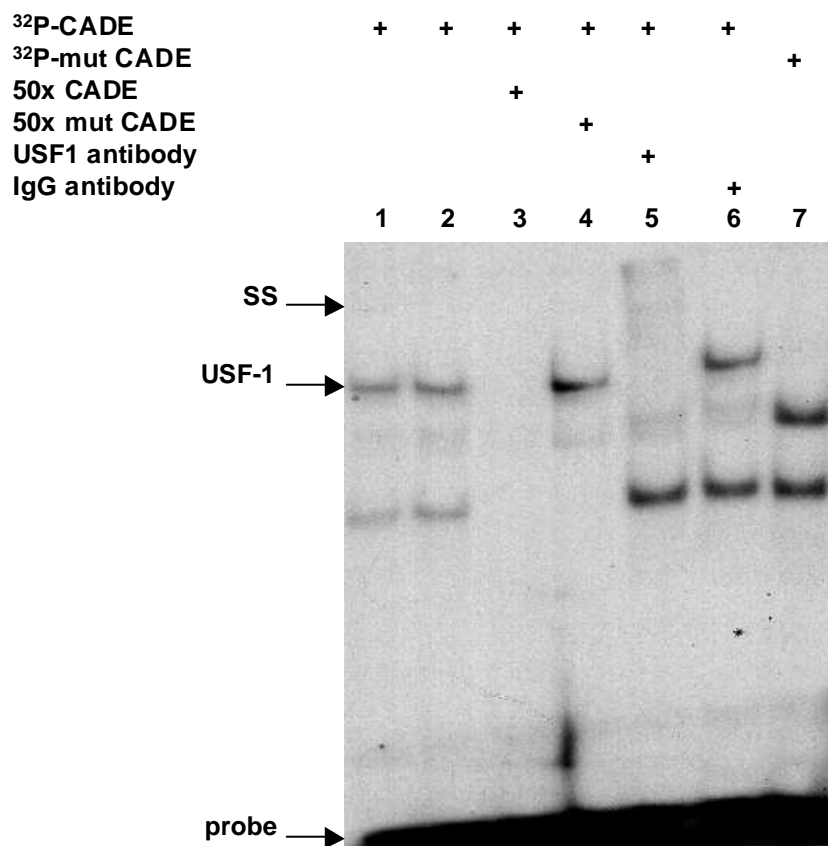


Figure 43. Binding of USF-1 to CAD promoter in MCF-7 cells. Nuclear extracts from MCF-7 cells treated with DMSO (lane 1) or 10 nM E2 (lane 2-7) were incubated with wild-type [³²P]CADE (+54 to +78) or [³²P]-labeled mutCADE and unlabeled wild-type or mutant E-box oligonucleotides, USF-1 antibody, or nonspecific IgG as described in Materials and Methods.

3.1.5 Role of Sp1 in E2-mediated transactivation of CAD gene promoter

Role of Sp1 in E2-mediated transactivation of CAD gene promoter was investigated using dominant negative Sp1 expression plasmid (pBGENSp1) vs. empty vector (pBGENO) on hormone inducibility in MCF-7 and ZR-75 cells (Figure 44). The empty vector alone slightly decreased hormone inducibility; however, the results show that dominant negative Sp1 significantly inhibited E2-induced transactivation in MCF-7 and ZR-75 cells transfected with pCAD1, thus confirming the role of Sp1 in this response.

We have further investigated the role of Sp1 in mediating hormone-induced CAD gene expression using an siRNA duplex that targets Sp1 mRNA resulting in down-regulation of both Sp1 mRNA and protein. Transfection of siRNA into MCF-7 or ZR-75 cells significantly decreased Sp1 protein in whole cell extracts (50–70%) and both basal and hormone inducibility in cells transfected with pSp1₃ (Abdelrahim et al., 2002). The results in (Figure 45) show that E2 induced luciferase activity in ZR-75 cells transfected with pCAD1, and both basal and induced responses were inhibited 60% by siRNAs targeted to the luciferase reporter gene (iGL2) and Sp1 (iSp1), whereas siRNA targeted to lamin B had no effect. These results further confirm the role of Sp1 in hormone-induced transactivation of CAD through interaction of the ER α /Sp1 complex with GC-rich motifs.

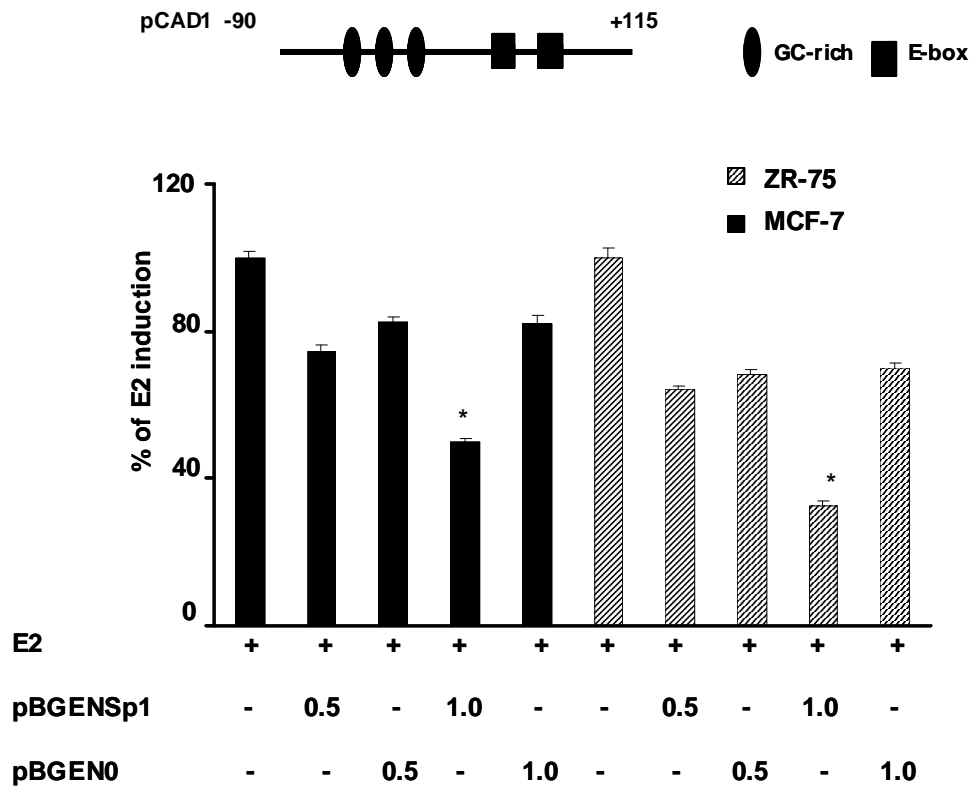


Figure 44. Effects of dominant negative Sp1 on E2-mediated transactivation of pCAD1. Cells were cotransfected with 1 μ g pCAD1 along with increasing amounts of either dominant negative Sp1 expression plasmid (pBGENSEP1) or empty vector pBGENO, treated with E2, and luciferase activity was determined as described in Materials and Methods. The ratio of pCAD1: pBGENSEP1 (1:1) resulted in significant ($P < 0.05$) * inhibition of E2-induced luciferase activity in both ZR-75 and MCF-7 cells.

ZR-75

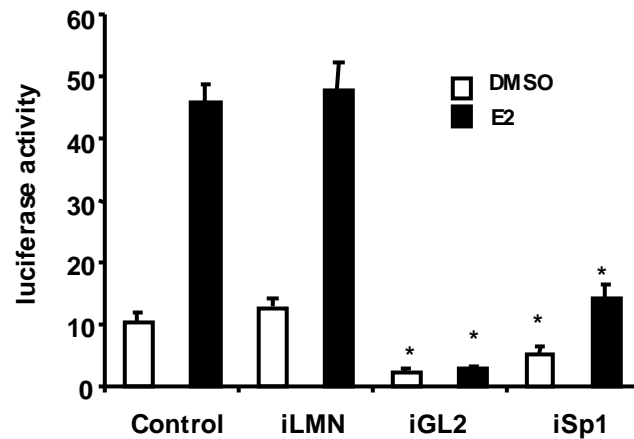


Figure 45. Inhibition of hormonal activation of pCAD1 by small inhibitory RNA for Sp1. ZR-75 cells were transfected with pCAD1 and siRNAs for lamin C (iLMN), luciferase (iGL2), and Sp1 (iSp1), treated with 10 nM E2 or DMSO and luciferase activity determined as described in Materials and Methods. Results are expressed as means \pm SD for at least three replicate determinations for each treatment group and significant ($P < 0.05$) inhibition in cells transfected with iGL2 and iSp1 compared with cells transfected with control or iLMN is indicated with an *asterisk*.

3.1.6 Interactions of ER α and Sp1 proteins with the GC-rich region of CAD gene promoter

Confirmation of ER α /Sp1 interactions with the CAD gene promoter were investigated using the chromatin immunoprecipitation assays (ChIP) and PCR primers directed to the -176 to -19 region of the promoter. Sp1 antibodies immunoprecipitated the CAD promoter in untreated ZR-75 cells and in cells treated with 10 nM E2 for 30, 60, and 90 min (Figure 46). ER α (H184) antibodies gave weak to nondetectable bands where the ER α (G20) antibody showed a hormone-induced increase in ER α interactions with the CAD gene promoter. Previous studies have reported interaction of the Brahma-related gene 1 (BRG-1) with hormone-responsive gene promoters (DiRenzo et al., 2000; Shang et al., 2000), and in this study we detected BRG-1 associated with the CAD gene promoter in the presence or absence of hormone.

As a control, we also investigated the interactions of ER α , Sp1, and Brg-1 with the +2465 to +2605 region (E2 nonresponsive) of the cathepsin D gene promoter by PCR. Only minimal to nondetectable bands were observed on the cathepsin D gene promoter (Castro-Rivera et al., 2001; Wang et al., 2001b). These data confirm interactions of ER α and Sp1 with the human CAD gene promoter in ZR-75 cells, and current studies are investigating interaction of other nuclear cofactors required for ER α /Sp1 action.

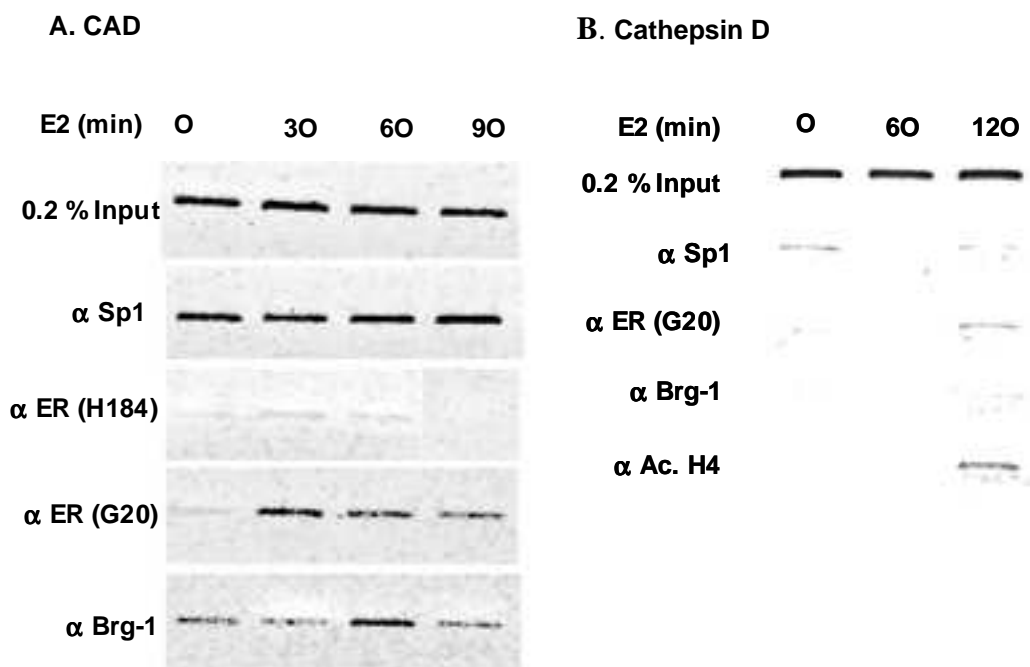


Figure 46. Analysis of ER α and Sp1 interactions with the CAD gene promoter by ChIP. A, CAD gene promoter. ZR-75 cells were treated with 20 nM E2 for different time points and after immunoprecipitation of cross-linked complexes, the chromatin was analyzed by PCR as described in Materials and Methods. B, Cathepsin D gene promoter. As a control, we also investigated the interactions of ER, Sp1, and Brg-1 with the +2465 to +2605 region [E2 nonresponsive] of the cathepsin D gene promoter by PCR as described in Materials and Methods. Only minimal to nondetectable bands were observed.

3.2. Molecular Mechanisms of AhR-ER α /Sp1 Crosstalk in Breast Cancer Cells

3.2.1. *Inhibitory effects of TCDD on CAD gene expression in ZR-75 and MCF-7*

breast cancer cells

Results described in section 3.1 show that E2 induced CAD gene expression in ZR-75 and MCF-7 cells and this response was mediated through interaction of ER α /Sp1 with proximal GC-rich motifs (-90 to +25). This study used the CAD gene as a model for investigating the mechanisms of inhibitory AhR-ER α crosstalk in which ER α /Sp1 was the hormone-activated transcription factor complex. We first investigated the effects of TCDD on E2-induced CAD gene expression. Results in Figure 47A show that E2 induced CAD mRNA levels in ZR-75 cells and this response was inhibited by the antiestrogen ICI 182,780 and the AhR agonist TCDD. E2 induction was observed at 12 h and 24 h in this study (Figure 47A) whereas in the previous study E2 induction was observed only at 12 h (Figure 33). This could be due to changes in serum and different passage of cells used. Results in Figure 47B also show that treatment of MCF-7 cells with 10 nM E2 also induced CAD mRNA levels, whereas treatment with TCDD alone or in combination with E2 resulted in CAD mRNA levels similar to that observed in cells treated with DMSO (solvent control). These data demonstrate inhibitory AhR-ER α crosstalk associated with hormonal regulation of CAD gene expression in ER-positive ZR-75 and MCF-7 cells.

