FUNCTIONAL DIFFERENCES BETWEEN ESTROGEN RECEPTORS ALPHA AND BETA IN A RAT FIBROBLAST CELL LINE

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LIST OF ABBREVIATIONS

ACTR	activator of the thyroid and retinoic acid receptor
AF-1	transcriptional activation function at A/B domain
AF-2	transcriptional activation function at LBD
AhR	aryl hydrocarbon receptor
AIB1	amplified in breast cancer 1
AP-1	activator protein-1
ARNT	AhR nuclear translocator
ATF	activating transcription factor
8-Br-cAMP	8-Bromo-cyclic AMP
CaM	calcium-calmodulin
cAMP	cyclic AMP
CAT	chloromphenicol acetyltransferase
CBP	CREB binding protein
CBP/p300	CBP and related p300 proteins
CMV	human cytomegalovirus immediate early promoter
CRE	cAMP response element
CREB	CRE binding protein
CREM	CRE modifier
CS-FBS	dextran-charcoal stripped FBS

CT	cholera toxin
DBD	DNA binding domain
DBSF	DNA binding stimulatory factor
DMEM	Dulbecco's modified Eagle's medium
DRE	dioxin-responsive element
E_2	estradiol-17β
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
ER	single estrogen receptor
ERα	ER-alpha
ERβ	ER-beta
ERE	estrogen response element
ERK	extracellular signal-regulated kinase
αERKO	ERα knockout
βERKO	ERβ knockout
FBS	fetal bovine serum
FRK	fos-regulating kinase
GAL4 DBD	GAL4 DNA binding domain
GAL4 TA	GAL4 transcription activation
G protein	GTP-binding protein
GR	glucocorticoid receptor
GRE	glucocorticoid response element
Grb2	growth factor receptor binding protein-2

G_3PDH	glyceraldehyde-3-phosphate dehydrogenase
GRIP	glucocorticoid receptor-interacting protein
GTFs	general transcription factors
HAT	histone acetytransferase
HBSS	Hanks' buffered salt solution
HDACs	histone deacetylases
HSV-tk	thymidine kinase promoter from herpes simplex virus
hMTIIa	human metallothionein
Hsp90	90-kDa heat shock protein 90
4HT	4-hydroxytamoxifen
IBMX	isobutylmethylxanthine
INR	initiator
IP3	inositol trisphosphate
IR	insulin receptor
IRS	IR substrate
JNK	Jun NH_2-terminal kinase
k_a	association constant
k_d	dissociation constants
LAD	left anterior descending coronary artery
LBD	ligand binding domain
MAPK	mitogen-activated protein kinase
MAPKK	MAPK kinase
MAPKKK	MAPKK kinase

mSOS	son of sevenless
MTT	(3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide
NcoA	nuclear receptor co-activator
NCoR	nuclear receptor co-repressor
p140	140 kDa proteins
p160	160 kDa proteins
p300	300 kDa proteins
pCAF	CBP/p300 associated factor
p/CIP	CBP/p300 co-integrator associated protein
PIC	preinitiation complex
PI-3	phosphotidylinositol-3' kinase
PKA	protein kinase A
PKC	protein kinase C
PLCγ	phospholipase Cγ
PR	progesterone receptors
RAC3	receptor-associated co-activator 3
RARα	retinoic acid receptor α
Rat-1 cells	a rat embryo fibroblast cell line
RIP-140	receptor interacting-protein
RNAP	RNA polymerase
RNAPII	RNAP for class II genes
RTK	the receptor tyrosine kinase
RT-PCR	reverse-transcription polymerase chain-reaction

RXR	retinoid X receptor
SERMs	selective estrogen receptor modulators
SMC	smooth muscle cells
SMRT	silencing mediator for RXR and TR
SRC-1	steroid receptor coactivator 1
STAT	signal transducer and activator of transcription
TAE	tris-acetate (40 mM), EDTA (1 mM)
TAFs	TBP-associated factors
TBP	TATA-binding protein
TCDD	2,3,7,8-tetrachlorodibenzo-p-dioxin,
TFII	general transcription factors for class II genes
THC	tetrahydrochrysene
TIF-2	transcriptional intermediary factor 2
TK	thymidine kinase
TOT	trans-hydroxytamoxifen
TPA	12-O-tetradecanoylphorbol-13-acetate
TR	thyroid hormone receptor
TRAM-1	TR activator molecule
TRE	TPA response element
TRE	thyroid hormone response element
UBF120	cells from uterus of the bovine fetus at 110-120 gestation days
UBF180	cells from uterus of the bovine fetus at 180-200 gestation days
wt	wild-type

CHAPTER I

INTRODUCTION

Estrogen exhibits multiple functions in various physiological processes in animals. Its targets include the reproductive organs, mammary glands, cardiovascular system, central nervous system, and bone.

For thirty years estrogen action was thought to be mediated by single estrogen receptor alpha (ERα). In 1995, a second ER, called ER beta (ERβ), was discovered. ERα and ERβ have remarkable similarity, exhibiting similar binding affinity to estradiol and high homology in their DNA and ligand binding domains (Kuiper et al, 1996 and 1997). On the other hand, they have both overlapping and unique tissue distribution patterns, where ERα and ERβ are approximately equally distributed in some tissues while one of the ER subtypes is predominant in other tissues (Kuiper et al, 1997). Characterization of the functional properties and determination of similarities and differences in these two receptors will allow us better understanding of how estrogen acts to regulate the numerous physiological processes in which it participates.

The objectives for this study were: 1) to establish stable, ectopic expression of ERβ in a rat fibroblast cell line (rat-1 cells), 2) to compare the effects of estrogen in rat-1+ERβ cells with rat-1+ERα (which stably express ectopic ERα) and rat-1 cells (which do not express ER) on transiently-transfected receptor gene activation and on selected

endogenous promoters as a model for the roles of ER in developmental processes, 3) to examine the effect of promoter context on estrogen action by comparing estrogen response element-mediated estrogen action and AP-1-mediated estrogen action, and 4) to examine the relationship between estrogen action and insulin in the context of receptor type, ERα or ERβ, and promoter context by comparing estrogen response element- and AP-1-mediated gene activation.

CHAPTER II

REVIEW OF LITERATURE

Introduction

The estrogen receptor (ER) is a member of the steroid receptor superfamily. It plays crucial roles in many physiological processes. Within an estrogen target cell, ER acts as a ligand-induced transcription factor, which interacts with the basal transcription machinery resulting in an alteration of the transcription rate of the target gene. The purpose of this review is to summarize the recently published information on ER, so as to create a focused picture of the mechanism of the transcriptional activation function of ER.

Transcription factors and control of gene expression

In eukaryotes, there are three types of RNA polymerase (RNAP): i.e. RNAPI, RNAPII, and RNAPIII. Genes transcribed by the cognate RNAP are called class I, II and III genes, respectively. Each RNA polymerase has its own set of transcription factors. RNA polymerase II (RNAPII) is responsible for synthesizing the mRNA precursors that result in the expression of the protein coding regions of the genome. In

this review, we focus only on RNAPII and class II gene related transcription factors and transcriptional activation.

A transcription factor is defined as "a regulatory molecule with a direct effect on transcription whose substrate is another macromolecule and not a small molecule" (Ouzounis and Papavassiliou, 1997). One feature that is common to all transcription factors is a DNA binding domain, although direct binding to DNA is not always necessary for the transcription factor to regulate gene expression. The transcription factor exerts its influence on gene expression by either direct binding to DNA or using another transcription factor as a bridge, thus exhibiting indirect binding to DNA.

All known transcription factors are proteins (Ouzounis and Papavassiliou, 1997). Transcription factors for class II genes generally fall into three classes: general transcription factors (GTFs), upstream transcription factors, and inducible transcription factors (Lewin, 1994). The first class of transcription factors is responsible for forming the initial transcription complex at the transcription initiation site of a target promoter. The result of the action of this complex is termed a basal transcription rate. The second class of transcription factors is responsible for binding to a site that is upstream of a transcription start site, where they interact with the basal transcription machinery thus altering the transcription level. The synthesis of the second class of transcription factors is well regulated, thus their presence varies within specific cells or tissues (Voet and Voet, 1995). However, their binding to a target is not regulated and depends only on the availability of the target site, thus they are constitutively active (Latchman, 1998). The third class of transcription factors has a large number of members. The binding of these transcription factors to the target is well controlled. They must be activated before they

are able to modulate target gene expression (Voet and Voet, 1995). The synthesis and activation of various members of this class of transcription factors may occur in response to hormones, growth factors, cytokines, antigens, oxidants, and ultraviolet light.

RNAPII is not able to initiate transcription by itself; instead, it requires the presence of GTFs (Lewin, 1994). The first step in the transcription initiation process is the association of RNAPII with GTFs to form the preinitiation complex (PIC). General transcription factors for class II genes include TFIIA, TFIIB, TFIID, TFIIE, TFIIF, TFIIH, and TFIIJ (Orphanides *et al*, 1996). TFIID has two major components: a TATA-binding protein (TBP) and at least eight tightly associated TBP-associated factors (TAFs) (Verrijzer and Tjian, 1996). The formation of the PIC goes as follows: first, TBP either recognizes and binds to the TATA box of the promoter of a class II gene, or (in the case of TATA-less promoter), TBP binds to a site called initiator (INR) of the promoter (Pugh and Tjian, 1991). Second, TAFs join to TBP to form the TFIID complex (Figure 1-a). Next, TFIIA joins to the TFIID complex (Figure 1-b). Then, acting as a bridge factor (Buratowski, 1989) TFIIB binds to the complex and recruits RNAPII, which was previously associated with TFIIF (Figure 1-c). Thus, TFIIB plays an important role in bringing the polymerase to the transcription initiation site. Finally, after RNAPII has associated with the complex, the TFIIE, TFIIH, and TFIIJ transcription factors bind, in that order, to form a large preinitiation complex (PIC) (Figure 1-d). The PIC may initiate transcription at a very low level, i.e., at the basal transcription rate (Burley and Roeder, 1996). Hence, PIC is also called the basal transcription machinery or apparatus (Lewin, 1994).

The basal transcription rate may be increased or decreased by upstream transcription factors. Sp1 is one example of this class of transcription factors. Its binding site is a GC box, which has the consensus sequence GGGCGGG and is located upstream of the transcription start site (Letovsky and Dynan, 1989). Sp1 has a DNA binding domain consisting of three zinc fingers. Sp1 binds to the GC-box, where it may interact with the PIC resulting in constitutive transcriptional activation. Sp1 is present in all cell types, but the concentration of Sp1 can vary 100-fold in different cell types and at various stages in development (Saffer *et al*, 1990; Saffer *et al*, 1991).

Using a knockout mouse model, Marin *et al* (1997) found that an Sp1-null embryo develops at a much slower rate than a wild type embryo and only survives through 9.5 days of gestation. Moreover, the Sp1-null embryo exhibits broad developmental abnormalities. Based upon these observations, Marin *et al* (1997) concluded that Sp1 was essential for early embryonic development.

Sp1 may also exhibit physical and/or functional interactions with other transcription factors to generate more complex patterns of regulation of gene expression. For instance, Gualberto and Baldwin (1995) reported that activation of an HIV-1 promoter required cooperative interaction between host cell proteins Sp1 and p53. Other evidence indicated that an interaction between Sp1 and E2F-1 affected the transcription of the mouse thymidine kinase gene (Karlseder *et al,*1996) and hamster dihydrofolate reductase gene (Lin *et al,* 1996). Furthermore, Sp1 was also found to physically and functionally interact with steroid receptors (Porter *et al*, 1997; Wang *et al*, 1998). This point is discussed in detail later in this review.

The basal transcription level can also be altered by the class of inducible transcription factors. Since this class of transcription factors is the largest group among the three classes, the next few paragraphs of this review address the CREB and AP-1 transcription factors as examples; steroid receptors will be discussed in more detail in a later section.

The transcription factor CREB is a cAMP response element (CRE) binding protein. The DNA binding domain of CREB has a leucine zipper dimerization motif and binds as a dimer to the CRE (5'-TGACGTCA-3') of cAMP inducible genes (Montminy, 1997b). This binding does not depend on cAMP and does not increase the basal transcription level. The CREB protein is converted into its active form when serine^{133} is phosphorylated by PKA (protein kinase A), which must be previously activated by cAMP (McKnight *et al*, 1988), calcium-calmodulin (CaM) kinase II or CaM kinase IV (Meyer and Habener, 1993).

To activate transcription, CREB needs to associate with a coactivator, CREB binding protein (CBP). The phosphorylation of CREB on serine^{133} results in the conformational change that allows CREB to associate with the CBP. Nakajima *et al* (1997) determined that CBP was a part of the basal transcriptional complex. Hence, as an adapter between CREB and the basal transcription complex, CBP plays an important role in CREB related transcription activation. Furthermore, the relationship of CREB and CBP may be more complicated since CREB was found to form heterodimers with other transcription factors such as ATF-1 and CREM (CRE modifier) (Habener, 1990), and CBP was found to interact with many transcription factors and coactivators (Montminy, 1997a; Goldman *et al*, 1997).

Activator protein-1 (AP-1) was initially identified as a transcription factor that bound to a 5'-TGANTCA-3' palindromic sequence in the human metallothionein (hMTIIa) and collagenase genes. Binding of AP-1 to DNA was inducible by phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA), thus the AP-1 binding site was referred to as the TPA response element (TRE) (Lee *et al*, 1987). Since that time, a similar binding site has been found in a large number of genes and the TRE sequence was determined to be 5'-TGACTCA-3' (Karin *et al*, 1997). It is now known that AP-1 actually consists of dimers of members of several groups of proteins, including Fos (v-Fos, c-Fos, FosB, Fra1, Fra2), Jun (v-Jun, c-Jun, JunB, JunD), and activating transcription factor (ATF2, ATF3/LRF1, B-ATF) (Karin *et al*, 1997). These are all basic-region leucine zipper (bZIP) proteins, whose bZIP domain enables two molecules to form a dimer and bind to the DNA target site. Jun and ATF family members (but not Fos) form stable homodimers among themselves. Jun family members also heterodimerize with Fos- and ATF-family members.

Activity of the AP-1 dimer is regulated at two levels: abundance, which is controlled by regulating expression of bZIP proteins, and protein conformation, which controls the switching into the active form (Karin *et al*, 1995). Many stimuli are capable either of increasing AP-1 abundance or activity, including growth factors, interleukins, hormones, tumor promoting agents, alkylating agents, organometals and electromagnetic radiation (Karin *et al*, 1995). The mechanism for switching AP-1 to the transcriptionally active form is the phosphorylation of the AP-1 components by mitogen-activated protein kinase (MAPK). So far, three different types of MAPKs have been found to be responsible for phosphorylation of AP-1 in response to various stimuli.

Specifically, these are: JNK (Jun NH2-terminal kinase) (Darnell *et al*, 1994), FRK (Fos-regulating kinase) (Treisman, 1992), and ERK (extracellular signal-regulated kinase) (Gille *et al*, 1992). Because the AP-1 family members can be regulated by numerous stimuli, the AP-1 family members have the ability to affect basal transcription rate in a time and tissue specific manner depending on the availability of the AP-1 dimer partners. This gives the AP-1 family a great flexibility in regulating the transcription rate of target genes.

Another important factor in gene transcription is the local environment in which transcription occurs. Eukaryotic genomic DNA is surrounded by chromatin proteins. The fundamental repeating unit of chromatin is the nucleosome, a protein core wrapped twice by DNA (Wolffe, 1995). The protein core is composed of the histone octamer, i.e., two copies each of histone proteins H2A, H2B, H3 and H4. A single unit of histone H1 binds to DNA where DNA enters and exits the histone core resulting in stabilization of the nucleosome. By ordering and folding the nucleosome array, DNA is further compacted and condensed (Rhodes, 1997). The chromatin structure impedes the formation of the PIC (Archer *et al*, 1992) by physically hindering the binding of the transcription factors to the DNA. Genes that are transcriptionally active or potentially active are generally found in the most flexible nucleosome structure. Their promoter and transcription factor binding areas are either free of nucleosomes or are able to undergo a rearrangement of the nucleosome array which makes the DNA accessible to the transcription factors before the transcription process is initiated (van Holde *et al*, 1995; Ramakarishnan, 1997; Pruss *et al*, 1995; Grunstein, 1990; Kornberg and Lorch, 1992; Fletcher and Hansen, 1996). These observations have led to a theory of the

chromatin remodeling process, which asserts that there are two major elements important in allowing the activation of transcription to occur. The first is histone acetylation, and the second is histone deacetylation.

Histone acetylation takes place in the lysine residues of the histone proteins and causes a reduction of the net positive charge in the histone molecule resulting in a destabilization of the histone-DNA interaction of the nucleosome (Hong, *et al*, 1993). Histone deacetylation has the reverse effect. There is evidence that hyperacetylation of histones is correlated with transcriptional activity and that hypoacetylation is associated with transcriptional repression (Loidl *et al*, 1994; Turner *et al*, 1995). Recently, proteins having histone acetyltransferase activity have been identified participating in interactions with specific transcription factors. Other proteins have likewise been identified with deacetylase activity that participates in transcriptional repression (Pazin and Kadonaga, 1997b; Tsukiyama and Wu, 1997). Hence, the activation and repression of transcription can depend on whether a protein factor possessing histone acetylase activity or a corepressor possessing histone deacetylase activity is recruited by a particular transcription factor.

Another example of a protein complex which acts as a chromatin remodeling factor and plays an important role in allowing the activation of transcription is the SW1/SNF complex (Pazin and Kadonaga, 1997a). Possessing the activity of both ATPase and DNA helicase (Eisen *et al*, 1995) the SW1/SNF complex was shown to be able to disrupt chromatin structure (Owen-Hughes *et al*, 1996) and increase transcription factor binding to DNA (Wang *et al*, 1996). After the alteration of

chromatin structure occurs, the SW1/SNF complex dissociates from the chromatin and leaves the exposed binding site open to transcription factors.

In summary, there is a complex three-stage process that regulates the activation of transcription. One part of the process (i.e., chromatin remodeling) is responsible for making the DNA accessible to the transcription factor proteins. Next, the PIC is formed, which establishes the basal transcription level or rate. Lastly, factors in classes II and III influence the basal transcription rate.

Integration of cell surface signal transduction with gene transcription

GTP-binding protein (G protein) linked receptors are the largest family of cell surface receptors. These receptors contain an extracellular domain, an intracellular domain, and a trans-membrane domain that consists of seven membrane-spanning α-helices (Helper and Gillman, 1992). Generally possessing three subunits (termed α, β and γ), the G protein resides at the inner surface of cell membrane and has its γ-subunit connected with the membrane receptor (Fung *et al*, 1981). The α-subunit binds to GDP when the three subunits α, β and γ, are unified. Extracellular ligand binding to the cell surface receptor triggers G protein binding to the receptor and causes a conformational change resulting in GDP dissociation and GTP association with the α-subunit (Samama *et al*, 1993). Replacement of GDP by GTP causes a secondary conformational change that allows the α-subunit to bind to various effectors such as adenylyl cyclase and phospholipase Cβ (Lohse *et al*, 1992; Attramadal, 1992).

Various isoforms of the α-subunit allow for the diversity of possible signals. The inhibiting-isoform of the G protein α-subunit ($G_{\alpha i}$) inhibits adenylyl cyclase or phospholipase Cβ; whereas the stimulating-isoform of the α-subunit ($G_{\alpha s}$) activates these effectors. Cyclic AMP (cAMP), the product of adenylyl cyclase, then activates the cAMP-dependent protein kinase A (PKA) (McKnight *et al*, 1988), which, in turn, phosphorylates the transcription factor CREB so as to trigger transcriptional regulation. IP3 (inositol trisphosphate), which is the product of phospholipase Cβ, increases the intracellular Ca^{++} concentration. Also, calcium may activate other effector proteins such as PKC, calmodulin, and CaM kinase II, which, in turn, are able to activate CREB (Birnbaumer, 1992).

The second-largest family of cell surface receptors is the receptor tyrosine kinase (RTK) family, which feature an intracellular domain possessing tyrosine kinase activity. This receptor family includes insulin receptor (IR) and epidermal growth factor receptor (EGFR). IR consists of two extracellular α-subunits and two intracellular β-subunits, which traverse the plasma membrane and exhibit tyrosine kinase activity in their cytosolic domains (Kahn and White, 1988). The β-subunits are linked by disulfide bonds to the α-subunits. EGFR has only a single polypeptide chain that includes both extracellular and intracellular domains. The intracellular domain of EGFR contains a tyrosine-specific protein kinase, which is similar to that of IR (Karlsson *et al*, 1979). Ligand binding to the receptor elicits a conformational change resulting in the dimerization and autophosphorylation of the receptor in the case of both the EGFR (Ueno *et al*, 1991; Kashles *et al*, 1991) and the IR (Treadway *et al*, 1992). These phosphorylated tyrosine residues serve as the docking sites for SH2 domain proteins.