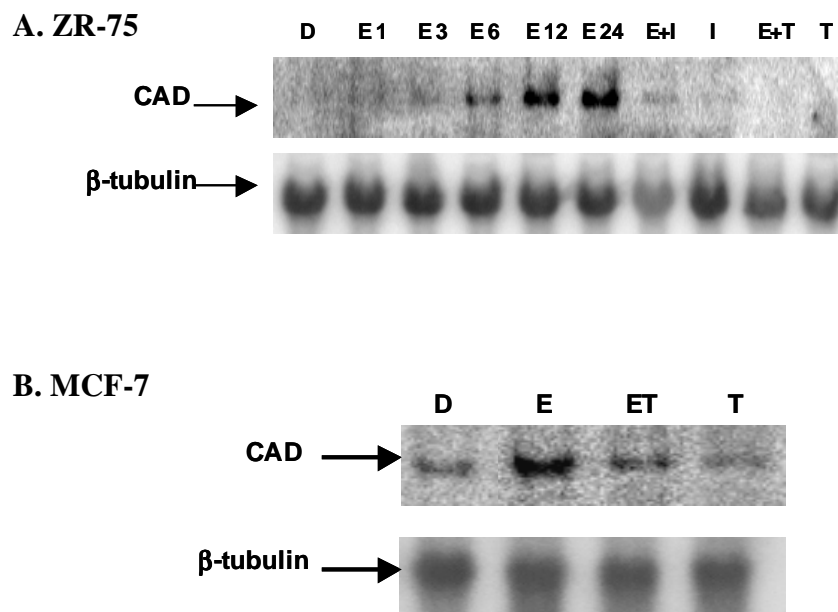


Figure 47. Regulation of CAD mRNA levels in ZR-75 and MCF-7 cells. ZR-75 cells [A] were treated with DMSO (D), 10 nM E2 (E) alone for 1-24 h, 1 μ M ICI 182,780 (I), or 10 nM TCDD (T) alone for 12 h, or in combination with E2 (E+I or E+T) for 12 h. CAD mRNA levels were determined by Northern blot analysis as described in the Materials and Methods. Using a similar approach, CAD mRNA levels were also determined in MCF-7 cells [B] treated with DMSO, 10 nM E2, 10 nM TCDD, or E2 plus TCDD for 6 h.

3.2.2. Inhibitory effects of TCDD on CAD gene promoter-reporter constructs

The inhibitory effects of TCDD on E2-induced transactivation was also investigated in ZR-75 cells transfected with constructs containing CAD gene promoter

inserts. E2 induced transactivation in cells transfected with pCAD1 and pCAD2 which contains the E2-responsive -90 to +115 and -90 to +25 promoter inserts, respectively. After cotreatment with E2 plus TCDD, the induced luciferase activity was significantly decreased (Figures 48A and 48B). In a similar set of experiments, ZR-75 cells were transfected with pCAD1 or pCAD2 and treated with DMSO, 10 nM E2, E2 plus ICI 182,780, or 1 μ M ICI 182,780 alone (Figures 49A and 49B). The results show that like TCDD, ICI 182,780 also inhibited E2-induced transactivation and the classical antiestrogen was a more effective inhibitor in the transient transfection studies. We also carried out a comparable set of experiments in MCF-7 cells transfected with pCAD1 or pCAD2 and treated with E2 alone or in the presence of 10 nM TCDD (Figures 50A and 50B) or ICI 182,780 (Figures 51A and 51B). The results were comparable to those observed in ZR-75 cells and both TCDD and ICI 182,780 inhibited E2-induced transactivation.

We then investigated inhibitory AhR-ER α crosstalk in ZR-75 and MCF-7 cells transfected with pCAD1 and constructs containing mutations in critical GC-rich sites and a potential iDRE motif containing a CACGC (Figure 52). Results presented in section 3.1 demonstrated that the upstream GC-rich sites #1 and #2 were the major E2-responsive motifs in the CAD gene promoter and the results in Figure 53A show that induction by E2 was increased in ZR-75 cells transfected with pCAD1m1 (mutated site #2), whereas in cells transfected with pCAD1m2, hormone-inducibility was not observed, indicating that GC-rich site #1 was sufficient for hormone inducibility. TCDD inhibited E2-induced transactivation in cells transfected with pCAD1m1.

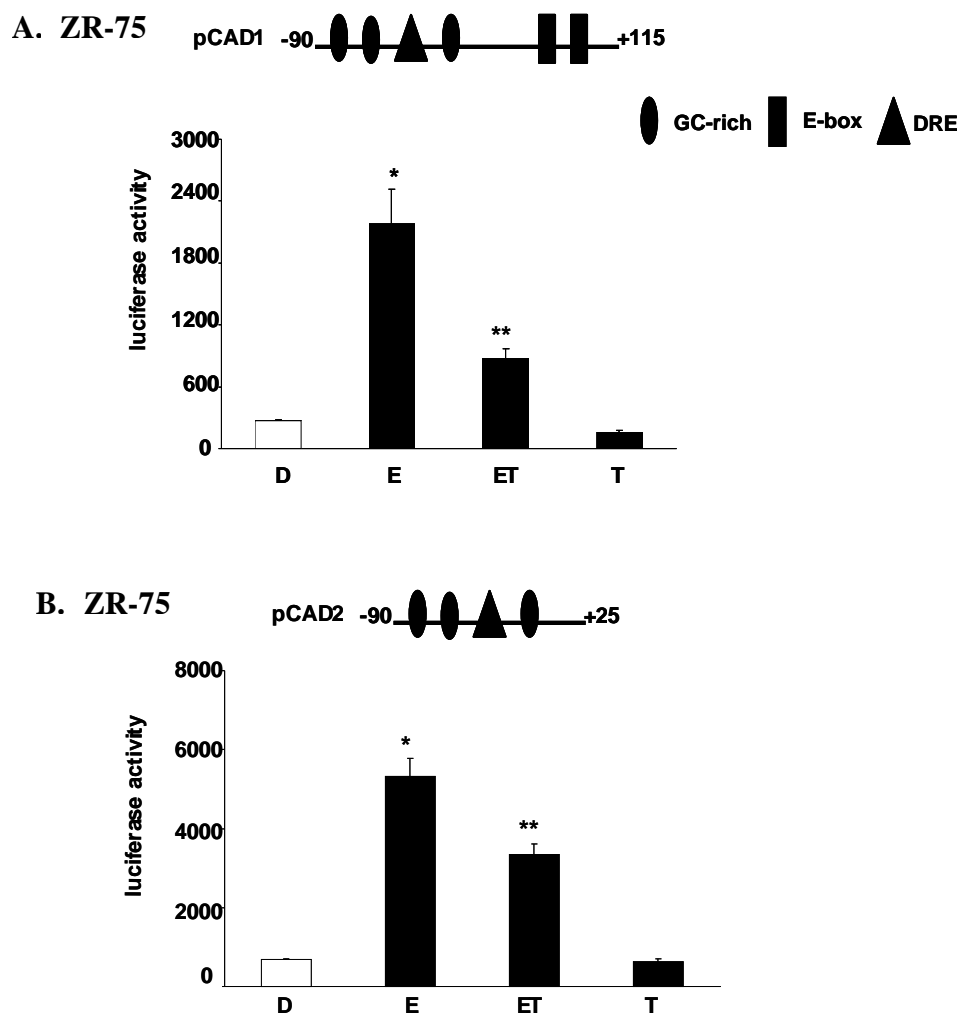


Figure 48. Regulation of CAD constructs by TCDD in ZR-75 cells. ZR-75 cells were transfected with 500 ng of pCAD1 [A] or pCAD2 [B] and 150 ng of ER α , treated with DMSO, 10 nM E2, E2 plus TCDD, or 10 nM TCDD [A and B] and luciferase activity determined as described in the Materials and Methods. Results are expressed as means \pm SE for three replicate determinations for each treatment group and significant ($p < 0.05$) induction by E2 (*) or inhibition of this response by TCDD (**) are indicated.

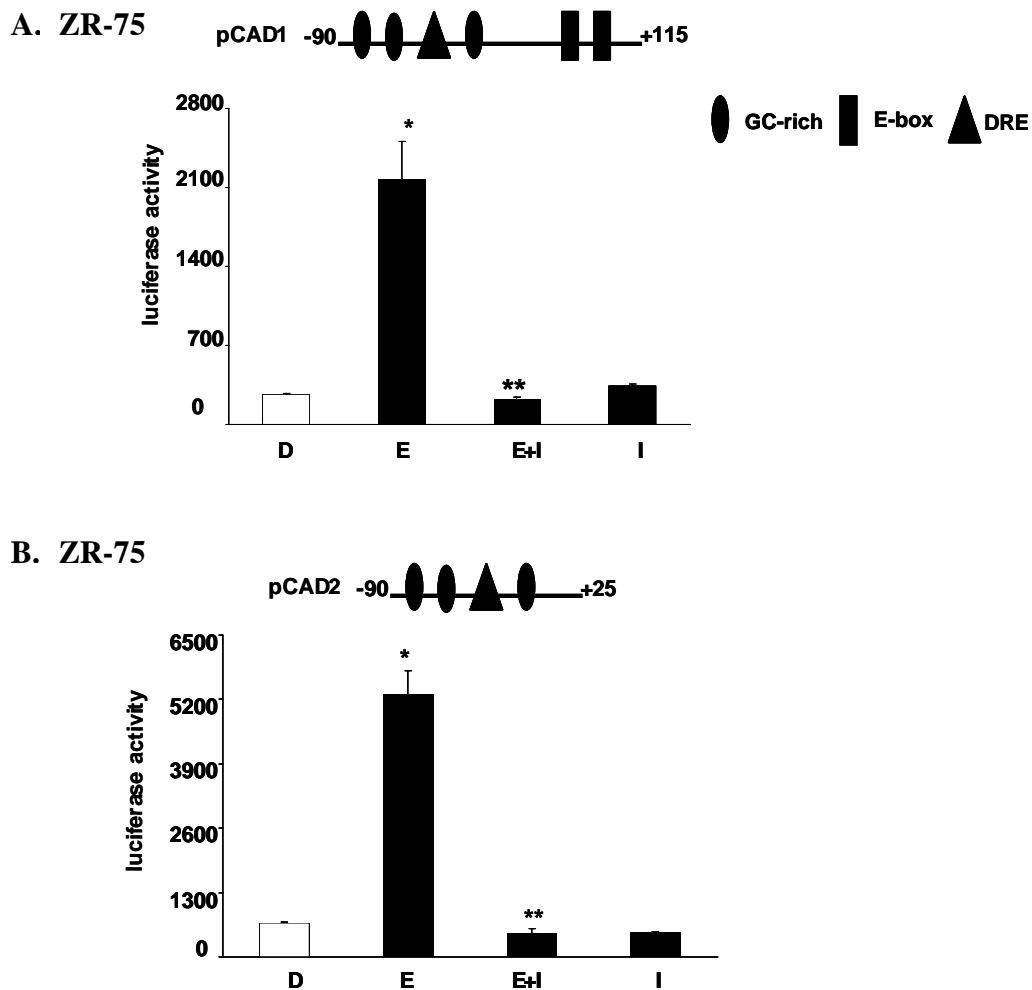


Figure 49. Regulation of CAD constructs by ICI 182,780 in ZR-75 cells. ZR-75 cells were transfected with 500 ng of pCAD1 [A] or pCAD2 [B] and 150 ng of ER α , treated with DMSO, 10 nM E2, E2 plus ICI 182,780, or 1 μ M ICI 182,780 [A and B] and luciferase activity determined as described in the Materials and Methods. Results are expressed as means \pm SE for three replicate determinations for each treatment group and significant ($p < 0.05$) induction by E2 (*) or inhibition of this response by ICI 182,780 (**) are indicated.

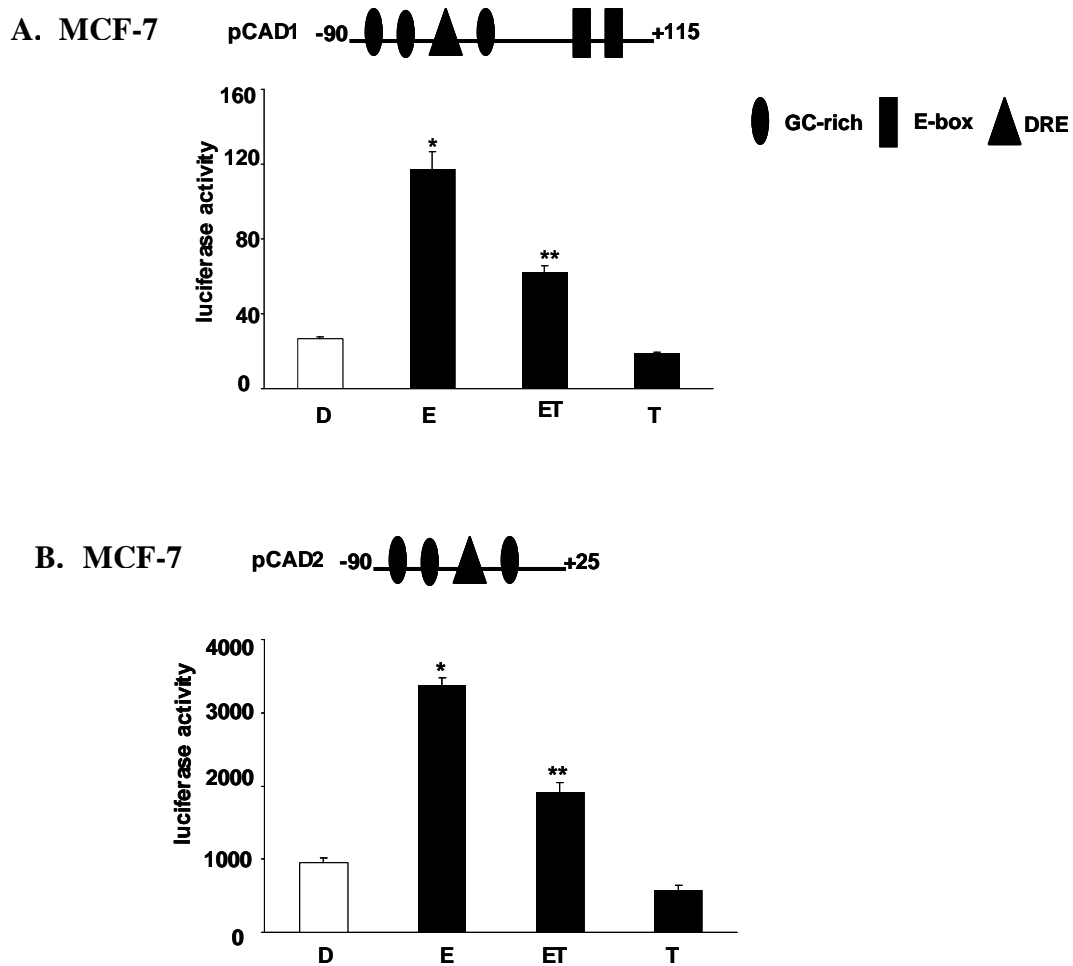


Figure 50. Regulation of CAD constructs by TCDD in MCF-7 cells. MCF-7 cells were transfected with 500 ng of pCAD1 [A] or pCAD2 [B] and 150 ng of ER α , treated with DMSO, 10 nM E2, E2 plus TCDD, or 10 nM TCDD [A and B] and luciferase activity determined as described in the Materials and Methods. Results are expressed as means \pm SE for three replicate determinations for each treatment group and significant ($p < 0.05$) induction by E2 (*) or inhibition of this response by TCDD (**) are indicated.