SH2 domain proteins contain a pocket for phosphotyrosine (Pawson, 1992). EGFR may dock with growth factor receptor binding protein-2 (Grb2) (Pelicci *et al*, 1992) and IR may recruit both insulin receptor substrate (IRS) and Grb2 (White and Kahn, 1994). In addition, IRS and Grb2 are both able to dock other SH2 domain proteins that possess specific effector activity. For instance, IRS can recruit phosphotidylinositol-3' kinase (PI-3) resulting in accelerated glucose uptake (Saltiel, 1996). Grb-2 can dock with the mammalian homologue of Drosophila mSOS (son of sevenless) which converts Ras into its active form (GTP-bound form) (Myers and White, 1996). Ras then activates raf protein, also called mitogen-activated protein (MAP) kinase kinase kinase (MAPKKK). Once MAPKKK has been activated, it then phosphorylates MAP kinase kinase (MAPKK). Finally, MAPKK phosphorylates and activates MAP kinase (MAPK) (Elion, 1998). There are three major subfamilies of MAPK: ERKs (which activate transcription factors such as Elk and Ets), JNKs (which activate transcription factors such as C-jun (Elion, 1998)), and FRKs (which activate Fos). Another important effector is phospholipase Cγ (PLCγ) that may dock to either IRS or Grb-2 via its SH2 domain (Kim *et al*, 1991). PLCγ catalyzes the reaction that results in the release of IP3, which triggers Ca^{++} mediated signal transduction (Birnbaumer, 1992).

Steroid receptor superfamily

The steroid receptor superfamily is the largest subgroup of inducible transcription factors. The group consists of the receptors for the steroid hormones, thyroid hormone, vitamin D, retinoic acid, some oncogenes, and several orphan receptors for which the

specific ligands are unknown. Members of the steroid receptor superfamily are also referred as nuclear receptors because they exert their activities in the nucleus. However, in some instances, the term nuclear receptor is restricted to those receptors where the unliganded form is localized to the nucleus (Simons, 1998). These proteins all share a common domain structure (Carson-Jurica *et al*, 1990), which generally exhibits six functional domains called A through F beginning at the amino-terminus (Figure 2) (Kumar *et al*, 1987; Laudet *et al*, 1992).

Subtypes and structure of the estrogen receptor

For almost thirty years, the classic ER had been believed to be the only receptor to mediate estrogen action. Then in 1996, a second ER was cloned from multiple species (Kuiper *et al*, 1996; Mosselman *et al*, 1996; Tremblay, 1997). To distinguish them, the second ER was named ER-beta (ERβ) and the classic ER renamed ER-alpha (ERα). The two subtypes of ER share the common structural characteristics of the steroid receptor superfamily. Investigators reported that in the rat, ERα mRNA was expressed in mammary glands, skeletal muscle, ovary, uterus, testis, pituitary gland, liver, kidney, thyroid, epididymis, adrenal gland, and bone, while ERβ mRNA was found to be expressed in the prostate, ovary, uterus, testis, lung, bladder, brain, hypothalamus, thyroid, epididymis, adrenal gland, and bone (Shughrue, 1996; Byers *et al*, 1997; Kuiper *et al*, 1997; Couse *et al*, 1997; Arts *et al*, 1997; Brandenberge *et al*, 1998; Pau *et al*, 1998). ERα mRNA has also been found in rat coronary artery smooth muscle cells (Bei *et al* 1996). In their study, Dotzlaw *et al* (1996) found that about 70% of forty human

breast cancer samples had expressed ERβ mRNA. In addition, their study found that two previously characterized ER-positive and two ER-negative breast cancer cell lines also expressed ERβ mRNA.

The amino-terminal domain (A/B domain) is the least conserved portion between the ER subtypes. In the rat, ERβ has a much shorter A/B domain than ERα. A transcriptional activation function (AF-1) is located within the A/B domain. The activity of AF-1 is independent of ligand binding and exhibits cell type specificity (Tora *et al*, 1989).

The C-domain, or DNA binding domain (DBD), is the most conserved region among the members of the steroid receptor superfamily. There is a 95% identity in the DBD between rat ERα and β (Kuiper *et al*, 1996). Similarly, there is 96% identity in the DBD between human ERα and β (Tremblay, 1997). The most conserved portion of the DBD, (called the core region) contains about 70 amino acids including two zinc-stabilized peptide loops that are termed "zinc fingers". Each zinc molecule is coordinated between sulfur residues of four cysteines (Schwabe *et al*, 1990; Schwabe *et al*, 1993). The ER zinc finger motif is responsible for locating the correct DNA binding site, called the estrogen response element (ERE). All members of the steroid receptor superfamily have similar zinc finger motifs in the DNA binding domain. The specificity of the DBD/DNA interaction results from the conformational adjustments that allow the zinc fingers to "fit" the ERE DNA sequence (Schwabe *et al*, 1990; Schwabe *et al*, 1993). By recognizing the correct binding site, the DBD determines which target gene the receptor is to act upon. For example, when the DBD of the glucocorticoid receptor (GR) is replaced by the ER DBD, the mutant GR protein will bind to an ERE and will

then activate adjacent estrogen target genes in the presence of glucocorticoid stimulation (Green and Chambon, 1987).

The so-called "hinge domain" (D domain) functions as a biochemical hinge between DBD and the ligand-binding domain (LBD) (Kumar *et al*, 1986). There is little conservation in the D domain between ERα and ERβ. In some members of the steroid receptor superfamily, this region has been found to participate in ligand-independent transcriptional repression (Baniahmad, 1993; Baniahmad, 1995). Relatively little information on ligand-independent transcriptional repression has been directly derived from studies of ER.

However, recent experiments conducted by Jackson *et al* (1997) showed that the steroid receptor hinge domain may take part in coactivator/corepressor mediated tissue specific transcription activation/repression. In one experiment, they coexpressed a coactivator (L7/SPA) with one of three truncated progesterone receptors (PR), i.e., hinge domain alone, LBD alone, and hinge domain plus LBD (hinge+LBD), and then treated transfected cells with an artificial ligand for PR, RU486. Reporter activity was similarly increased over control levels in the presence of RU486-hinge and hinge alone. The highest reporter activity was observed in the presence of RU486-occupied hinge+LBD. No reporter activity was observed in the presence of RU486 occupied LBD alone, unoccupied hinge+LBD, or unoccupied LBD. Thus, while there was no reporter activity in the absence of the hinge domain, there was always moderate reporter activity in the presence of the hinge domain regardless of whether or not ligand was also present. Jackson *et al*'s (1997) results suggest that L7/SPA directly interacts with the hinge domain, and that this interaction is inhibited by the unoccupied LBD. Their

results also indicated that RU486 somehow released the inhibition caused by the LBD. From this, they concluded that the hinge domain is involved in ligand-independent activation of transcription.

The ER ligand binding domain (LBD) consists of 12 anti-parallel α-helices (H1-H12), an Ω loop between H2 and H3, and two anti-parallel β strands between H5 and H6 (Figure 3) (Parker *et al*, 1997). These helices are arranged into a three-layer sandwich. The middle layer formed by H5, H6, H9, and H10 is centered between the "upper" layer of H1-H4 and the "lower" layer of H7, H8, and H11. H12 and the β-strands are arranged about the flanks of the three-layer structure of helices. A cavity surrounded by two β-strands, H12, H11, H8, H6 and H3 functions as the ligand-binding core. Estradiol binds to H11, H6 and H3 by means of hydrogen bonds (Brzozowski *et al*, 1997). There is 55% and 58% homology in LBD between ERα and ERβ in the rat and human, respectively. Rat ERα and β have a similar binding affinity to estradiol-17β (E_2) with 0.1 nM and 0.4 nM dissociation constants (K_d), respectively. A second transcriptional activation function (AF-2) was found within the ER LBD region (Lees *et al*, 1989). The full function of AF-2 depends on the formation of the estrogen-ER LBD complex. In addition, AF-2 is a region where ER interacts with coactivators or corepressors. A leucine-rich motif LXXLL (where L denotes leucine and X denotes any amino acid) is the binding site for ER AF-2. This motif is located at the centers of both transcriptional coactivator molecules (Torchia *et al*, 1997; Heery *et al*, 1997; Voegel *et al*, 1998; Ding *et al*, 1998; Kalkhoven, 1998) and transcriptional corepressor molecules (L'Horset *et al*, 1996; vom Baur, 1996; Heery *et al*, 1997).

The structure of AF-2 is important for the interaction of ER with coactivators. For example, Henttu *et al* (1997) reported that point mutation of lysine^{366} (Figure 2) of the ER into alanine reduced ER binding to steroid receptor coactivator 1 (SRC-1) but did not change ER binding to receptor interacting-protein (RIP-140). Interestingly, Webb and his co-workers (1998) found that AF-1 also was able to recruit transcriptional coactivators such as GRIP, SCR-1a and RAC3. The difference is that AF-1 interacts with coactivators at a site near the C terminus of the coactivator, rather than at the LXXLL site. Because the two transcription activation domains of the ER molecule (i.e. AF-1 and AF-2), are located in separate regions of the receptor and are in contact with separate surfaces of the coactivators, ER may exhibit differential responses to various cellular stimuli, thus allowing multiple patterns of gene regulation in the target cell. In addition, the poorly conserved AF-1 region and highly conserved AF-2 region between ERα and ERβ imply that the two subtypes can exert similar function through the AF-2 domain, or different function through the AF-1 domain.

The two ER subtypes have non-homologous F domains. Rat ERβ has a shorter F domain than ERα (Kuiper *et al*, 1996). Some members of the steroid receptor superfamily do not have the F domain at all (Koelle *et al*, 1991). The F domain of ER does not affect estradiol induced transcription activity in CHO and 231 cells. However, in the same type of cells, the presence of the F domain greatly decreased transcriptional activity in the presence of trans-hydroxytamoxifen or ICI 164 384 (Montano, 1995). Moreover, the F domain suppressed the transcriptional activity induced by estradiol binding in HeLa and 3T3 cells, although it had no effect in CHO and 231 cells. It seems

that the F domain permits a ligand-mediated effect on the activity of the receptor in a cell-specific manner.

Activation of estrogen receptors

A conformational change is believed to be an important step in the activation of ER. As far back as 1980, Bailey *et al* performed an experiment in which unliganded ER was extracted from cells by using a hypotonic buffer followed by a sucrose gradient centrifugation. In this extraction procedure, ER sedimented as a 9S complex of proteins. In contrast, occupied ER had to be extracted by hypertonic buffer (0.4-0.6 M KCl) and was found to sediment after a sucrose gradient centrifugation as a 5S molecule (Bailly *et al*, 1980). From this and other similar results (e.g. Grody *et al*, 1982) it is clear that, upon ligand binding, ER is transformed from a 9S into 5S form. The experiments reported in Bailly *et al* (1980) were among the first published evidence that estrogen induced a conformational change in ER. Later, it was found that the patterns of protease digestion are different between unliganded-ER and liganded-ER, which suggests that the solvent exposed region accessible to protease is changed upon ligand binding (Fritsch *et al*, 1993; Beekman *et al*, 1993).

Additionally, it has been found that liganded and unliganded ER can be separated by aqueous two-phase partitioning (Hansen and Gorski, 1986; Fritsch *et al*, 1997). In this process of affinity partitioning, the majority of unliganded ER tends to be recovered from the more hydrophobic phase while the ligand-bound receptor is recovered from more hydrophilic phase. This conversion indicates that a ligand-induced conformation

change occurred in the protein resulting in a significant decrease in surface hydrophobicity.

The most recent findings on the crystal structures of ER LBD and E_2-ER LBD revealed the rearrangement of H12 in ER LBD caused by the ligand binding. Specifically, it was found that H12 forms a lid on top of the ligand binding cavity of the LBD by positioning itself over the cavity in the E_2-ligand complex, while it flanks the cavity in the absence of the ligand (Brzozowski *et al*, 1997). It is of critical importance that slight differences occur in the conformation of ER LBD when bound to different ligands, such as estrogen and raloxifene (Brzozowski *et al*, 1997).

It has generally been believed that estrogen binding to ER induces dimerization and that this, in turn, results in DNA binding. This concept has been questioned because it has been shown that estrogen is not necessary for ER dimerization (Gordon and Notides, 1985) and DNA binding *in vitro* (Tora *et al*, 1989). Similar results were also reported by Redeuilh *et al* (1987) and Salomonsson *et al* (1994). To determine whether or not ligand binding is necessary for ER-dimerization, Wang *et al* (1995) used a yeast two-hybrid system to investigate *in vivo* dimerization. In their experiment, they inserted the ER cDNA into two expression vectors, GAL4 DNA binding domain (GAL4 DBD) vector and GAL4 transcription activation (GAL4 TA) vector. This resulted in recombinant fusion proteins GAL4 DBD-ER and GAL4 TA-ER. Transformed yeast successfully expressed a LacZ reporter gene under control of the GAL1 promoter only when the GAL4 DBD and GAL4 TA were in apposition. It is important to note that this result could only have been achieved if the two ER formed a dimer to bring GAL4 DBD and TA together. It was found that reporter activity was increased in the presence of E_2

but was not increased in the absence of E_2 in the yeast after it had been co-transformed with GAL4 DBD-ER and GAL4 TA-ER. Moreover, it was also found that both tamoxifen and ICI 182 780 (separately) increased the β-galactosidase reporter activity. Taken together, all of these results strongly suggest that estradiol binding (as well as other ligand binding) results in ER dimerization in vivo.

Heat shock proteins are believed to be indirectly related to ER activation. The 9S form of ER derived from sucrose gradient centrifugation is a heterocomplex structure, which includes ER and 90-kDa heat shock protein 90 (Hsp90) (Pratt and Toft, 1977). In COS cells transfected with the ER LBD segment, ER LBD and Hsp90 formed a less stable complex than GR or PR LBD (Scherre *et al*, 1993). Since Hsp90 was demonstrated to bind to the ER LBD, it has been asserted that the stable ER Hsp90 complex might require an additional binding site in ER (Chambraud *et al*, 1990). The fact that steroid receptors in the 9S form are not able to dimerize or to bind to DNA (Hsu *et al*, 1992; Alnemri, 1991; Polenz *et al*, 1994) suggests that one possible function of Hsp90 is maintaining the unliganded steroid receptor in an inactive state. On the other hand, in the absence of Hsp90, it was found that GR was not able to bind steroids (Bresnick *et al*, 1989). It has also been shown that the GR binding to steroids can be restored by incubating with Hsp90 (Scherer *et al*, 1990). Further, a linear relationship was found to exist between ligand binding and the number of GR-Hsp90 complexes (Hutchison *et al*, 1992). Additionally, it has been shown that steroid binding caused the dissociation of Hsp90 from the receptor (Sanchez *et al*, 1987; Mendel *et al*, 1986; Denis *et al*, 1988). These data suggest that Hsp90 may act to stablize the unliganded receptor in an inactive state.

Evidence suggests that phosphorylation of steroid receptors regulates their activity (Boulikas, 1995; Karin and Hunter, 1995). For example, in 1992 Denton and his colleagues pre-labeled MCF-7 cells with [^{32}P]-phosphate, and found that the phosphorylation of estrogen receptor increased by 4-fold within 20 minutes of exposure to estradiol. In addition, calf uterine slices showed considerable elevation of ER phosphorylation following E_2 treatment (Denton *et al*, 1992). Also, it was shown that the phosphorylation rate was as fast as the nuclear retention of the receptor in MCF-7 cells (Denton *et al*, 1992). Moreover, all the phosphorylated residues of ER recovered from HCl digestion of ER and subsequently resolved on a two-dimensional acrylamide gel were found to be serine (Denton *et al*, 1992). Specifically, these residues were identified as serine^{104}, serine^{106} , serine^{118} (LeGoff *et al*, 1994) and serine^{167} (Arnold *et al*, 1994). All these serine residues were found to be located within the A/B domain, and it was also found that ER without the A/B domain did not exhibit E_2-dependent phosphorylation (Arnold *et al*, 1994). A triple mutant, replacing serine at 104, 106 and 118 with alanine, resulted in a 40% decrease in E_2-induced transcription activation in COS-1 cells, while single mutation of any one of the three serine residues resulted in a 15% reduction in transcriptional activation compared to wild type receptor (Le Goff *et al* 1994). Phosphorylation at serine^{167} was found to enhance ER binding to DNA (Arnold *et al*, 1995b).

Another phosphorylation site, tyrosine^{537}, was identified in hER from both Sf9 and MCF-7 cells (Castoria *et al*, 1993; Arnold *et al*, 1995a). The phosphorylation of tyrosine^{537} has been shown to be important for both ER dimerization (Arnold *et al*, 1995a) and for binding to the estrogen response element (ERE) (Arnold *et al*, 1995c;

Auricchio, 1996). The phosphorylation of tyrosine^{537} was shown to be independent of E_2 treatment; therefore the tyrosine^{537} site was considered to be a basal phosphorylation site (Arnold *et al*, 1995). Two mutants of ER (specifically, Y537A and Y537S – i.e., tyrosine^{537} replaced by alanine and serine, respectively) were found to be independent of ligand in activating transcription to levels of 20% and 100% of E_2-induced transcription activity of wild type ER, respectively (Weis *et al*, 1996). Because these constitutively active ER were able to recruit the coactivators RIP-140 (White *et al*, 1997) and SRC-1 (Weis *et al*, 1996), White *et al* (1997) proposed that tyrosine^{537} was responsible for keeping the receptor inactive in the absence of the ligand.

Estrogen receptor interaction with DNA

The consensus estrogen response element (ERE) is a palindromic DNA sequence, 5'-AGGTCAnnnTGACCT-3' (Klock *et al*, 1987). Acting as an enhancer, it can be located upstream, downstream or within a promoter region. The specificity of the steroid hormone response element is determined by three things: the nucleotide sequence, the spacers (non-specific nucleotides) between the two half-sites (above denoted by nnn), and the inverted repeat. For example, EREs and TREs (thyroid hormone response elements) have identical half-sites. However, if two half-site nucleotide sequences are arranged in an inverted repeat without the spacer (Glass *et al*, 1988), the resulting sequence is a TRE and not an ERE. Similarly, two half-site nucleotide sequences arranged in a direct repeat with four nucleotides in the spacer results in another TRE (Umesono *et al*, 1991; Naar *et al*, 1991). Additionally, it has

been shown that a palindrome 5'-AGAACAnnnTGTTCT-3' sequence which has only a two nucleotide difference from the ERE sequence functions as a GRE (glucocorticoid response element) (Naar *et al*, 1991).

Kumar and Chambon (1988) transfected HeLa cells with vectors expressing wild type hER (HE0) or one of several truncated hERs. The cell extract then was incubated with [^{32}P]-labeled ERE oligonucleotide sequence. In the gel retardation assay, the retarded bands (ER-ERE complex) were found to appear in extracts from the cells which were transfected with HE0 or LBD-truncated hER, i.e., no LBD, and treated with estradiol before harvesting. In cell extracts without estradiol treatment before harvesting, there was no retarded band found suggesting that no protein-DNA binding occurred. The band for LBD-truncated hER-ERE was weaker than HE0-ERE, suggesting that the HE0-ERE complex was more stable than LBD-truncated ER-ERE complex. When extract from HE0 transfected cells was combined with extract from LBD-truncated ER transfected cells, three retarded bands were observed. The band with the highest molecular weight was identical to that seen in HE0 transfected cells alone. The band with lowest molecular weight was similar to that seen in LBD-truncated-ER transfected cells alone. The molecular weight of the third retarded band was intermediate suggesting formation of the heterodimer of HE0 and LBD-truncated ER. These results indicated that ER bound to the ERE as a dimer in an E_2-dependent manner (Kumar and Chambon, 1988). Similarly, genomic footprinting revealed that ER binding to the ERE of the apo VLDLII gene promoter requires ligand (Wijnholds *et al* 1988).