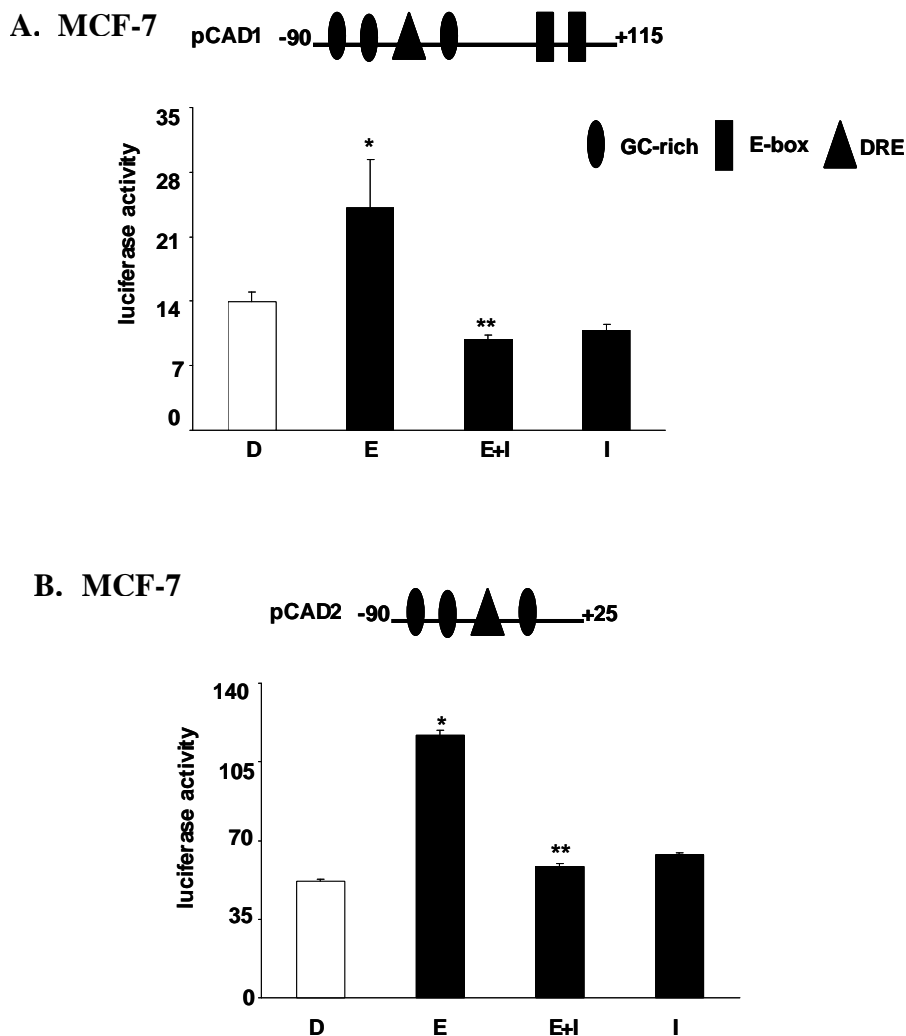


Figure 51. Regulation of CAD constructs by ICI 182,780 in MCF-7 cells. MCF-7 cells were transfected with 500 ng of pCAD1 [A] or pCAD2 [B] and 150 ng of ER α , treated with DMSO, 10 nM E2, 1 μ M ICI 182,780, or E2 plus ICI 182,780 [A and B] and luciferase activity determined as described in the Materials and Methods. Results are expressed as means \pm SE for three replicate determinations for each treatment group and significant ($p < 0.05$) induction by E2 (*) or inhibition of this response by ICI 182,780 (**) are indicated.

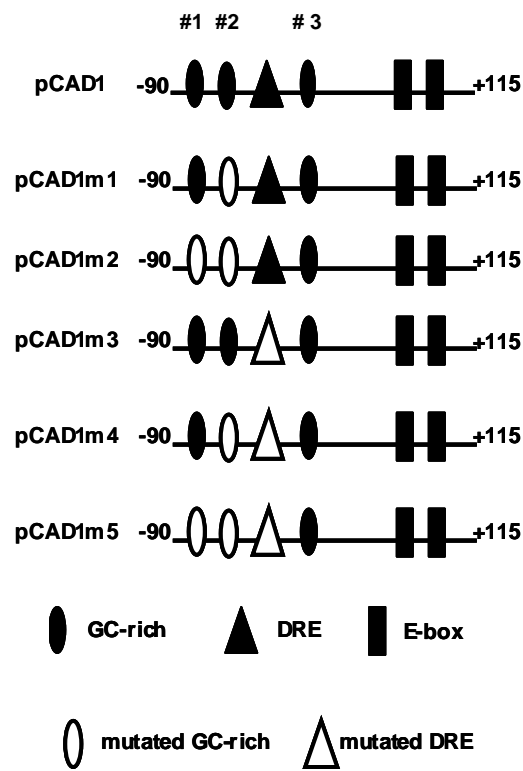


Figure 52. Summary of CAD constructs and their cis-elements used to study inhibitory AhR-ER α crosstalk

Inhibitory AhR-ER α crosstalk for cathepsin D, heat shock protein 27 and c-fos has been linked to direct interactions of the AhR complex with "inhibitory DREs" (iDREs) containing the core CACGC motif that binds the AhR complex (Krishnan et al., 1995; Gillesby et al., 1997; Duan et al., 1999; Porter et al., 2001). The CAD promoter also contains a CACGC motif at -45; however, E2 induced luciferase in ZR-75 cells transfected with pCADm3 (mutated DRE), and in cells cotreated with E2 plus TCDD, the induced response was significantly inhibited (Figure 53B). Transfection of plasmids which contain the mutant iDRE and also mutations of GC-rich sites #1 (pCADm4) or #1 and #2 (pCADm5) resulted in loss of E2-responsiveness and the effects of TCDD on this activity. These results suggest that the indirect antiestrogenic activity of TCDD in ZR-75 cells was iDRE-independent. A parallel set of experiments were carried out in MCF-7 cells transfected with pCAD1, pCADm1 and pCAD1m2 (Figure 54A), pCAD1, pCAD1m3, pCAD1m4 or pCAD1m5 (Figure 54B). Although pCAD1m1 was hormone-inducible and this response was inhibited by cotreatment with TCDD (Figure 54A), the magnitude of the induction response was significantly decreased suggesting a more important role for GC-rich site #2 in mediating activation by E2.

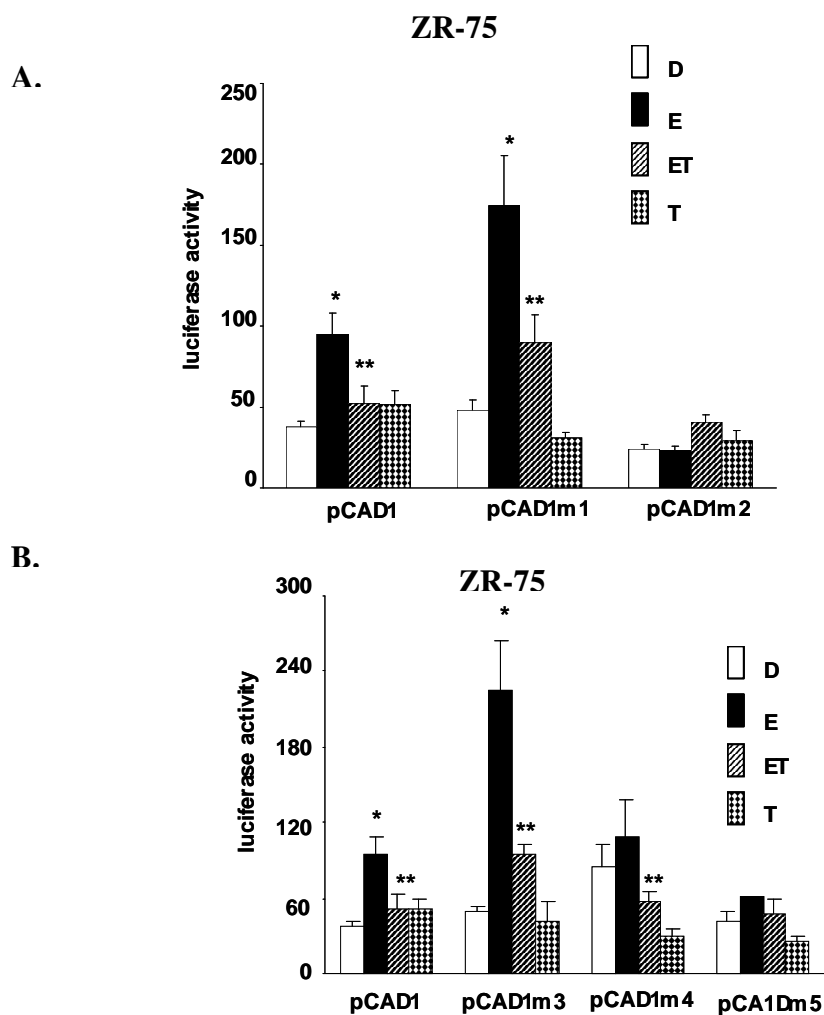
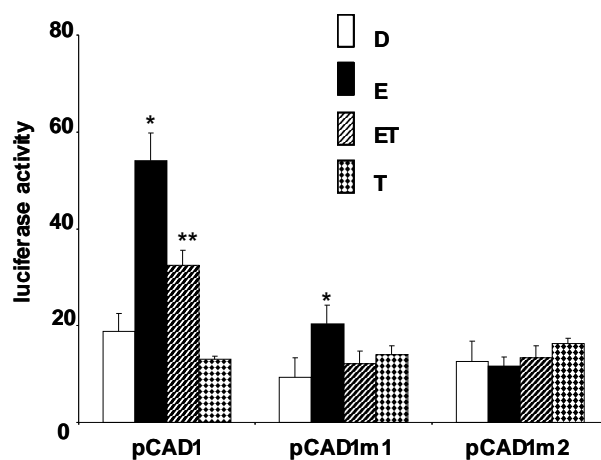


Figure 53. Inhibitory AhR-ER α crosstalk in ZR-75 cells transfected with wild-type or mutant CAD constructs. ZR-75 cells were transfected with 500 ng each of wild-type or mutant CAD constructs [A and B] and 150 ng of ER α , treated with DMSO, 10 nM E2, 10 nM TCDD, or E2 plus TCDD for 36 h, and luciferase activity determined as described in the Materials and Methods. Results are expressed as means \pm SE for three replicate determinations for each treatment group and significant ($p < 0.05$) induction by E2 (*) or inhibition by E2 plus TCDD (**) are indicated.

A. MCF-7



B. MCF-7

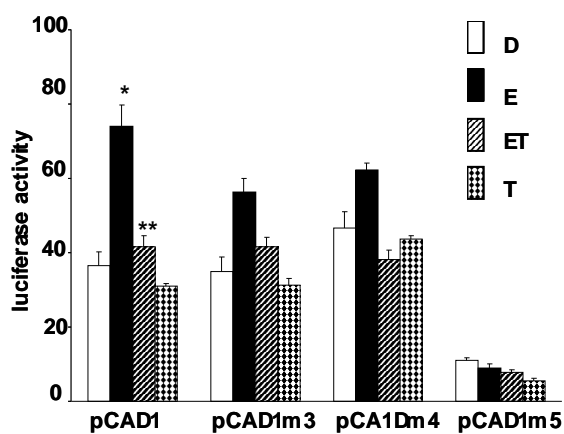


Figure 54. Inhibitory AhR-ER α crosstalk in MCF-7 cells transfected with wild-type or mutant CAD constructs. MCF-7 cells were transfected with 500 ng each of wild-type or mutant CAD constructs [A and B] and 150 ng of ER α , treated with DMSO, 10 nM E2, 10 nM TCDD, or E2 plus TCDD for 36 h, and luciferase activity determined as described in the Materials and Methods. Results are expressed as means \pm SE for three replicate determinations for each treatment group and significant ($p < 0.05$) induction by E2 (*) or inhibition by E2 plus TCDD (**) are indicated.

Compared to results in ZR-75 cells (Figure 53B) hormone-induced transactivation was greatly decreased in MCF-7 cells transfected with pCAD1m3 (Figure 54A), suggesting that in MCF-7 cells, the CACGC sequence may also influence hormone-induced transactivation through the GC-rich site #2. This observation was not unprecedented since a previous study in MCF-7 cells showed that hormone-induced transactivation of a GC-rich motif in the cathepsin D promoter was also dependent on a proximal CAGGC site (Wang et al., 1998). The pCAD constructs are clearly activated by E2 in ZR-75 and MCF-7 cells and ER α /Sp1 activation is a critical component of this process. However, results with the mutant constructs demonstrate, that cell context also plays a role in differential activation of specific promoter elements. Despite these differences in hormone-induced transactivation of wild-type and mutant CAD promoter constructs in ZR-75 and MCF-7 cells, inhibitory AhR-ER α crosstalk was observed for both gene and reporter gene expression in both cell lines.