However, not all of the published data support the assertion that E_2 is required for ER binding to DNA, and, as a result, this assertion is controversial. For instance, E_2-

independent formation of the ER-ERE complex was found *in vitro* (Walter *et al*, 1985; Reese and Katzenellenbogen, 1991), in COS-1 cells (Arbuckle *et al*, 1992; Dauvois *et al* 1992), in Sf9 cells (Arbuckle *et al*, 1992), and *in vivo* (Reese and Katzenellenbogen, 1992). A recent experiment (Cheskies *et al*, 1997), in which the interaction between ER and ligand was kinetically monitored, gave a most persuasive answer to this controversy. In essence, their technical approach was based on the physical chemistry concept that increased binding could be detected by the real-time monitoring of the changes in the refractive index of a transparent substrate coated with a thin layer of optically dispersive media. In this case, the "front surface" of an optical sensor chip was a thin layer of ERE sequences that were applied as a coating onto the active surface of the sensor chip. In the presence of ER, if the ER-ERE concentration was indeed increased, there would necessarily be a corresponding increase of the refractive index that could be monitored in real-time. The surface plasmon resonance corresponding to changes in the refractive index was monitored optically. Injecting ligand-free or ligand-bound ER over the sensor chip surface, they found that ligand-bound ER-ERE complex had a higher association constant ($k_a = 9.62 \times 10^4\ M^{-1}\ S^{-1}$) than ligand free ER-ERE complex ($k_a = 8.17 \times 10^3\ M^{-1}\ S^{-1}$), which demonstrated that ligand binding resulted in a rapid formation of ER-ERE complex. Surprisingly, the ligand-bound ER-ERE complex was also found to have had a higher dissociation constant ($k_d = 1.86 \times 10^{-3}\ S^{-1}$) than ligand-free ER-ERE complex ($k_d = 5.06 \times 10^{-4}\ S^{-1}$). This indicated that the ligand-bound ER-ERE complex was less stable than ligand-free complex. Interestingly, the surprising instability of the ligand-bound ER-ERE complex appears to be confirmed by the fact that the relative agonist activity mediated through ERE seems correlated with

both the formation rate and the stability of ligand-ER complex. E_2 has full agonist activity through ERα at the ERE site and possesses the fastest rate of complex formation, while ICI 182 780 has low agonist activity (it is a functional antagonist) accompanied by the slowest rate of complex formation (Cheskies *et al*, 1997). ERβ has also been found to bind to ERE as a homodimer in response to E_2 stimulation in a transient transfection assay (Pettersson *et al*, 1997; Paech *et al*, 1997).

Ligand-independent activation

Although ER is classically activated by estrogen-dependent phosphorylation, there is vast evidence that ER can be activated in the absence of a ligand. It was originally found that 8-Bromo-cyclic AMP (8-Br-cAMP) was able to activate PR mediated gene expression in the absence of a ligand (Denner *et al*, 1990). Phosphorylation of PR was found to be essential for PR activation by dopamine in the absence of progesterone (Power *et al*, 1991). Shortly thereafter it was demonstrated that ER dependent gene expression can be activated by several chemical agents including 8-Br-cAMP, CT/IBMX (CT plus IBMX; CT, cholera toxin; IBMX, isobutylmethylxanthine) (Aronica and Katzenellenbogen, 1993) and 12-O-tetradecanoylphorbol-13-acetate (TPA) (Ignar-Trowbridge *et al*, 1996). Importantly, the neurotransmitter dopamine, and a number of growth factors such as IGF-1, EGF, TGF-α and insulin (Smith *et al*, 1993; Ignar-Trowbridge *et al*, 1993; Bunone *et al*, 1996; Patrone *et al*, 1996; El-Tanani *et al*, 1997) were all found to activate ER in the absence of estrogenic ligand.

Ligand-independent activation of ER is accompanied by the phosphorylation of the ER protein. As an activator of protein kinase A (PKA), 8-Br-cAMP increases the phosphorylation of endogenous ER in rat uterine cells (Aronica and Katzenellenbogen, 1993). Similarly, CT is a protein kinase A activator (Aronica and Katzenellenbogen, 1993) and TPA is a protein kinase C (PKC) activator (Ignar-Trowbridge *et al*, 1996). These both elevate the phosphorylation of unliganded ER. Moreover, the growth factors IGF-1 and EGF have also been shown to induce hyperphosphorylation of ER (Aronica and Katzenellenbogen, 1993; Ignar-Trowbridge *et al*, 1996). These data suggest that ER-mediated gene expression can be activated by multiple signal transduction pathways and that phosphorylation of ER is crucial for ligand-independent activation of ER by chemicals and growth factors.

Ligand-independent activation of ER may involve phosphorylation in different domains or residues depending on the particular signaling pathway being activated. Serine^{118} located in the AF-1 domain has been identified as a ligand-independent phosphorylation site responsible for ER activation in ligand-independent pathways. Mutation of serine^{118} to alanine has been found to completely abolish the ability of EGF to activate ER (Ignar-Trowbridge *et al*, 1996). In addition, hER phosphorylation of hER resulting from TPA has also been found to occur in serine^{118} (Joel *et al*, 1995).

Further mutation studies revealed that in order to activate ER, different stimuli require different ER domains. For example, the AF-1 domain was found to be essential for IGF-1 and EGF activation of ER (Ignar-Trowbridge *et al*, 1993; Ignar-Trowbridge *et al*, 1996; El-Tanani and Green, 1997). In contrast, it was shown that AF-2 does not play a role in EGF-mediated ER activation (Bunone, 1996). However, CT/IBMX induced

ligand-independent activation of ER was shown to be dependent on AF-2 and was independent of AF-1 (El-Tanani and Green, 1997). Similarly, dopamine was shown to require full AF-2 function to activate unliganded ER (Smith *et al*, 1993).

Among all ligand-independent ER stimuli examined to date, insulin is unique since it appears to require both AF-1 and AF-2 domains of ER to fully activate the receptor. Deletion of AF-2 completely abolishes the ability of insulin to activate ER, while deletion of AF-1 significantly reduces the ability of insulin to activate ER (Patrone *et al*, 1996).

The ligand-independent activation pathway varies between effectors. Aronica and Katzenellenbogen (1993) reported that H8, a PKA inhibitor, abolished the ability of E_2, CT/IBMX and IGF-1 to activate ER. It was shown that bisindolylmaleimide, a PKC inhibitor, did not affect the activity of E_2, IGF-1, and TGFα to activate ER, but completely inhibited the effect of TPA on ER activation (Ignar-Trowbridge *et al*, 1996). Another PKC inhibitor, H7, diminished EGF activation of ER by 40-50% but did not affect TGFα-mediated activation (Bunone *et al*, 1996). These results suggest that the E_2–independent activation of ER may be mediated through either the PKA or PKC pathway.

Bunone *et al* (1996) found that overexpression of an inactive mutant form of Ras in SK-Br-3 and HeLa cells resulted in 40% reduction in activation of ER induced by EGF. Furthermore, overexpression of an inactive mutant form of MAPKK considerably reduced the EGF activation of the ER in SK-Br-3 cells and almost completely blocked the EGF effect on ER activation in HeLa cells. Cotransfection of wild type ER with a mutant MAPKK possessing constitutive activity resulted in the activation of ER in the

absence of any stimuli. Therefore, it is highly likely that the EGF-induced ER activation pathway is mediated via a MAPK pathway (Bunone *et al*,1996).

Estrogen receptor interaction with alternative DNA response elements

After ER has been activated, it functions as a transcription factor to activate the transcription of target genes through a variety of different DNA response elements. The classic pathway is mediated by ER binding to an ERE (5'-AGGTCAnnnTGACCT-3') adjacent to a target gene. Kumer and Chambon (1988) and Theulaz *et al* (1988) described ER interactions with EREs (5'-AGGTCActgTGACCT-3') in the Xenopus vitellogenin genes. However, in many cases ER achieves transcriptional activation through interaction with an imperfect palindromic sequence. For instance, the human pS2 gene has an ERE with sequence of 5'-GGTCAcgcTGGCC-3' (Berry *et al*, 1989) while rabbit uteroglobin gene has an ERE sequence of 5'-GGTCAccaTGCCC-3' (Lopez de Haro *et al*, 1990). Another example was reported by Darwish *et al* (1991) where it was shown that the ERE (5'-AGGTCAgggTGATCT-3') of the rat calbindin D-9K gene differed from the vitellogenin ERE by only one nucleotide.

Mukherjee and Chambon (1990) concluded that ER interaction with an ERE may require one or more accessory proteins that do not bind to the ERE, but instead facilitate the ER binding to the ERE. Their conclusion was based on the observation that purified ER is not able to bind to an ERE but does associate with ERE in the presence of cell extracts from either HeLa cells or yeast. DBSF (DNA binding stimulatory factor), p45

and p48 were specific proteins identified by Mukherjee and Chambon (1990) and by Landel *et al* (1994) as the accessory proteins.

ER has been believed to act as one of the regulators of IGF-I since Ernst *et al* (1989) confirmed that estrogen elevates IGF-1 mRNA expression in long bones of ovariectomized rats. It has been repeatedly shown that there is no conventional ERE sequence in the IGF-1 gene (Kim *et al*, 1991; Kajimoto and Rotwein, 1991; Hall *et al*, 1992). This suggests that other pathways are involved in estrogen-stimulated transcriptional activation.

There is increasing evidence that ER may activate transcription through associating with other transcription factors. This association can either be via a pure protein-protein interaction without ER-DNA contact, or can be via a more complex interaction, such as a half-site ERE plus protein-protein interaction.

Gaub and his co-workers (1990) observed ER interaction with a Fos-Jun heterodimer complex in the human ovalbumin gene promoter. They observed that the ovalbumin gene had a half ERE-site responsible for c-Fos and c-Jun heterodimer binding independently of ER. Cotransfection assays revealed that ER, c-Fos and c-Jun together activated ovalbumin expression, and that the activation did not depend on a direct ER-DNA interaction. Later, similar results were observed in MCF-7 cells transiently transfected with a reporter that contained four AP-1 sites, a herpes thymidine kinase (TK) and the chloromphenicol acetyltransferase (CAT) coding region, (AP-1)4-TK-CAT (Philips *et al*, 1993). It was shown that the CAT activity was increased 4- to 5-fold upon stimulation with IGF-1, was increased two-fold upon E_2 stimulation, and there was a synergistic effect on reporter activity when both IGF-1 and E_2 were present.

Philips *et al* (1993) also demonstrated that the effect of estradiol on reporter activity resulted from AP-1 transcriptional activation rather than from AP-1 synthesis. Umayahara and co-workers (1994) reported that transient transfection of a plasmid containing the IGF-1 promoter and a luciferase reporter into HepG2 cells, which express exogenous ER, resulted in increased luciferase activity in response to estrogen treatment. Although direct ER binding to DNA was not observed, an AP-1 site located within the IGF-I promoter region was found necessary for ER to mediate transcriptional activation, indicating that ER interaction with AP-1 was involved in the transcriptional activation.