3.2.3 Characterization of CFP and YFP fusion proteins used in fluorescence resonance energy transfer (FRET)

FRET has been used to study interactions of nuclear receptors and co-regulatory proteins or peptides in living cells (Llopis et al., 2000; Tamrazi et al., 2002; Weatherman et al., 2002; Bai and Giguere, 2003), and using YFP/CFP chimeras of ER α and Sp1, ligand-induced interactions of ER α with Sp1 in MCF-7 cells have been reported (Kim et al., 2005). Figure 55 summarizes the YFP/CFP chimeras used in this study in MCF-7 cells.

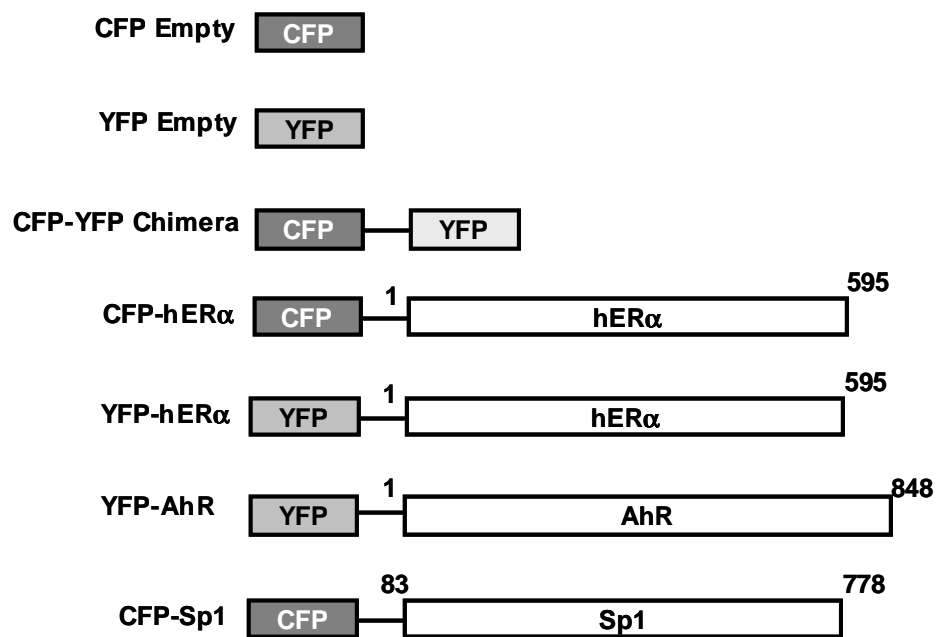


Figure 55. Summary of chimeric constructs used for FRET studies.

ZR-75 cells exhibit lower transfection efficiencies and were not used for the FRET studies. In a previous report (Kim et al., 2005), it was shown that the YFP/CFP-ER α and Sp1 chimeras were functional in transactivation assays. Figure 56A summarizes the effects of DMSO, E2, and E2 plus TCDD on distribution of transfected YFP-AhR in MCF-7 and COS-1 cells. In MCF-7 cells treated with DMSO and E2, the AhR was detected in the cytosolic and nuclear fractions, whereas after treatment with E2 plus TCDD or TCDD alone, the receptor was localized exclusively in the nucleus and exhibited a punctate staining pattern. In a parallel experiment with COS-1 cells that do not express ER or AhR, the transfected YFP-AhR was both cytosolic/perinuclear and nuclear in cells treated with DMSO or E2, whereas E2 plus TCDD or TCDD alone induced formation of a nuclear AhR complex as observed in MCF-7 cells. The functionality of the YFP-AhR chimera was also investigated in COS-1 cells treated with DMSO or 10 nM TCDD and transfected with pDRE₃ which contains three tandem consensus DREs linked to firefly luciferase (Figure 56B). In the absence of YFP-AhR, TCDD did not induce luciferase activity; however, induction by TCDD was observed after cotransfection of YFP-AhR indicating that the chimeric YFP-AhR protein was functional.

3.2.4 Direct interactions between CFP-ER α and YFP-AhR determined by FRET in MCF-7 cells

Ligand activation of the AhR inhibits induction of ER α /Sp1-dependent activation of CAD gene/gene promoter expression in MCF-7 and ZR-75 cells (Figures 47-54). This led us to investigate the direct interactions of CFP-ER α and YFP-AhR by

FRET in MCF-7 cells. In solvent (DMSO)-treated MCF-7 cells transfected with YFP-AhR and CFP-ER α , both receptors were primarily localized in the nucleus and this was also observed in cells treated with 10 nM E2, 10 nM TCDD, or E2 plus TCDD (Figure 57A). Cells were pretreated with TCDD for 15 min and then treated with E2 (alone or in combination with TCDD) for an additional 8 min. A punctate nuclear pattern was observed in all the ligand treated groups and was most pronounced in cells treated with E2 plus TCDD. Excitation of CFP-ER α at 410 nm and emission at 488 illustrates the blue fluorescent emission of the nuclear CFP-ER α . The yellow fluorescence was detected in the FRET channel at 525 nm by exciting CFP-ER α using 820 nm pulsed laser and this represents the CFP-YFP interaction and energy transfer. The emission intensities in the FRET channel were enhanced in the treated cells.

The results in Figure 57B quantitate the FRET efficiencies in the various treatment groups, and there was a significant increase in FRET efficiencies in cells treated with E2, TCDD and E2 plus TCDD. The overlay of the CFP and FRET signals are shown in Figure 56A and confirm the enhanced emission observed in the FRET channel for the treatment groups. These results demonstrate for the first time that AhR and ER α interact in living cells, and this observation is consistent with *in vitro* studies that also show interactions between these proteins (Klinge et al., 1999; Ohtake et al., 2003; Wormke et al., 2003). A parallel set of experiments was also carried out in COS-1 cells that do not express endogenous AhR or ER α .

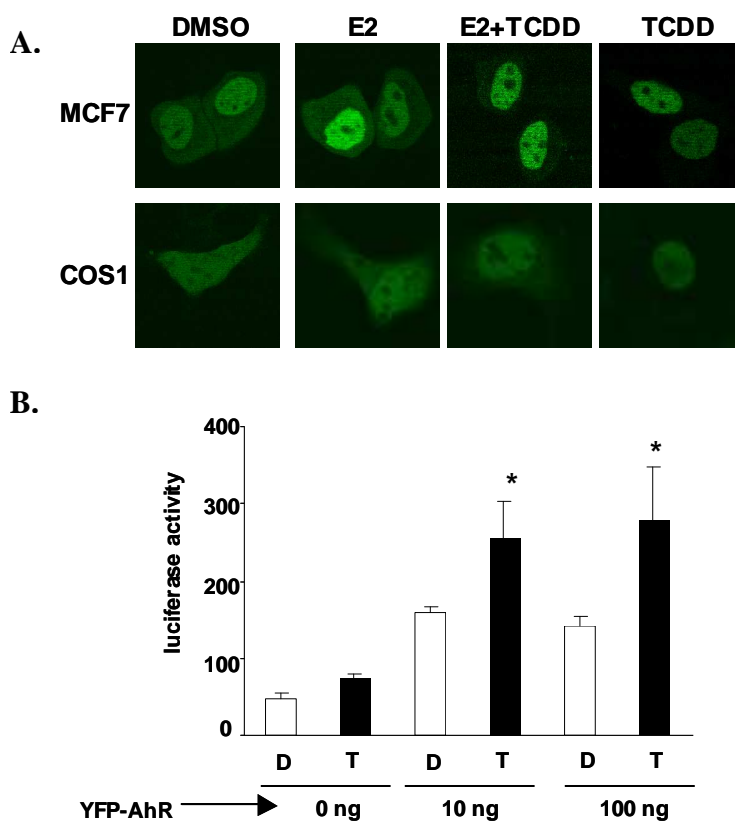


Figure 56. Activity of YFP-AhR in MCF-7 and COS-1 cells. A. Ligand-dependent subcellular trafficking of YFP-AhR. MCF-7 or COS-1 cells were transfected with 250 ng of YFP-AhR for 16 h, treated with DMSO, 10 nM E2, 10 nM TCDD, or E2 plus TCDD for 30 min, and localization of YFP-AhR was determined as described in the Materials and Methods. B. Ah-responsiveness of COS-1 cells transfected with YFP-AhR. COS-1 cells were transfected with 150 ng pDRE₃ and different amounts of YFP-AhR expression plasmid, treated with DMSO or 10 nM TCDD, and luciferase activity determined as described in the Materials and Methods. Results are expressed as means \pm SE for three replicate determinations for each treatment group and significant ($p < 0.05$) induction by TCDD is indicated (*).

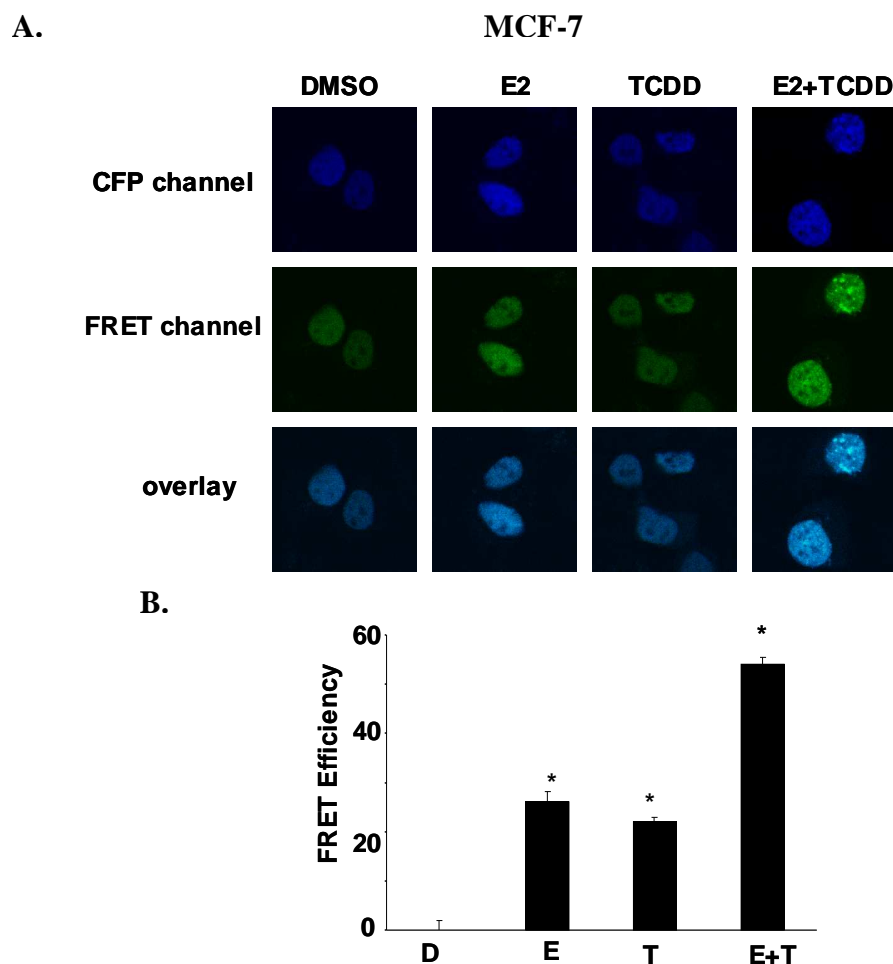


Figure 57. Ligand-dependent interactions of YFP-AhR and CFP-ER α in MCF-7 cells.

MCF-7 cells were transfected with YFP-AhR and CFP-ER α , pretreated with 10 nM TCDD for 15 min before treating with DMSO or 10 nM E2 for 8 min, and representative FRET images in each treatment group were acquired from 8-18 min after treatment. For each treatment group, 10-15 images were acquired and each image contained 1-5 cells which were subsequently analyzed for FRET efficiencies in MCF-7. Significant ($p < 0.05$) induction of FRET efficiency in the various treatment groups is indicated by an asterisk.

In COS-1 cells transfected with CFP-ER α and YFP-AhR (Figure 58A), the results of excitation/emission studies were similar to those observed in MCF-7 cells and FRET efficiencies were significantly increased in COS-1 cells treated with E2, TCDD and E2 plus TCDD (Figure 58B).

3.2.5 Direct interactions between CFP-Sp1 and YFP-ER α as determined by FRET in MCF-7 cells

Direct interactions of chimeric Sp1 and AhR proteins were not observed in the FRET assay, and this is not unexpected due to the high molecular weights of these proteins which probably preclude adequate distance (1-10 nm) and dipole-dipole orientation between the fluorophores to observe energy transfer. We therefore investigated the effects of the liganded AhR complex on hormone-dependent activation of ER α /Sp1 in MCF-7 cells which express endogenous AhR. Cells were transfected with CFP-Sp1 and YFP-ER α and treated with solvent (DMSO) control, E2, TCDD or E2 plus TCDD. Cells were pretreated with TCDD for 15 min prior to addition of E2 for 8 min (Figure 59A). Cells treated with DMSO or TCDD exhibit low FRET efficiencies, whereas after treatment with E2, there was a significant increase in the FRET signal. This ligand-dependent increase was consistent with the recent FRET study showing ER α -Sp1 interactions in breast cancer cells (Kim et al., 2005). However, the intensity of the E2-induced FRET emission is significantly decreased in cells treated with E2 plus TCDD, and quantitation of the FRET efficiencies summarized in Figures 59B confirms this observation.