Additionally, the human collagenase promoter contains an AP-1 site and can be activated by estrogen, tamoxifen and ICI 164 384 (Webb *et al*, 1995). Transient transfection of a reporter that contained the human collagenase promoter resulted in activation of reporter gene activity by E_2, tamoxifen and ICI 164 in various cell lines including HeLa, NIH-3T3, HEP G2, SHM, SY5Y, CEF, CV-1, MDA453, CHO and $F9^b$. However, E_2, tamoxifen, and ICI did not exhibit agonist activity in F9 cells, known to express a low level of AP-1 proteins. To address the importance of both the AP-1 site and AP-1 proteins in transcription activation, they co-transfected this reporter gene along with a c-Fos expression plasmid. They found that c-Fos restored the ability of E_2, tamoxifen, and ICI to activate the collagenase promoter activity in F9 cells.

Webb *et al* (1995) demonstrated that the ER-AP-1 interaction is achieved by ER binding to c-Jun rather than c-Fos. Interestingly, a transient transfection assay revealed that ERα and ERβ exhibited different responses when acting through the classic ERE-mediated pathway versus the AP-1-mediated pathway. In HeLa cells, increased ERE-

reporter activity was observed following transient expression of either ERα or ERβ after E_2 treatment (Paech *et al*, 1997). However, reporter activity was stimulated by E_2 following transient expression of ERα in AP-1-mediated activation but not when ERβ was expressed (Paech *et al*, 1997).

Another alternative pathway for transcriptional activation effects of ER is the interaction between ER and Sp1. In early 1992, two groups reported that E_2-dependent activation involved an ERE half-site and a GC-rich region (Sp1 site) in promoters of the rat creatine kinase B gene (Wu-Peng *et al*, 1992) and the c-myc oncogene (Dubik and Shiu, 1992). Rishi and his co-workers (1995) found that an ERE half-site$(N)_{10}$ Sp1 motif located in the -82 to -62 region of the retinoic acid receptor α (RARα) promoter functioned as an E_2-inducible enhancer in human breast carcinoma cells. In HepG2 cells, a region of the RARα promoter that is known to be responsible for E_2-induced transcriptional activation is located in the -49 to -79 sequence that contains an Sp1 site. Using the gel-shift assay technique, it has been demonstrated that ER does not directly contact DNA in this region, which indicated that ER-induced transcription activation occurs through an ER-Sp1 interaction (Elgort *et al*, 1996). Further investigation of the same promoter in HepG2 cells by Sun *et al* (1998) confirmed that ER-induced transactivation depended on the formation of an ER-Sp1 protein complex. ER-Sp1 interaction has also been identified in another E_2-responsive gene, cathepsin D. So far, at least three motifs within the cathepsin D promoter have been identified as regions that mediate E_2-induced transcriptional activation in the MCF-7 cell line (Wang *et al*, 1998). The first of these, located in the -199 to -165 region, is an Sp1$(N)_{23}$ half-ERE motif that is a cognate DNA site for the ER-Sp1 complex to bind (Krishnan *et al*, 1994; 1995).

The second of these is an imperfect ERE site located in the -119 to -107 region at which the ER dimer can bind (Wang *et al*, 1997a). The third of these is located in the region -145 to –126, where there are two Sp1 sites. These two sites allow the ER-Sp1 complex to bind. Within this region, there is also a dioxin-responsive element (DRE), which is a binding site for the complex of aryl hydrocarbon receptor (AhR) and AhR nuclear translocator (ARNT). One of above Sp1 sites and the DRE have adjacent locations and consequently they ultimately form a complex of ER dimer, Sp1, AhR and ARNT. The formation of this complex depends on not only E_2 stimulation, but also on the ligand of AhR, 2,3,7,8-tetrachlorodibenzo-p-dioxin, TCDD (Wang, 1998).

In summary, it has been shown that the mechanism of ER-mediated transcriptional activation is complex and involves multiple DNA elements. To date, at least three of these pathways have been intensively studied. The classical pathway is through ER interactions with an ERE, or an imperfect ERE, or a half-site ERE. The second pathway involves ER-AP-1 interactions. Finally, the third pathway involves ER-Sp1 interactions (Figure 4).

The key elements for estrogen receptor induced transcriptional activation

As discussed earlier, assembly of general transcription factors and RNAPII to form the basal transcription machinery is the first step in activation of transcription. There is accumulating evidence that ER interacts with the basal transcription machinery. In the early 1990's, a cell-free system was used to investigate steroid receptor modulation of transcription initiation. It was found that ER activated RNAPII in an ERE containing

promoter (Elliston *et al*, 1990). Later, ER and several other steroid receptors were found to have direct contact with TFIIB through their AF-2 functional domain (Ing *et al*, 1992). As mentioned previously, TFIIB is referred to as a "bridge protein" because it plays an important role in the assembling of PIC by bringing RNAPII and TFIID together. An *in vitro* experiment showed that the AF-1 and AF-2 of ER bound to TBP (which is a component of TFIID) and that TBP overexpression resulted in an increased transcription rate. This increased rate of transcription was induced by the ER through the ERE site independently of the distance between the ERE and the promoter (Sadovsky *et al*, 1995). Jacq and his co-workers (1994) determined that ER AF-2 binding to $TAF_{II}30$ (which is a component of TFIID) is required for ER mediated transcriptional activation. In addition, Brou *et al* (1993) showed that AF-1 and AF-2 of ER each separately interact with a different TAF (TAFs are other components of TFIID).

Interaction with the PIC is a necessary but not a sufficient condition for ER (as well as other steroid receptors) to modulate transcriptional activation. To achieve efficient hormone-mediated transcription control, ER requires other cofactors that are termed coactivators. Therefore, basal transcription factors and coactivators are both essential to the processes that specify and stabilize the basal transcription machinery (Goodrich *et al*, 1993). On the other hand, several steroid receptors have been identified as transcriptional repressors in the absence of hormone. Two proteins are recognized as corepressors that interact with the steroid receptor AF-2 domain to mediate transcriptional repression. One of these is NCoR (nuclear receptor co-repressor) and the other is SMRT (silencing mediator for RXR and TR). Although ER has not been

identified as functioning as a transcriptional repressor in the absence of estrogen, the presence of corepressor NCoR or SMRT does change ER agonist/antagonist activity. For instance, microinjection of an NCoR expression plasmid into HepG2 cells converts trans-hydroxytamoxifen (TOT, a potent agonist of ER in HepG2 cells) into an antagonist (Lavinsky *et al*, 1998). Smith *et al* (1997) showed that overexpression of SRC-1 enhanced both E_2- and 4-hydroxytamoxifen (4HT)- stimulated reporter activity while the overexpression of SMRT diminished 4HT agonist activity in a dose-dependent manner. This suggests that the relative amounts of coactivators and corepressors can affect ER activity in the presence of certain ligands. It is possible that the conformation of the ER/E_2 complex may allow ER to only recruit coactivators. However, the conformation of the ER when bound with some other ligand such as TOT or 4HT allows ER to recruit either coactivators or corepressors so that the agonist/antagonist activity of the ligand depends on what is available to be recruited in the particular cells or tissue.

To date, at least ten coactivators and three corepressors have been identified (Horwitz *et al*, 1996; Glass *et al*, 1997; Shibata, 1997). SRC/NCoA (nuclear receptor co-activators) were initially recognized as 160 kDa proteins (p160). So far, the p160 family is known to include three members: SRC-1/NCoA-1, TIF2/GRIP1/NCoA-2, and p/CIP/ACTR/AIB1/RAC3/TRAM-1. CBP and related p300 proteins (CBP/p300) have roles as coactivators and, more importantly, serve as a platform for transcription factors and coactivators to form a coactivator complex (Shibata *et al*, 1997). Generally, CBP/p300 binds to pCAF (CBP/p300 associated factor) that harbors p/CIP (CBP/p300 co-integrator associated protein). Other coactivators such as SRC-1/NCoA-1 bind to

p/CIP, while CREB, AP-1, and steroid receptors directly bind to CBP/p300. CBP/p300, pCAF p/CIP and SCR-1 all have intrinsic histone acetytransferase (HAT) activity (Torchia *et al*, 1998). NCoR/SMRT interacts with two related corepressor proteins, mSin3A and mSin3B, (Schreiber-Agus *et al*, 1995; Ayer *et al*, 1995) that recruit HDACs (histone deacetylases) (Taunton *et al*, 1996; Yang *et al*, 1997) to form a corepressor complex which leads to transcriptional silencing. A current model for the mechanism of ER activation or repression of transcription is as follows:

- when ERs bind to antagonists they recruit a corepressor complex (such as NCoR/SMRT) which has histone deacetylase activity; this results in a transcription suppressed status.
- when ERs bind to E_2 or are activated by an alternative pathway such as PKA or MAPK, the activated ER interacts with a coactivator complex resulting in transcriptional activation. This coactivator complex may include SRC-1/NCoA-1, p/CIP, p/CAF CBP/p300, which possess HAT activity (Figure 5; Torchia *et al*, 1998).

Selective estrogen receptor modulators and ER activity

Anti-estrogens were originally designed to inhibit ER activity in the treatment of ER-positive breast or uterine cancer (William *et al*, 1997). However, the fact that the same antiestrogen compounds can act as antagonists in some tissues/cells but as agonists in other tissue/cells changes the original concept of the estrogen antagonist. To date, all antiestrogens, including ICI 164 384 that had been believed to be pure

antagonists (William *et al*, 1997), have a mixed agonist/antagonist function (Paech *et al*, 1997; Cosman *et al*, 1999). Pharmacologists have attempted to exploit this mixed agonist/antagonist function to design new compounds that have selective estrogen agonist and antagonist activities. These compounds are known as selective estrogen receptor modulators (SERMs). Understanding the mechanism of action of SERMs will not only provide information of clinical significance but also expand our knowledge of the mechanism of ER function. Although the mechanism of SERMs has not yet been completely understood, some information is available concerning the ER-SERM complex.

As reported by Brozozowski and co-workers (1997), the conformation of the ER LBD differs upon binding to estrogen and raloxifene (a SERM). The ER has a unique conformation while it binds to a given ligand. The conformation of the ER/SERM complex allows recruitment of either coactivators or corepressors. The ultimate effect on transcription is determined by whether the coactivators or the corepressors are recruited. In some circumstances, ER is able to recruit both coactivators and corepressors (Smith *et al*, 1997). In that case, availability of the coactivator or corepressor is the key for transcription. Transcription is determined by the final ratio of coactivators over corepressors recruited by the ER.

The context of the promoter also plays an important role in ER-mediated transcription. In transient transfection assays, tamoxifen is an agonist at an ERE site but it is an antagonist when the ERE is replaced by an AP-1 site in HeLa, NIH-3T3, HEP G2, SHM, SY5Y, CEF, CV-1, MDA453, CHO and $F9^b$ cells (Webb *et al*, 1995).