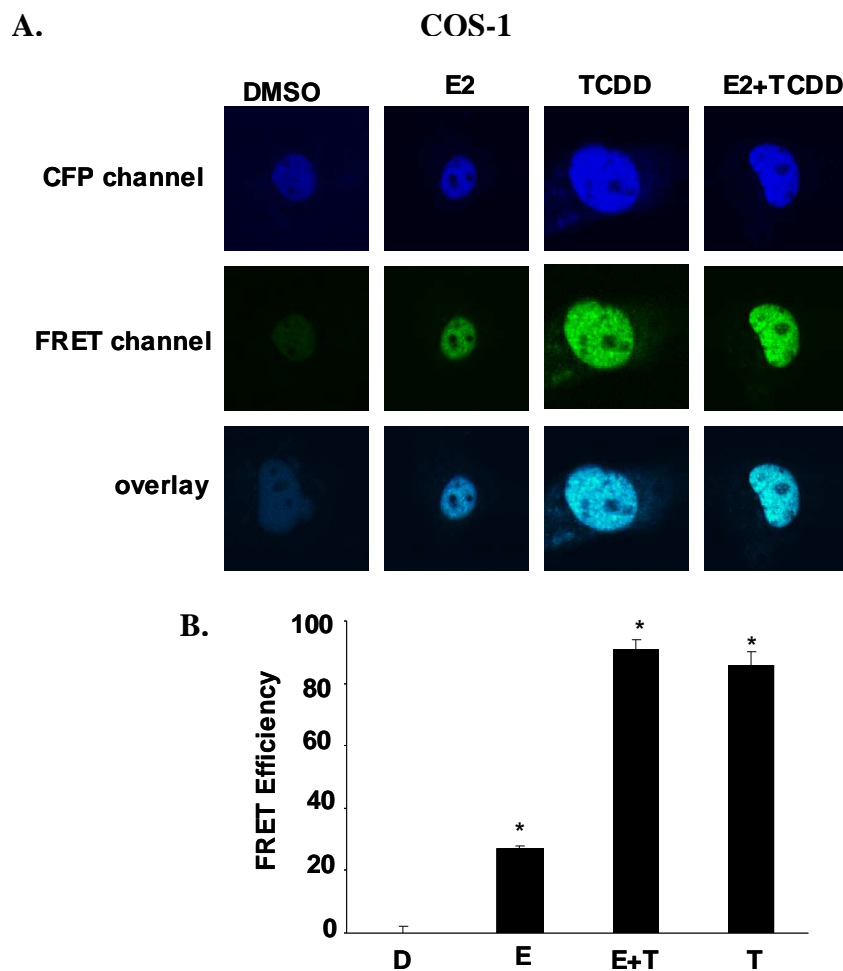


Figure 58. Ligand-dependent interactions of YFP-AhR and CFP-ER α in COS-1 cells.

COS-1 cells were transfected with YFP-AhR and CFP-ER α , pretreated with 10 nM TCDD for 15 min before treating with DMSO or 10 nM E2 for 8 min, and representative FRET images in each treatment group were acquired from 8-18 min after treatment. For each treatment group, 10-15 images were acquired and each image contained 1 - 5 cells which were subsequently analyzed for FRET efficiencies in MCF-7. Significant ($p < 0.05$) induction of FRET efficiency in the various treatment groups is indicated by an asterisk.

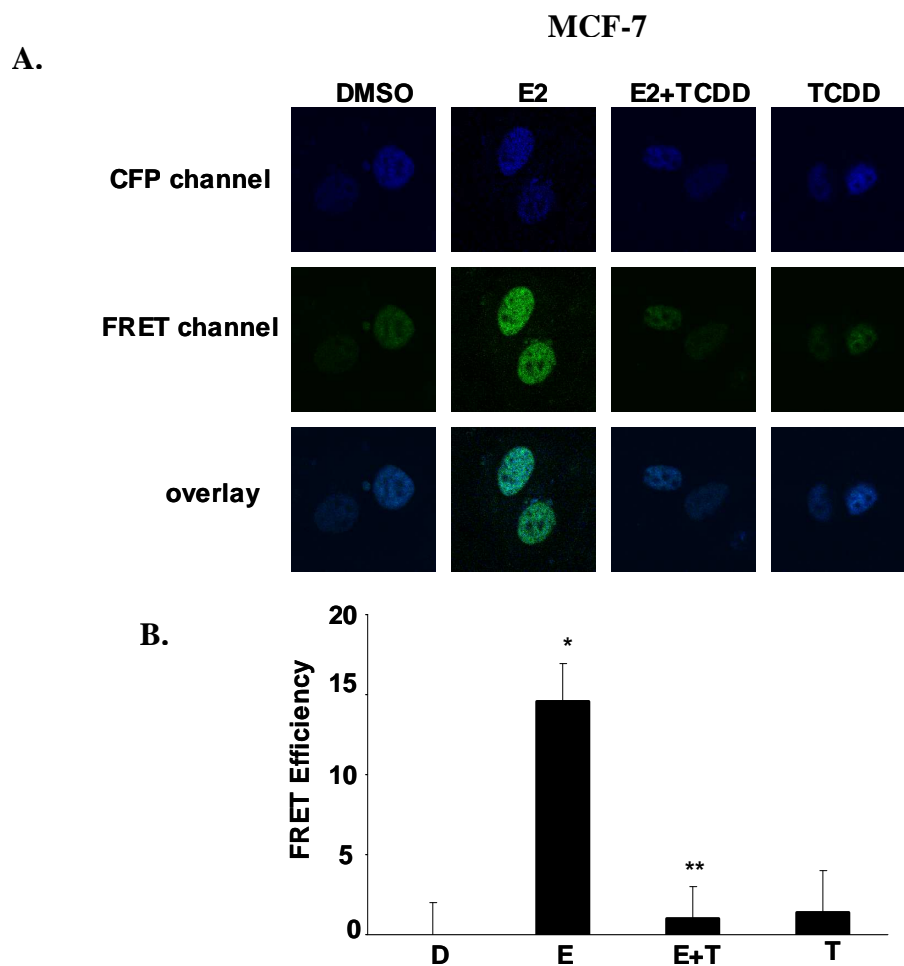


Figure 59. Ligand-dependent interactions of YFP-ER α and CFP-Sp1 in MCF-7 cells. MCF-7 cells were transfected with YFP-ER α and CFP-Sp1, pretreated with 10 nM TCDD for 15 min before treating with DMSO or 10 nM E2 for 8 min, and representative FRET images in each treatment group were acquired from 8-18 min after treatment. For each treatment group, 10-15 images were acquired and each image contained 1-5 cells which were subsequently analyzed for FRET efficiencies (B). Significant ($p < 0.05$) induction of FRET efficiency by E2 (*) and inhibition of this response by cotreatment with TCDD (**) are indicated.

These results demonstrate that the liganded- AhR complex induces a rapid change in ER α /Sp1 interactions which correlates with the observed inhibitory AhR-ER α crosstalk on the CAD gene/gene promoter (Figures 48-55). We further investigated ER-AhR interactions in MCF-7 cells treated with DMSO, E2, TCDD, E2 plus TCDD and transfected with FLAG-AhR. Cell lysates were immunoprecipitated with non-specific IgG or FLAG antibodies and analyzed for ER α by Western blot analysis (Figure 60A). ER α was detected in IgG precipitates, and the low levels were observed in the E2 plus TCDD treatment group. Interactions of ER α with the AhR were determined in the FLAG antibody immunoprecipitates in which higher levels of ER α were observed in the TCDD and E2 plus TCDD treatment groups. These results were consistent with the enhanced AhR-ER α interactions observed by FRET in MCF-7 and COS-1 cells treated with TCDD and E2 plus TCDD (Figure 57). Levels of Sp1 protein did not show any consistent treatment-related effects in the immunoprecipitation in COS-1 cells transfected with pDRE₃ and FLAG-AhR studies. Results in Figure 60B show that TCDD induced transactivation (compared to DMSO), thus confirming that the FLAG-AhR chimera was functional.

There is also evidence that proteasome-dependent degradation of ER α in breast cancer cells cotreated with E2 plus TCDD may result in limiting levels of ER α and thereby inhibit expression of E2-responsive genes (Wormke et al., 2003). Therefore we investigated the effects of proteasome inhibitor MG132 on CAD mRNA levels in presence of different ligands.

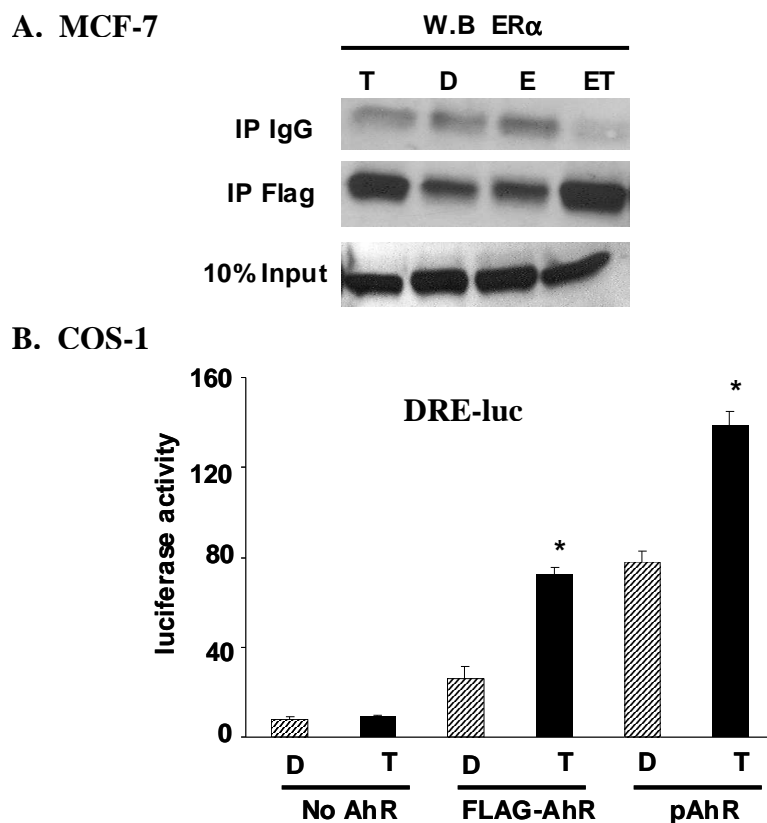


Figure 60. Flag AhR-ER α interactions and function. [A] FLAG-AhR ER α interactions. MCF-7 cells were transfected with FLAG-AhR for 16 h, treated with DMSO, 10 nM E2, 10 nM TCDD, and E2 plus TCDD for 30 min, and interactions of FLAG-AhR and ER α were determined by immunoprecipitation and Western blot analysis as described in the materials and Methods. [B] Functionality of FLAG-AhR. COS-1 cells transfected with pDRE₃, FLAG-AhR or pcDNA3.1-AhR (pAhR), treated with DMSO or 10 nM TCDD, and luciferase activity determined as described in the Materials and Methods. Significant ($p < 0.05$) induction by TCDD is indicated.

Results in Figure 61 show that E2 induced CAD mRNA levels in ZR-75 cells and this was downregulated in cells co-treated with E2+TCDD. However, in cells pre-treated with MG132, CAD mRNA levels were decreased in all the treatments including the control. Thus in this study it was not possible to determine whether the low levels of ER α in cells cotreated with E2 plus TCDD were limiting since the proteasome inhibitor MG132 itself directly decreased CAD mRNA levels (Figure 61). Thus, it is possible that decreased and possibly limiting ER α levels in MCF-7 and ZR-75 cells cotreated with E2 plus TCDD may contribute to the antiestrogenic effects of the latter compound.

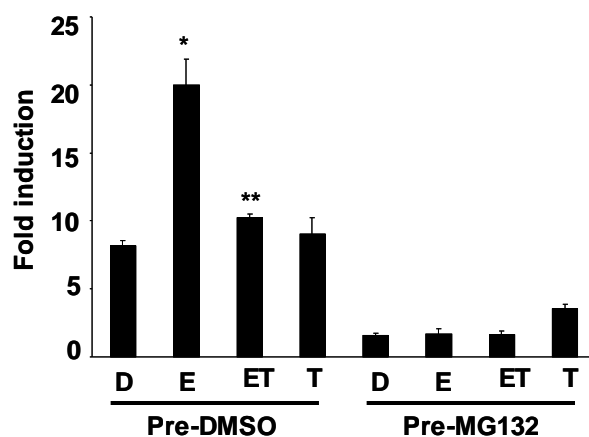


Figure 61. Effects of proteasome inhibitor MG132 on CAD mRNA levels in ZR-75 cells.

ZR-75 cells were pretreated with DMSO (D) or 10 μ M MG132 before treating with DMSO, 10 nM E2 (E), 10 nM TCDD (T) or E+T for 12 h. CAD mRNA levels were determined by Real-time PCR analysis as described in the Materials and Methods.

Significant ($p < 0.05$) induction by E2 (*) and inhibition of this response by TCDD (**) are indicated.

These results suggest that the AhR complex either forms a transcriptionally-inactive AhR:ER/Sp1 complex where the AhR corepresses ER/Sp1 or the AhR competitively dissociates ER α from interactions with Sp1. The latter pathway is supported, in part, by recent studies showing that treatment of cells with TCDD alone or in combination with E2 recruits the ER/AhR complex to promoters of Ah-responsive genes such as CYP1A1 (Beischlag and Perdew, 2005; Matthews et al., 2005). We therefore investigated simultaneous interactions of AhR/Arnt and ER α on the endogenous CAD and CYP1A1 gene promoters (Figure 62) using a ChIP assay.

Initial studies examined interactions of ER α , Sp1, AhR and Arnt with the CAD gene promoter after treatment with DMSO, 10 nM E2, 10 nM TCDD, and E2 plus TCDD for 1 h (Shengxi Liu, unpublished results). There was evidence that all of these transcription factors were associated with the E2-responsive (GC-rich) region of the CAD promoter in the solvent (DMSO)-treated group. Arnt and Sp1 levels exhibited minimal changes in band intensities in the various treatment groups. The ER α band increased and decreased in cells treated with TCDD and E2 plus TCDD, respectively, and in cells treated with TCDD, there was a decrease in AhR interaction with the CAD promoter. These results show some treatment-related differences at one specific time point (1 h) and, in order to more accurately define AhR/ER α interaction with the CAD promoter during conditions of inhibitory AhR-ER α crosstalk (i.e. E2 plus TCDD), we determined the time-dependent interactions of transcription factors with the CAD promoter in cell cotreated with E2 plus TCDD (Figure 62).