Finally, the ER subtypes, ERα and ERβ, exhibit unique interactions with these

compounds. Raloxifene, tamoxifen, and ICI 164 384 all are antagonists bound to both ERα and ERβ at an ERE site in HeLa cells (Paech *et al*, 1997). However, at an AP-1 site, the responses of ERα and ERβ are quite different. Bound to ERα at an AP-1 site, tamoxifen has agonist activity similar to E_2, while raloxifene and ICI 164 384 have much weaker agonist activity than E_2 in HeLa cells. Bound to ERβ at an AP-1 site, raloxifene has stronger agonist activity than either tamoxifen or ICI 164 384, but E_2 does not exhibit agonist activity at all (Paech *et al*, 1997). Most recently, it was shown that showed that an aryl-substituted pyrazol stimulated ERE-coupled reporter activity in HEC-1 cells at a 120-fold higher rate in the presence ERα compared to ERβ (Sun *et al*, 1999). Similar results was also observed in CHO and HeLa cells (Sun *et al*, 1999). Another compound, tetrahydrochrysene (THC), is an agonist when bound to ERα but a pure antagonist when bound to ERβ. However, the S, S-enantiomer of THC acts as an agonist when bound to both ERα and ERβ (Sun *et al*, 1999). Thus, tissue distribution of ERα and ERβ become critical when selecting use of these compounds.

Taken together, SERM activity depends on the ER subtype, the availability of coactivators and corepressors in the tissues/cells, and the promoter context of a given target gene.

Effects of estrogens on cell proliferation

The effect of estrogen on cell proliferation in the rodent uterus was recognized decades ago (Bullough, 1946; Epifanova, 1958). E_2 injection into uteri of 20-day-old rats stimulated the rate of DNA synthesis and enhanced the content of RNA and protein

(Kaye *et al*, 1972). Kimura *et al* (1978) also observed estrogen-stimulated cell proliferation in mouse uterine epithelium. Further, long-term cell culture demonstrated that E_2 stimulated DNA synthesis in both endometrial and myometrial cells (Chen *et al*, 1973) and increased overall cell numbers (Pavlik and Katzenellenbogen, 1978). By examining incorporation of radiolabeled ATP into isolated nuclei, Stack and Gorski (1984) demonstrated that estrogen had a direct mitogenic effect on the uterus. By measuring cell generation time, Quarmby and Korach (1984) demonstrated that E_2 stimulated cellular proliferation in both stroma and epithelium in the immature mouse uterus but only in epithelium in the adult uterus.

In addition, the growth of mammary cells was also found to be estrogen responsive. Proliferation of mammary ductal epithelium was stimulated by E_2 (Daniel *et al*, 1987) and this effect could be blocked by antiestrogen (Silberstein *et al*, 1994). In cell culture systems many investigators observed that E_2 enhanced cell proliferation and DNA replication in MCF-7 cells, a human breast cancer derived estrogen-responsive cell line (Aitken and Lippman, 1982; Sutherland *et al*, 1983; Osborne *et al*, 1984; Jiang and Jordan 1992; Musgore *et al*, 1993; Thomas and Thomas, 1994; Wilcken *et al*, 1996; Foster and Wimalasena, 1996).

In estrogen-responsive rat pituitary tumor cells, E_2 failed to stimulate cell proliferation both in vivo and in cell culture (Sorrentino *et al*, 1976; Kirkland *et al*, 1976). Stable expression of exogenous human ER_{400val} cDNA (a mutant type) into HeLa cells (ER negative tumor-derived cell line) resulted in a negative effect of estrogen on the cell growth (Touitou *et al*, 1990; Maminta *et al*, 1991). A similar result was observed in a human osteosarcoma cell line stably transfected with ER_{400val} (Watts

et al, 1989). In CHO cells, overexpression of ER_{400val} not only inhibited cell proliferation but also caused cell death upon E_2 treatment (Kushner *et al*, 1990). To address whether the negative effect of ER on cell replication was caused by mutant ER, Jiang and Jordan (1992) established stable expression of either wild type human ER or ER_{400val} in an ER-negative breast cell line, MDA-MB-231. They found that E_2 inhibited cell growth in MDA-MB-231 cells transfected with either a mutant or wild type ER and this inhibition could be blocked by the antiestrogen ICI 164 384. Similarly, Wang and co-workers (1997b) found that E_2 (10 nM) diminished cell proliferation and DNA synthesis in MDA-MB-468 cells (ER-negative breast cancer cells) transfected with wild type ER. They also noticed that E_2 treatment resulted in increased number of cells in the G_0/G_1 phase and decreased numbers of cells in S and G2/M phases of the cell cycle.

The negative effect of ER on cell growth was also observed in the incidence of non-tumor-derived cells or tissues. The earliest observations were that estrogen suppressed surgically-induced vascular intimal hyperplasia in rabbits (Rhee *et al*, 1977) and prevented myointimal proliferation in rabbit cardiac and aortic allografts (Foegh *et al*, 1987; Cheng *et al*, 1991). Later, the proliferation of smooth muscle cells (SMC) induced by balloon injury of the arteries of animals (an experimental model for early development of atherosclerosis) was found to be inhibited by E_2 (Foegh *et al*, 1993; Sullivan *et al*, 1995; Chen *et al*, 1996). In a tissue culture system, Vargas and co-workers (1993) observed that E_2 significantly decreased ^{3}H-thymidine uptake by segments of pig left anterior descending coronary artery (LAD) suggesting that E_2 inhibited SMC proliferation in pig LAD segments.

It seems clear that estrogen effects on cell proliferation are tissue/cell type or developmental stage-specific events. These differences may be due to differential expression of factors that may interact with ER resulting in a specific response to estrogen, i.e., coactivators and corepressors. Although the mechanism of ER effects on cell proliferation remains unclear, some evidence shows that differential expression of the two ER subtypes might play a role.

Studies of ERα knockout (αERKO) and ERβ knockout (βERKO) mouse models showed that βERKO mice appeared to have normal uteri (Krege *et al*, 1998), while αERKO mice exhibited hypoplastic uteri that were about half the weight of wild-type mice (Lubahn *et al*, 1993). These findings suggest that ERα, but not ERβ, is important in cell proliferation in the mouse uterus. Additionally, the uterus of αERKO mice exhibited no incorporation of ^{3}H-thymidine in response to E_2 suggesting that cells have lost responsiveness to E_2 (Couse *et al*, 1995; Korach *et al*, 1996). Moreover, using recombinant tissue (a chimeric stroma+epithelium unit), Cooke and co-workers (1997) showed that ERα function is critical in uterine epithelial growth. E_2 stimulated epithelial cell proliferation in wild-type (wt) stroma+αERKO-epithelium and in wt-stroma+wt-epithelium units but not in αERKO-stroma+wt-epithelium or αERKO-stroma+αERKO-epithelium units. These data indicate that ERα in stroma is required for epithelial cell growth. ERα is not required in the epithelium, however. These results confirmed the role of ERα in the cell growth in uterus tissue.

In similar experiments, Cunha *et al* (1997) reported that ERα in stroma was essential for mammary epithelium to grow in stroma+epithelium recombinant units

from mammary ductal epithelium and stroma tissue of wild type and αERKO mice. Bocchinfuso and Korach (1997) found a dramatic underdevelopment in mammary glands of αERKO female mice as adult αERKO females exhibited the ductal structure similar to newborn wild-type female mice. In contrast, the mammary glands of adult βERKO female mice appeared to have normal ductal structure (Couse *et al*, 1997). Therefore, ERα seems to play the predominant role in mediation of mammary gland growth.

However, it seems that ERα does not play a role in the estrogen-induced inhibition of cell proliferation. A recent experiment showed that the SMC proliferation in injured blood vessels was markedly inhibited by E_2 in both wild type and αERKO mice (Iafrati *et al*, 1997) suggesting that the inhibitory effect of E_2 on SMC proliferation is independent of ERα. Although direct data in this regard are not available yet, it is possible that ERβ somehow mediated E_2 induced suppression of cell growth.

Nevertheless, estrogen has multiple roles in cell proliferation. It promotes cell proliferation in both stroma and epithelium in the immature uterus but only in epithelium in the mature uterus (Matin, 1980; Quarmby and Korach, 1984). On the other hand, estrogen inhibits the growth of certain cell lines and of SMC in injured vessels. These effects are tissue specific and may be mediated separately by ERα and ERβ.