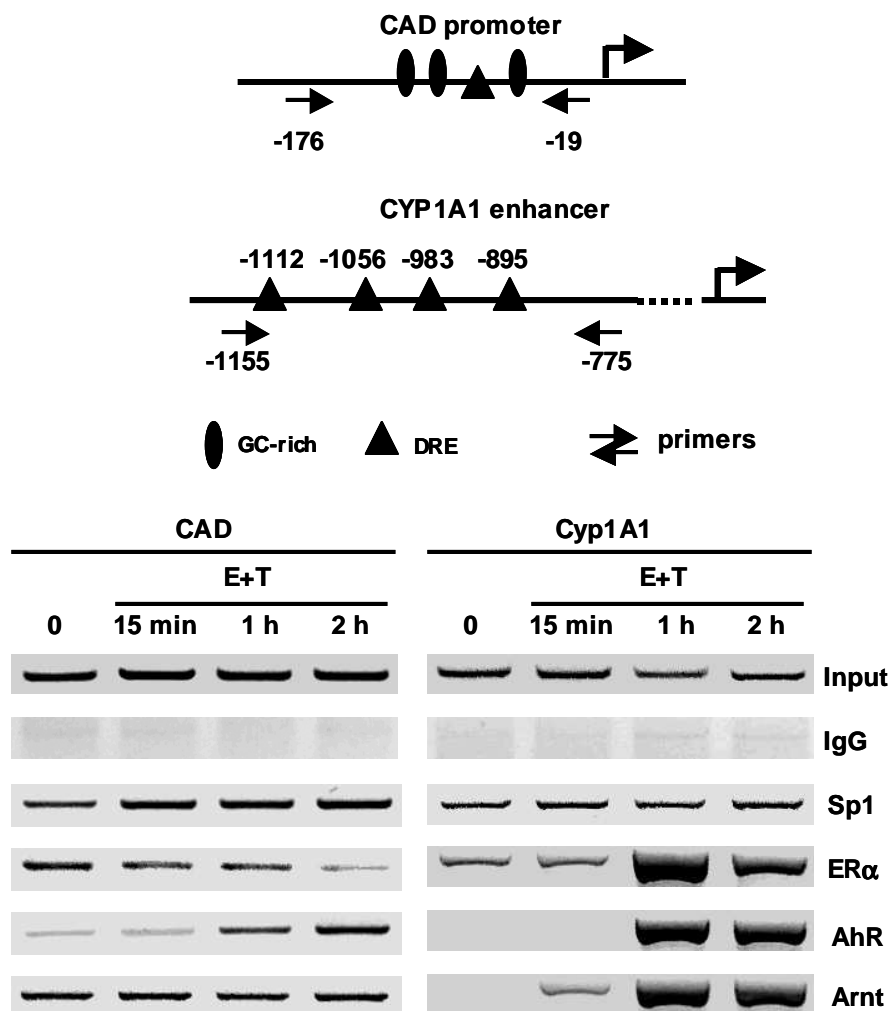


Figure 62. Analysis of protein interactions with CAD and CYP1A1 promoters by ChIP. A. Schematic representation of CAD promoter and CYP1A1 enhancer. B. Analysis of protein interactions with the CAD gene promoter and CYP1A1 enhancer by ChIP in ZR-75 cells. ZR-75 cells were treated with E2 plus TCDD (E+T) for 0, 15 min, 1 h and 2 h and after immunoprecipitation, the chromatin was analyzed by PCR as described in Materials and Methods.

Band intensities associated with Arnt and Sp1 were similar at all time points (0, 15, 60 or 120 min), whereas after 60 or 120 min, there was increase in bands associated with the AhR and a decrease in the ER α band. As a positive control for this experiment, we also showed that treatment with TCDD plus E2 recruited AhR, Arnt and ER α to the Ah-responsive region of the CYP1A1 promoter. Thus, inhibitory AhR-ER α /Sp1 crosstalk in cells cotreated with E2 plus TCDD may involve ligand-induced disruption of ER α /Sp1 by the AhR and decreased ER α interactions with the CAD gene promoter.

Previous studies in this laboratory reported that E2 did not affect Ah-responsiveness (i.e. CYP1A1 inducibility by TCDD) in MCF-7 cells (Hoivik et al., 1997), and recent studies reported that E2 either enhanced (Matthews et al., 2005) or suppressed CYP1A1 inducibility (Beischlag and Perdew, 2005). We therefore investigated the effects of E2 on TCDD-induced transactivation of DRE-luc reporter activity. Results in Figure 63 show that E2 (100 nM) decreased 2 nM TCDD -induced transactivation in MCF-7 and ZR-75 cells transfected with pDRE₃ (Figure 63). Currently we are investigating the effects of E2 on TCDD-induced CYP1A1 mRNA and protein to re-examine whether ER α modulates Ah-responsiveness in MCF-7 cells and ZR-75 cells.

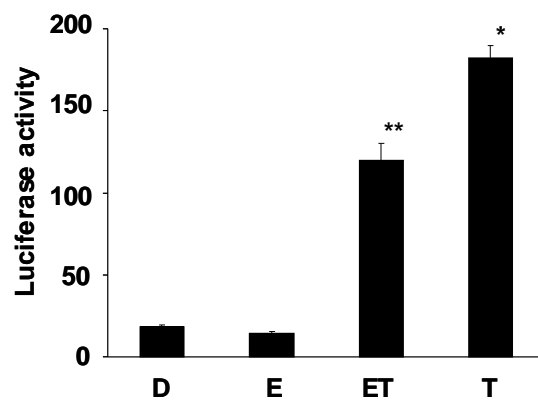
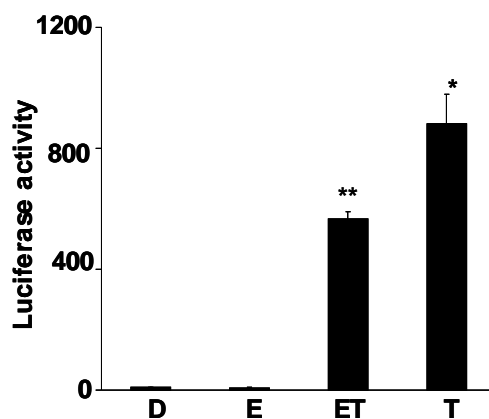
A. MCF-7**B. ZR-75**

Figure 63. Effects of E2 on TCDD-induced transactivation of pDRE₃ in MCF-7 and ZR-75 cells. MCF-7 [A] or ZR-75 [B] cells were transfected with pDRE₃, treated with DMSO [D], 100 nM E2 [E], E2 plus TCDD [E+T], or 2 nM TCDD [T], and luciferase activity determined as described in the Materials and Methods. Significant ($p < 0.05$) induction by TCDD (*) or inhibition of this response by E2 (**) are indicated.

CHAPTER IV

DISCUSSION AND CONCLUSIONS

4.1 Mechanism of Hormonal Regulation of CAD Gene Expression in MCF-7 and ZR-75 Breast Cancer Cells

The CAD gene encodes enzymes required for the first three steps in *de novo* pyrimidine synthesis, and previous studies in MCF-7 cells showed that E2 activated two of these enzyme activities, carbamylphosphate synthetase and aspartate carbamyltransferase (Aitken and Lippman, 1983). Hormonal activation of CAD is consistent with the mitogenic activity of E2 in breast cancer cells and the increase of purine and pyrimidine pools required for DNA synthesis and cell division. Previous studies have reported that serum or mitogenic stimulation of various cancer cell lines was accompanied by a parallel increase in CAD gene expression at the G₁/S phase boundary of the cell cycle (Boyd and Farnham, 1997; Boyd et al., 1998; Boyd and Farnham, 1999; Mac and Farnham, 2000).

Farnham and co-workers have extensively investigated regulation of the hamster CAD gene and the growth-responsive proximal region of the CAD gene promoter (Boyd and Farnham, 1997; Boyd et al., 1998; Boyd and Farnham, 1999; Mac and Farnham, 2000). Their results indicate that growth-dependent regulation of the hamster CAD gene promoter is linked to the proto-oncogene *c-myc* and formation of Myc-Max heterodimers that bind to the E-box motif (consensus sequence is CACGTG) and activate transcription.

The E-box motif is also the binding site for other proteins including transcription factor TF3, TFEB, upstream stimulatory factor (USF) as well as Max heterodimers paired with Mad family of proteins such as Mad, Mxi1, Mad3 and Mad4. Human and hamster CAD gene promoters are similar and both contain upstream GC-rich sites; however, the human promoter contains two E-boxes (5' and 3'), whereas only the 5' E-box is present in the hamster promoter. Recent studies in NIH3T3 cells indicate that the 5' E-box in the human promoter is the major growth-responsive element and transfected c-Myc preferentially activates CAD gene promoter constructs through this motif (Mac and Farnham, 2000). CAD is one of the few proposed c-Myc target genes whose expression is decreased in c-Myc null cells (Bush et al., 1998).

In this study we show that E2 activates CAD mRNA levels in MCF-7 and ZR-75 cells (Figures 32 and 33) and also reporter gene activity in cells transfected with pCAD1, which contains the growth-responsive -90 to +115 region of the CAD gene promoter (Figures 36 and 37). Previous studies have demonstrated that E2 transiently induces *c-myc* gene expression in MCF-7 cells, and a synthetic antisense *c-myc* phosphorothioate oligonucleotide inhibited *c-myc* protein expression and partially inhibited E2-induced growth of MCF-7 cells (Dubik et al., 1987; Watson et al., 1991). Another study indicated the *c-myc* and other protooncogenes (*c-fos* and *c-jun*) were not growth rate limiting in MCF-7 cells (Wosikowski et al., 1992).

Deletion and mutation analysis of the CAD gene promoter in MCF-7 and ZR-75 cells clearly demonstrates that E-box motifs that bind Myc-Max are not essential for basal or hormone-induced transactivation (Figure 38-40). Previous studies have

demonstrated that the E-box motif within the major late promoter element in the proximal -120 to -101 region of the cathepsin D gene promoter binds USF1/2, which are highly expressed in nuclear extracts of MCF-7 cells (Xing and Archer, 1998; Vyhldal et al., 2000). Not surprisingly, the +54 to +78 E-box in the CAD gene promoter also forms a USF1/2-DNA retarded band complex after incubation with nuclear extracts from MCF-7 cells (Figure 43). Therefore, the high expression of USF1/2 in MCF-7 cells and subsequent binding to the CAD promoter E-boxes may competitively inhibit hormone-induced myc complexes from binding and activating CAD gene expression from the E-box motif.

Deletion analysis of the CAD gene promoter demonstrates that the GC-rich motifs are required for hormone-induced transactivation in ER-positive MCF-7 and ZR-75 cells. The pattern of activation by wild-type and variant ER α was comparable in both cell lines in which deletion of the DNA binding domain (HE11) did not result in loss of hormone inducibility in both cell lines transfected with pCAD1 (Figure 36 and 37). These results are consistent with previous studies on other GC-rich promoters activated by ER α /Sp1 because transactivation does not require the DNA binding domain of ER α (Duan et al., 1998; Sun et al., 1998; Saville et al., 2000). The role of ER α /Sp1 in activation of CAD gene expression was further supported by the inhibitory effects of both dominant negative Sp1 and siRNA for Sp1 (Figure 45). Studies in this laboratory have demonstrated that iSp1 selectively decreases Sp1 protein in MCF-7 and ZR-75 cells and blocks basal and hormone-induced transactivation in cells transfected with a GC-rich (pSp1₃) construct (Abdelrahim et al., 2002). The pattern of responses for

activation/inactivation of pSp1₃ (Abdelrahim et al., 2002) and pCAD1 in MCF-7 cells cotransfected with iSp1 were identical (Figure 45), thus confirming that Sp1 protein is required for hormone-induced transactivation in cells transfected with pCAD1. Promoter analysis has demonstrated that many genes activated through genomic ER α /Sp1 are also coordinately upregulated via nongenomic pathways of E2 action. For example c-fos, cyclinD1, E2F-1 and bcl2 are regulated by hormone activation of cAMP, phosphatidylinositol-3-kinase (PI3-K), and mitogen-activated protein kinase (MAPK) pathways (Dong et al., 1999; Castro-Rivera et al., 2001; Duan et al., 2001; Ngwenya and Safe, 2003). In this study treatment of MCF-7 or ZR-75 cells with E2 alone did not induce pCAD1 reporter gene activity suggesting that nongenomic hormonal activation of kinases may not be required for transactivation of the growth-responsive CAD gene promoter (Figure 34).

Farnham and coworkers have proposed that the mechanism by which Myc activates the CAD promoter in NIH3T3 cells is via recruitment of P-TEFb which stimulates elongation by phosphorylating the CTD domain of RNAPII. This allows the release of a paused RNAP II and subsequent transcription elongation (Figure 64). In this study we show that Sp1 protein interacts with the GC-rich motifs within the CAD gene promoter (Figure 41-42), and further analysis by ChIP confirms interaction of both ER α and Sp1 with the proximal region of the CAD promoter (Figure 46). ChIP has previously shown that ER α and Sp1 proteins also bind GC-rich regions of other E2-responsive genes (Castro-Rivera et al., 2001; Wang and Hankinson, 2002), and we are currently investigating the temporal interactions of ER α , Sp1, and other cofactors with their

respective GC-rich motifs. On the basis of the results presented in this study we propose a model for hormonal regulation of CAD gene expression in breast cancer cells (Figure 65). We suggest that unliganded ER may associate with Sp1 in the absence of ligand. Upon binding E2, ER α undergoes a conformational change and interacts with not only DNA-bound -Sp1 proteins but also with other nuclear proteins. For example, cofactors such as HATs, chromatin remodeling complexes and mediator complexes (DRIP205) are then recruited and this results in transcriptional activation of the CAD gene.

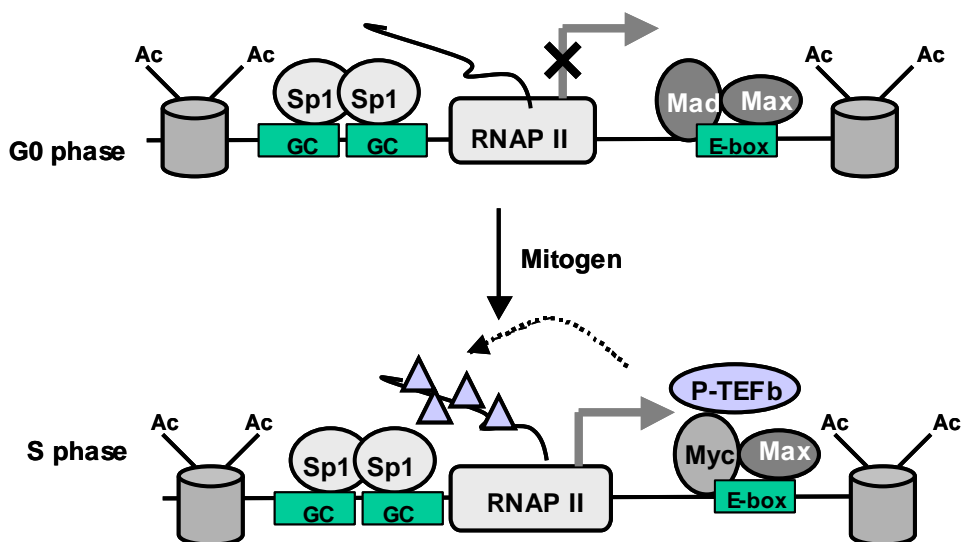


Figure 64. Model for Myc-mediated transcriptional activation of CAD gene expression in NIH 3T3 cells. In G0 phase, Mad/Max, Sp1, and RNAP II are bound to the CAD promoter and the nucleosomes have high levels of acetylated histones. In S phase, Myc/Max is bound to the CAD promoter and recruits P-TEFb, which phosphorylates the RNAP II CTD and allows elongation of CAD transcripts (Eberhardy., et al, 2000).

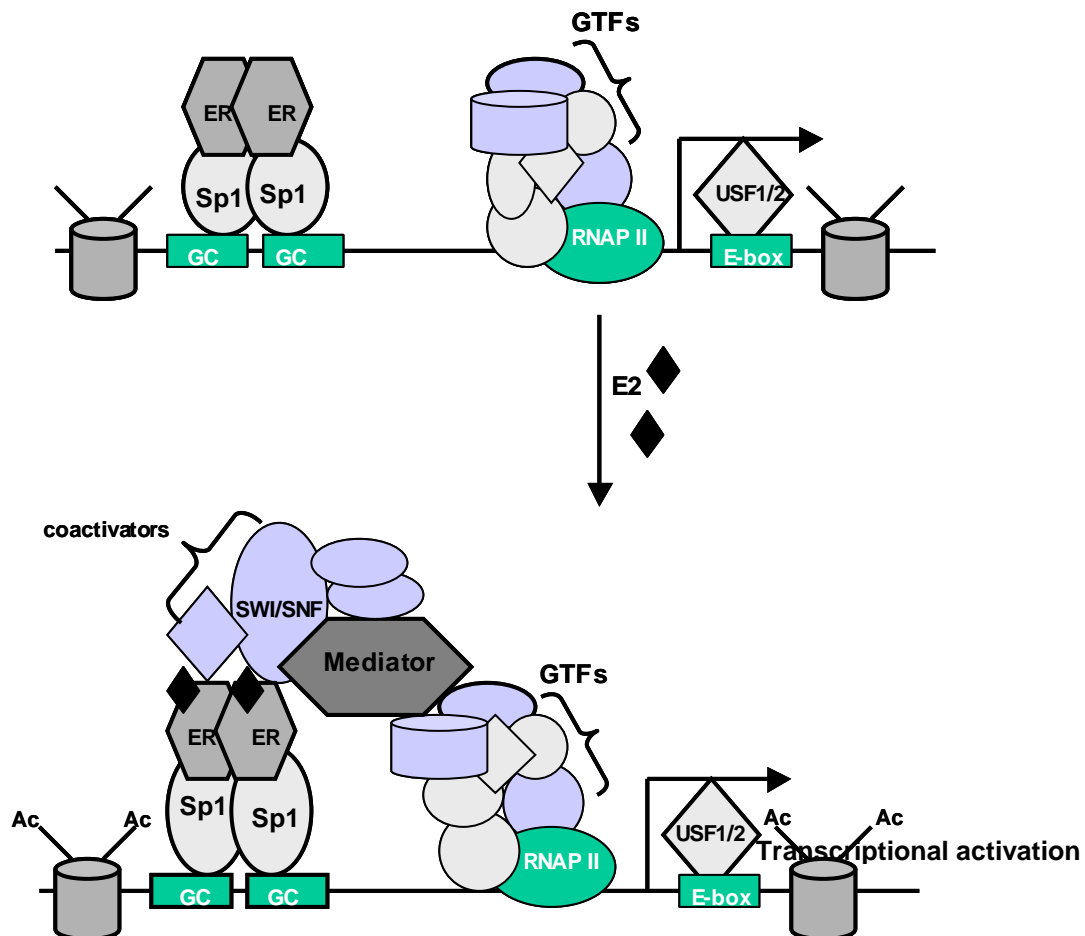


Figure 65. Hormone-induced transcriptional activation of CAD gene involves ER α /Sp1 in MCF-7 and ZR-75 cells. In the absence of hormone, Sp1 proteins are bound to upstream GC-rich sites in the CAD gene promoter. Upon ligand binding ER forms a homodimer and interacts with GC-rich bound Sp1 proteins. Coactivators and mediator complexes are subsequently recruited and this results in transcriptional activation of CAD gene.

In summary, results of this study have demonstrated that the reported hormone-dependent increase in CAD activity in breast cancer cells (Aitken and Lippman, 1983) is accompanied by induced gene expression (Figure 32) that is linked to ER α /Sp1 interactions with GC-rich motifs. Many of the genes regulated by ER α /Sp1 in ER-positive breast cancer cells play a role in purine/pyrimidine biosynthesis (CAD, thymidylate synthase) and metabolism (adenosine deaminase) and cell proliferation (cyclin D1, E2F1, *c-fos*, and *bcl-2*). These observations are consistent with the report showing that siRNA for Sp1 inhibits hormone-induced cell cycle progression in MCF-7 cells (Abdelrahim et al., 2002).

Activation of ER α through interaction with ERE motifs is primarily AF2 dependent, whereas previous studies in this laboratory have shown that ER/Sp1 depends, in part, on the AF1 domain of ER α (Saville et al., 2000). Coactivators such as SRCs that interact with ER α and other NRs through their LXXLL motifs (NR boxes) do not coactivate ER α /Sp1 in breast cancer cells (Kim et al., 2003). Their predominant effects on ER α /Sp1 are inhibitory and resemble the action of corepressors. Research in this laboratory has identified several mediator complex proteins, such as vitamin D interacting protein 205 (DRIP 205), DRIP 150 and DRIP 130, and the RING protein SNURF, as coactivators of ER α /Sp1 and their mechanisms of coactivation are currently being investigated. Current studies are investigating molecular mechanisms of ER α /Sp1 action and coactivator/coregulatory proteins required for this hormone-regulated pathway.

Graves et al. (2000) reported that CAD is phosphorylated at Thr-456 in the CPS II domain by MAPK in response to growth factors such as EGF and PDGF in vivo which relieves UTP inhibition and stimulates PRPP activation of CPSase II resulting in increased rate of pyrimidine biosynthesis. We hypothesize that estrogen will affect CAD phosphorylation in a manner similar to that of EGF and PDGF and this may result in an increased rate of pyrimidine biosynthesis by increasing CPS II activity. Future studies will investigate the effects of E2 on phosphorylation of CAD and the role of kinase pathways in regulation of CAD protein in breast cancer cells.

Research in this laboratory has recently identified other Sp proteins such as Sp3 and Sp4 that could play an important role in regulation of GC-rich gene promoters. For example E2-induced transactivation of a VEGF promoter construct containing proximal GC-rich motifs was decreased in ZR-75 cells by silencing Sp1 or Sp3. These results suggested that E2-induced transactivation of VEGF in ZR-75 cells requires both ER α /Sp1 and ER α /Sp3 (Stoner et al., 2004). Preliminary studies in MCF-7 cells and ZR-75 cells using siRNA for Sp3 and Sp4 show that both basal and E2-induced transactivation of CAD gene promoter is decreased by silencing Sp3 and Sp4 suggesting that Sp3 and Sp4 may play a critical role in regulation of CAD gene expression. Recent studies in this lab (Wu F., unpublished results) show that the functional role of Sp1, Sp3 and Sp4 varies with ligand-, cell- and promoter context and current studies are further investigating the role of Sp3 and Sp4 in basal and E2-induced transactivation of CAD and other E2-responsive GC-rich gene promoters in breast cancer cells.

4.2 Molecular Mechanisms of Inhibitory AhR-ER α /Sp1 Crosstalk in Breast Cancer Cells

The classical mechanism of E2-dependent induction of gene expression involves initial formation of a liganded nuclear ER homodimer which binds to consensus or nonconsensus EREs in hormone-responsive gene promoters (Kumar and Chambon, 1988; O'Malley, 2005). The discovery of ER β as a second ER subtype suggested that in some cell contexts ER β /ER α heterodimers may also be functional since both proteins can interact in *in vitro* assays (Cowley et al., 1997; Pace et al., 1997). There is also evidence that one or more ERE half-sites alone or in cooperation with other transcription factors such as Sp1 are functional hormone-responsive motifs, and E2 also activates gene expression through interaction of ER with other DNA-bound transcription factors such as AP-1 and Sp1 (Paech et al., 1997; Safe and Kim, 2004). Research in this laboratory has identified several genes involved in nucleotide synthesis and proliferation of breast cancer cells that are activated through ER α /Sp1-mediated pathways (Safe and Kim, 2004).

Hormone-induced transactivation can be inhibited by antiestrogens and also through crosstalk with other ligand-activated receptors including the retinoic acid, vitamin D and Ah receptors (Eisman et al., 1987; Muller et al., 2002; Safe and Wormke, 2003). Research in this laboratory has identified SAhRMs that are highly effective as inhibitors of rodent mammary tumor growth (Safe 2005), and we have also focused on determining the mechanisms of inhibitory AhR-ER α crosstalk (Safe and Wormke, 2003). The mechanism underlying AhR/ER crosstalk is complex and may involve interactions

of AhR with iDREs in promoters of E2 responsive genes, proteasome dependent degradation of ER and induction of inhibitory factors such as HES-1. In this study, we used the E2-responsive CAD gene and CAD gene promoter constructs to investigate AhR-ER α /Sp1 interactions in ZR-75 and MCF-7 cells.

TCDD inhibited induction of CAD mRNA levels by E2 (Figure 47) and reporter gene activity induced by E2 in cells transfected with pCAD constructs was also inhibited by TCDD (Figure 48 and Figure 50) suggesting that inhibitory AhR/ER crosstalk is functional on the CAD gene promoter. Previous studies have identified functional iDREs (CACGC) in promoters of four E2-responsive genes (heat shock protein 27, cathepsin D, c-fos and pS2) (Krishnan et al., 1995; Gillesby et al., 1997; Duan et al., 1999; Porter et al., 2001). However the mechanisms of inhibition are gene promoter specific (Figure 66). For example constructs containing the upstream GC(N)₁₉ERE1/2 motif of the cathepsin D gene promoter that binds ER/Sp1 were activated by E2 in reporter assays, and these induced responses were inhibited by TCDD. This region of the cathepsin D promoter also contains an iDRE between the ERE1/2 and Sp1 sites. TCDD-mediated inhibition was not observed using a cathepsin D promoter GC(N)₁₉ERE1/2 construct with a DRE mutation. Gel mobility shift assays using the GC(N)₁₉ERE1/2 construct showed that the core DRE was targeted by the AhR complex and this interaction blocked formation of the transcriptionally active ER/Sp1 complex, resulting in inhibition of E2-induced transactivation (Krishnan et al., 1995).

E2-responsiveness of the pS2 gene was primarily associated with an AP-1 motif (-518 to -512), which overlaps an iDRE at -527 to -514. Thus, inhibitory AhR-ER cross-

talk on the pS2 gene promoter may be due to competitive interactions of the AhR and AP-1 complexes for binding to the same region of the promoter (Gillesby et al., 1997).

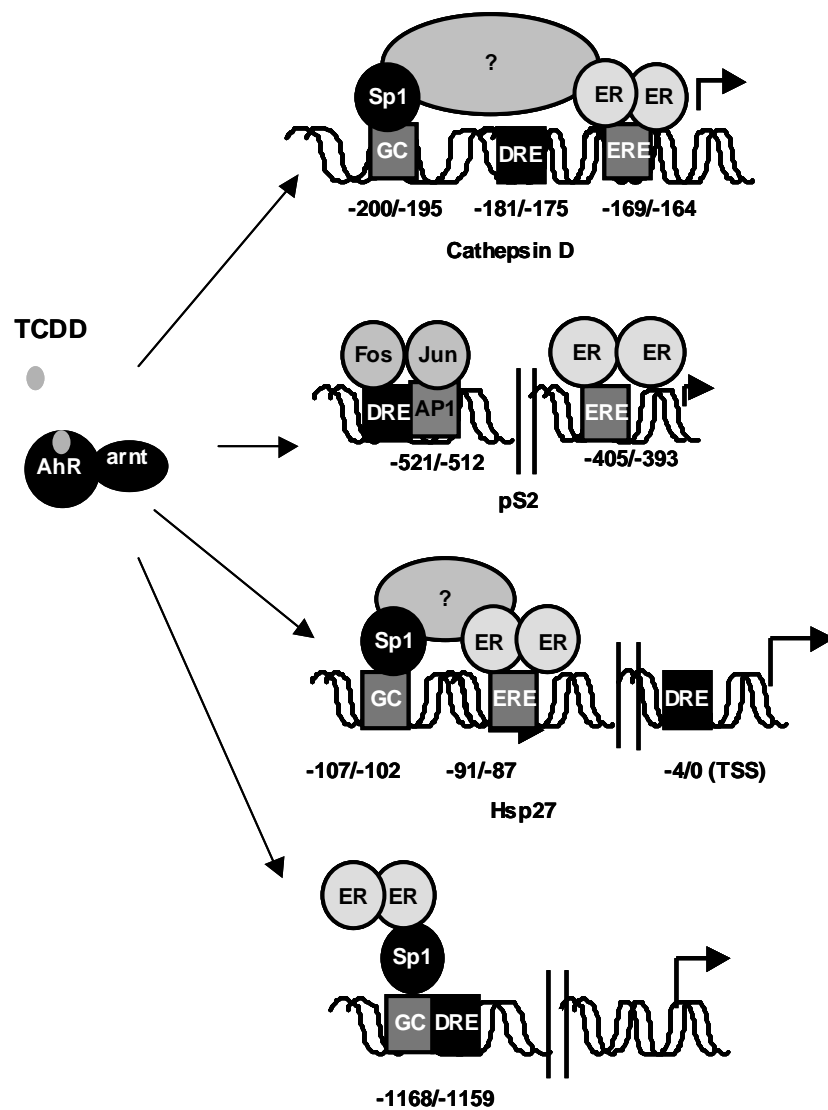


Figure 66. Functional iDREs in promoters of the ^{c-Fos}cathepsin D, c-fos, heat shock protein 27, and pS2 genes (Safe and Wormke, 2003).

A similar inhibitory interaction has been observed for the c-fos gene promoter that contains overlapping iDRE and GC-box motifs in the distal region of the promoter. Both the AhR and the ER/Sp1 complex may compete for the same binding site and this results in inhibition of E2-mediated transactivation (Duan et al., 1999). The functional iDRE in the Hsp27 promoter is located at the start site suggesting that the AhR complex may interfere with assembly of the basal transcription machinery (Porter et al., 2001). The CAD gene promoter has an iDRE located at -45 between the second and third Sp1 sites. However E2-mediated transactivation of the CAD gene promoter construct containing mutations in the iDRE was inhibited by TCDD in MCF-7 and ZR-75 cells, suggesting that the inhibitory AhR-ER α /Sp1 crosstalk was independent of the iDRE in the CAD gene promoter (Figure 53 and 54).

There is also evidence that low levels of ER α in breast cancer cells cotreated with E2 plus TCDD may be limiting and thereby inhibit expression of E2-responsive genes (Wormke et al., 2003). In this study it was not possible to determine whether the low levels of ER α in cells cotreated with E2 plus TCDD were limiting since the proteasome inhibitor MG132 itself directly decreased CAD mRNA levels (Figure 61). Thus, it is possible that decreased and possibly limiting ER α levels in MCF-7 and ZR-75 cells cotreated with E2 plus TCDD may contribute to the antiestrogenic effects of the latter compound.

Studies in this laboratory recently reported ligand-dependent interactions of ER α and Sp1 proteins in living MCF-7 and COS-1 cells using FRET (Kim et al., 2005), and these same constructs, and YFP-AhR (Figure 55) were used to investigate AhR-

ER α /Sp1 interactions. Direct interactions of AhR and Sp1 using the FRET assay were not observed due to the high molecular weights of both proteins. However a previous study has shown that both proteins directly interact. However, E2, TCDD and E2 plus TCDD increased FRET efficiency in MCF-7 and COS-1 cells transfected with CFP-ER and YFP-AhR (Figures 57 and 58). Moreover, these same treatments also enhanced colocalization of CFP-ER and YFP-AhR in MCF-7 cells, confirming that ligand-dependent interactions of ER α and AhR which have also been reported in other studies (Klinge et al., 1999; Ohtake et al., 2003; Wormke et al., 2003).

The molecular mechanisms of inhibitory AhR-ER α /Sp1 on GC-rich E2-responsive promoter such as CAD could involve several pathways which may be solely responsible for or contribute to this response. The AhR interacts not only with ER α but also Sp1 (Kobayashi et al., 1996) proteins. Therefore, AhR could repress ER α /Sp1 through formation of a transcriptionally-inactive AhR/ER α /Sp1 complex or squelch ER α /Sp1-mediated transactivation through competitively displacing ER α from binding Sp1 and forming an AhR/ER α complex. This latter possibility is supported by recent studies showing that in cells cotreated with E2 plus TCDD, there is an increased formation of AhR/ER α bound to promoter regions of Ah-responsive genes such as CYP1A1 (Beischlag and Perdew, 2005; Matthews et al., 2005). Since direct interaction of chimeric Sp1 and AhR interaction could not be determined by FRET due to the high molecular weights of both proteins, we investigated the effects of TCDD on ER α -Sp1 interactions in living cells by FRET (Figure 59). Compared to cells treated with DMSO or TCDD alone, treatment with E2 alone significantly increase FRET efficiency and this

enhanced interaction of ER α and Sp1 was totally blocked in cells cotreated with E2 plus TCDD (Figure 59). Disruption of hormone-activated ER α /Sp1 by the liganded AhR complex in living cells (Figure 59) correlated with inhibition of ER α /Sp1-mediated activation of CAD gene/reporter gene expression (Figures 47-54). Thus, in cells cotreated with E2 plus TCDD, there was increased association of AhR and ER α (Figure 60) and this was coupled with decreased association of ER α /Sp1 based on FRET efficiencies.

The ligand-dependent retention and/or loss of these transcription factors on the CAD gene promoter was further investigated in a ChIP assay which compared protein-DNA interactions on the CAD and CYP1A1 gene promoters after treatment with E2 plus TCDD (Figure 62A) (in collaboration with Shenxi Liu). In ZR-75 cell lines, treatment with E2 plus TCDD decreased ER α and increased AhR interactions with the CAD gene promoter, and this was accompanied by increased association of AhR, Arnt and ER α with the Ah-responsive region of the CYP1A1 promoter (Figure 62B). Similar results were obtained in MCF-7 cells (Liu, unpublished results). These results suggest that the mechanism of inhibitory AhR-ER α /Sp1 crosstalk in MCF-7 and ZR-75 cells cotreated with E2 plus TCDD results in loss of ER α bound to GC-rich E2-responsive promoter regions (in the CAD gene) and recruitment of ER α to the Ah-responsive region of the CYP1A1 gene promoter. Presumably ER α /AhR complexes are also formed on promoters of other Ah-responsive genes. Previous studies in this laboratory reported that E2 did not affect Ah-responsiveness (i.e. CYP1A1 inducibility by TCDD) in MCF-7

cells (Hoivik et al., 1997), and recent studies reported that E2 either enhanced (Matthews et al., 2005) or suppressed CYP1A1 inducibility (Beischlag and Perdew, 2005). Our results indicate that E2 (100 nM) decreased 2 nM TCDD -induced transactivation in MCF-7 and ZR-75 cells transfected with pDRE₃ (Figure 63). Currently we are investigating the effects of E2 on TCDD-induced CYP1A1 mRNA and protein to re-examine whether ER α modulates Ah-responsiveness in MCF-7 cells and ZR-75 cells.

In summary, these results demonstrate that inhibitory AhR-ER α /Sp1 crosstalk on the CAD gene promoter in breast cancer cells cotreated with E2 plus TCDD involves decreased ER α /Sp1 interactions as determined by FRET analysis. In addition, the loss of ER α from the CAD gene promoter is due, in part, to recruitment of ER α to AhR-responsive promoters (e.g. CYP1A1) and formation of ER α /AhR complexes. Thus, inhibitory AhR-ER α /Sp1 crosstalk involves competitive displacement of ER α from the ER α /Sp1 complex by the ligand-activated AhR complex (Figure 67) and current studies are investigating the validity of this model for other ER α /Sp1-regulated genes in breast cancer cells. In addition, we are also investigating the molecular mechanisms underlying the antiestrogenic actions of SAhRMs in breast cancer cells.

TCDD has been shown to activate/inhibit multiple kinase pathways in various cell lines. For example TCDD activates tyrosine kinases such as c-Src kinase in MCF10A mammary epithelial cells and this has been implicated in the inhibitory effects of TCDD on insulin signaling (Mazina et al., 2004; Park et al., 2004).

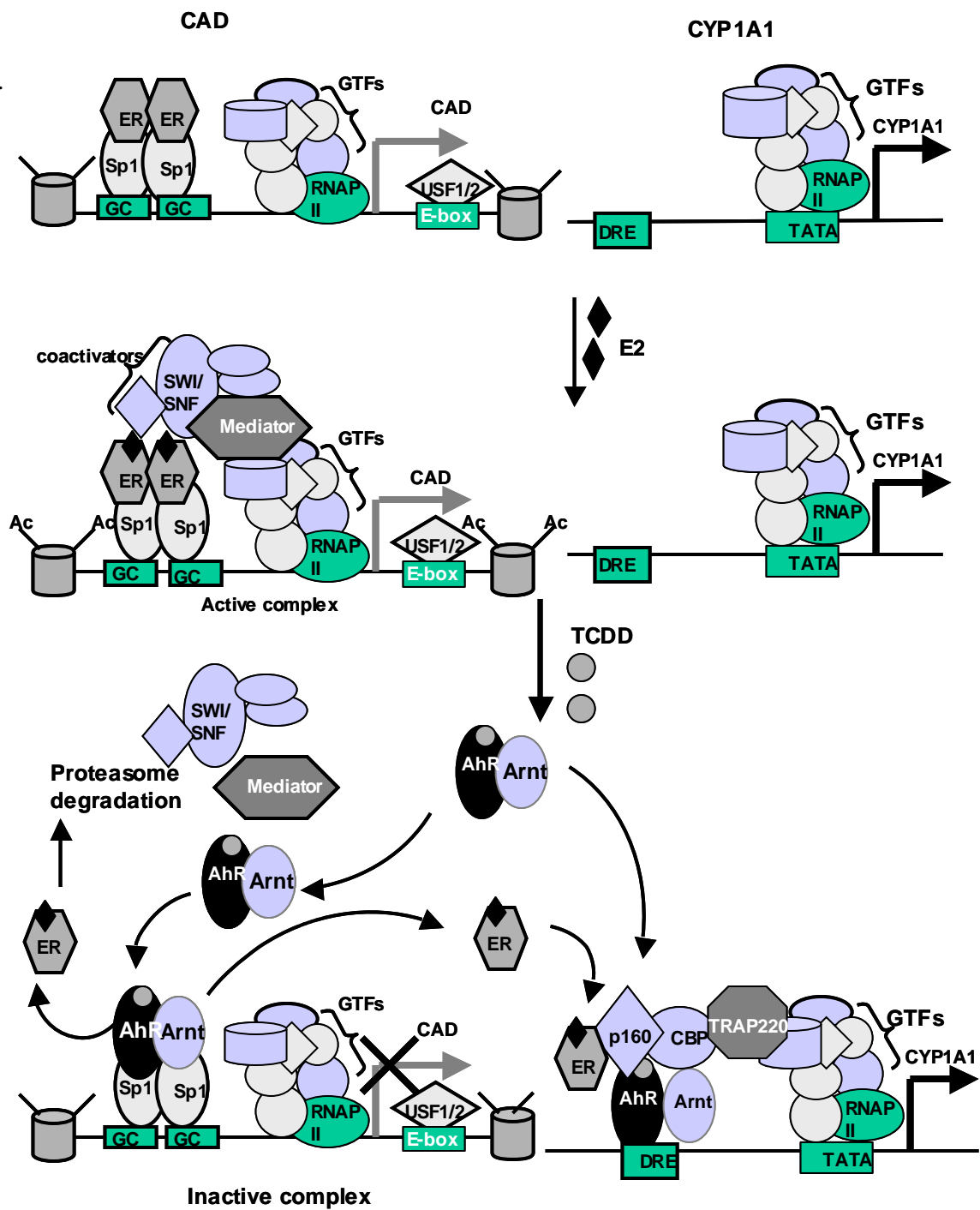


Figure 67. Proposed model for inhibitory AhR-ER/Sp1 crosstalk on CAD gene promoter.

TCDD also inhibits E2-dependent activation of cAMP/PKA which results in the failure to activate NFY proteins and subsequently inhibits E2-mediated transactivation of the E2F1 gene promoter containing the NFY sites in ZR-75 cells (Ngwenya., et al unpublished results). Future studies will investigate the effects of TCDD on non-genomic actions of E2 that are mediated by the membrane/cell surface ER and the role of kinase pathways in inhibitory AhR/ER crosstalk.

In summary the results of this research have delineated several important mechanistic aspects of estrogen-induced CAD gene expression and of inhibitory AhR-ER α /Sp1 crosstalk and these are summarized below.

1. The reported hormone-dependent increase in CAD activity in breast cancer cells is also accompanied by induced CAD mRNA levels.
2. Promoter analysis showed that E-box motifs are not essential for basal or hormone-induced transactivation of CAD gene promoter constructs in MCF-7 and ZR-75 cells. GC-box 2 at -47 is the most essential element for high basal activity of CAD promoter construct in MCF-7 and ZR-75 cells.
3. E2-responsiveness is primarily associated with GC-boxes 1 and 2 (at -67 and -47 respectively) in MCF-7 and ZR-75 cells and hormone-induced transactivation of CAD gene promoter does not require the DNA binding domain of ER α in MCF-7 or ZR-75 cells.
4. Sp1 protein is required for both basal and E2-induced transactivation of CAD gene promoter construct and hormone-induced CAD gene expression is linked to

ER α /Sp1 interactions with the GC-rich motifs in the CAD gene promoter in MCF-7 and ZR-75 cells.

5. The inhibitory AhR/ER crosstalk is functional on CAD gene promoter in MCF-7 and ZR-75 cells and is iDRE-independent.
6. Results of this study demonstrate for the first time that AhR and ER α interact in living MCF-7 and COS-1 cells and this interaction between AhR and ER α is enhanced in presence of E2 plus TCDD. Inhibitory AhR-ER α /Sp1 crosstalk in breast cancer cells cotreated with E2 plus TCDD involves decreased ER α /Sp1 interactions as determined by FRET analysis. CHIP data suggests that the loss of ER α from the CAD gene promoter may involve recruitment of ER α to AhR-responsive promoters (e.g. CYP1A1) and formation of ER α /AhR complexes. However additional CHIP experiments are warranted to confirm the loss of ER α from the CAD gene promoter. Thus, inhibitory AhR-ER α /Sp1 crosstalk may involve competitive displacement of ER α from the ER α /Sp1 complex by the ligand-activated AhR complex or may result in formation of inactive AhR-ER α /Sp1 complex.

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