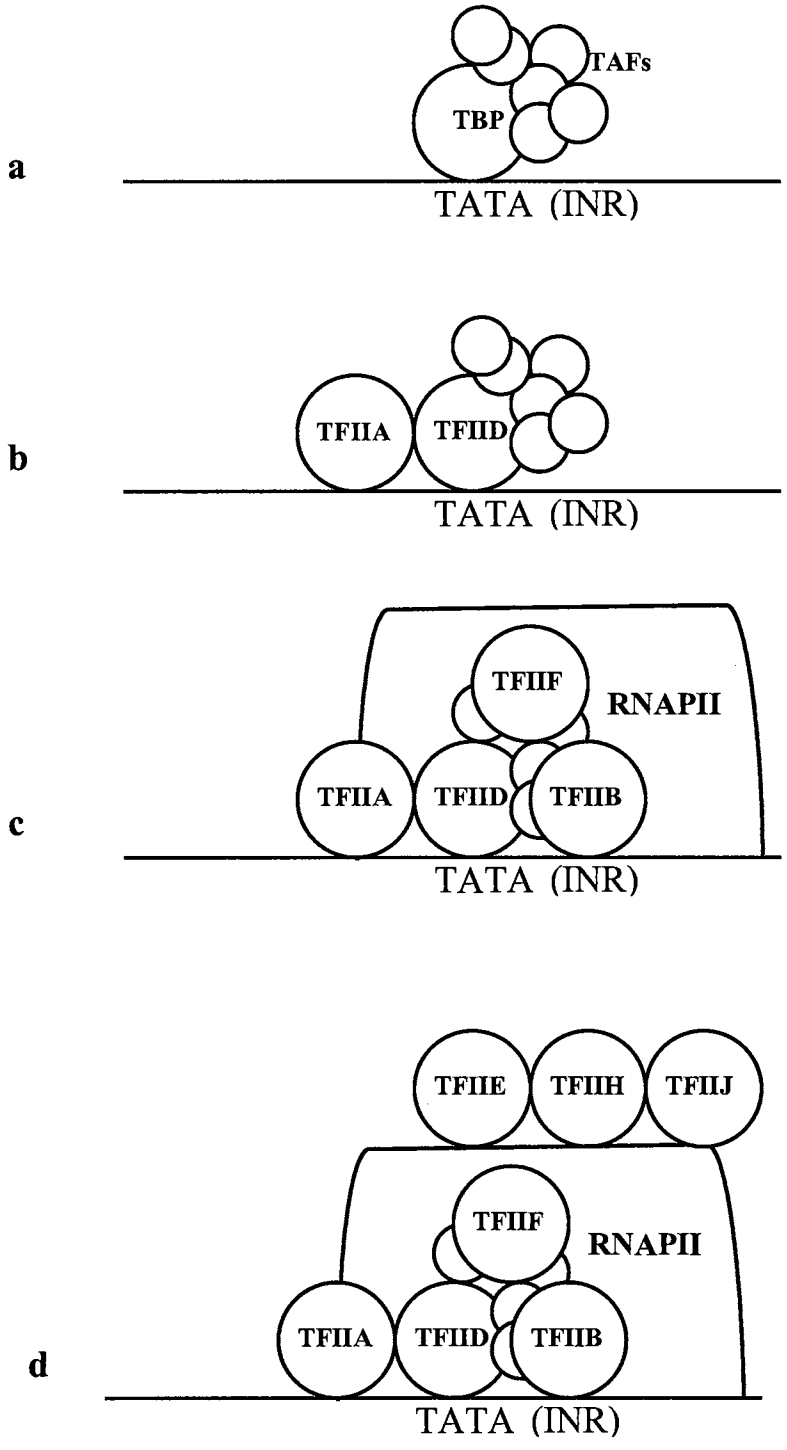

Figure 1. The assembly of basal transcription machinery (preinitiation complex).

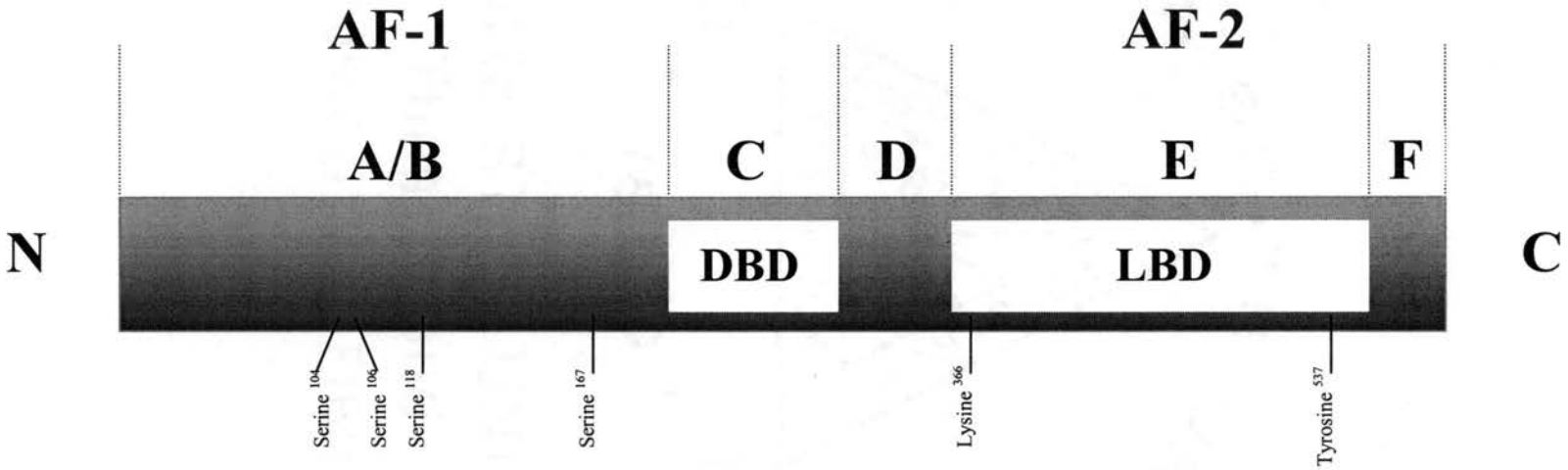

Figure 2. Domain structure of steroid receptor superfamily. Indicated amino acid residues are conserved between estrogen receptor subtype alpha and beta.

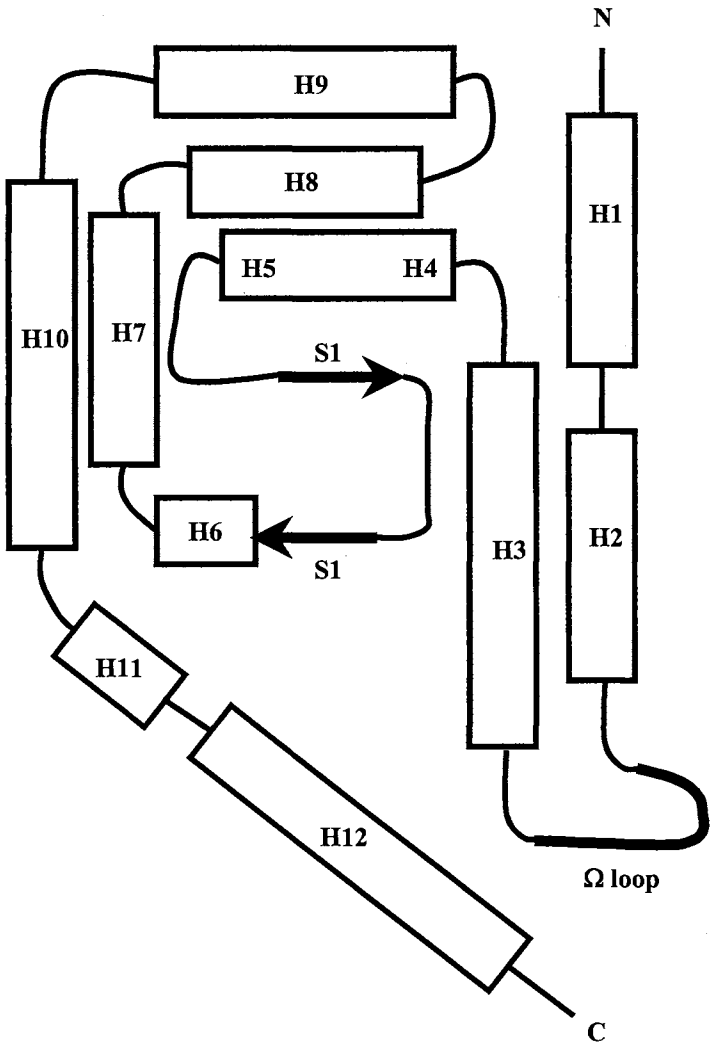

Figure 3. Schematic picture of estrogen receptor ligand binding domain (adapted from Parker, 1997).

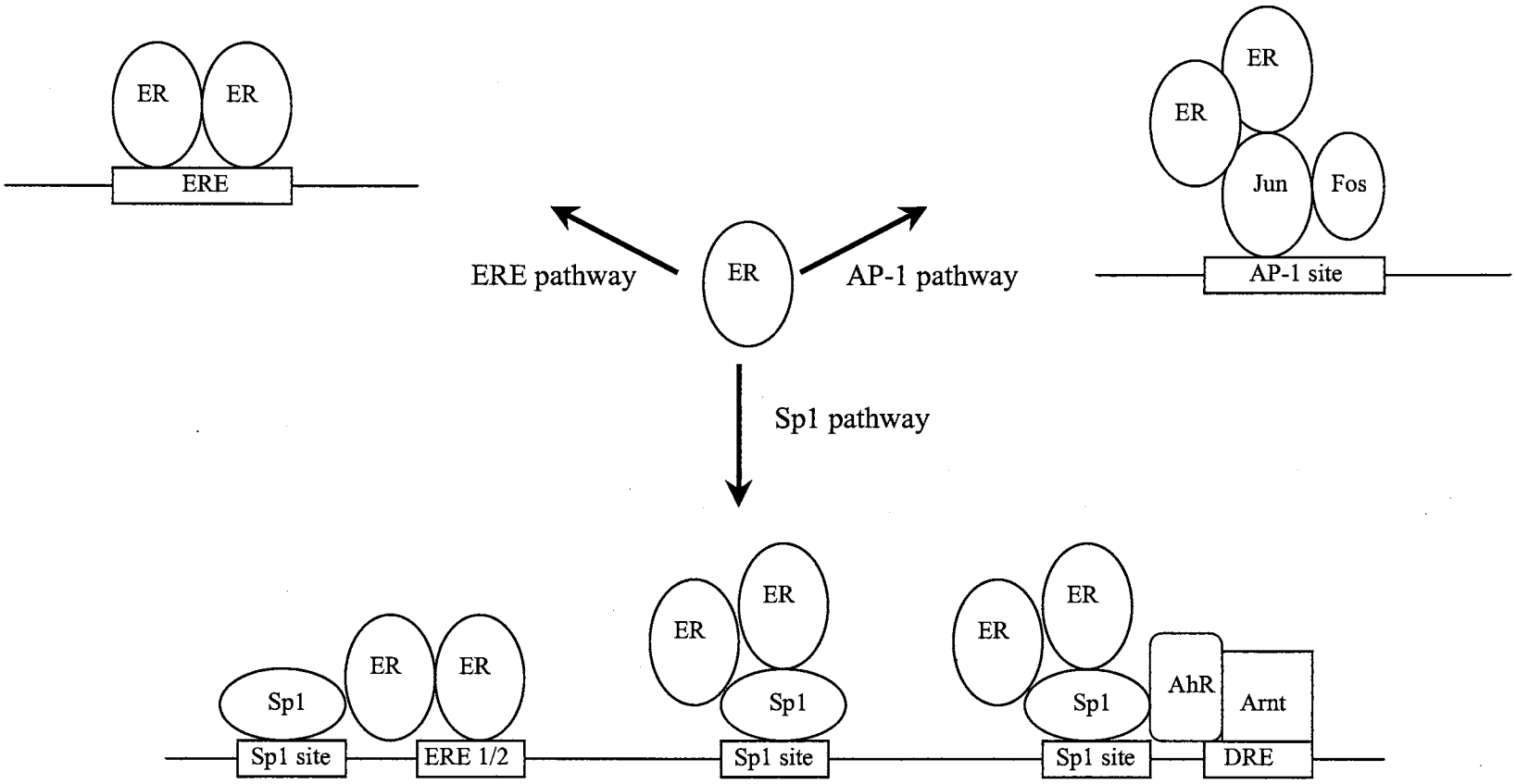

Figure 4. Transcriptional function of estrogen receptor involves multiple pathways

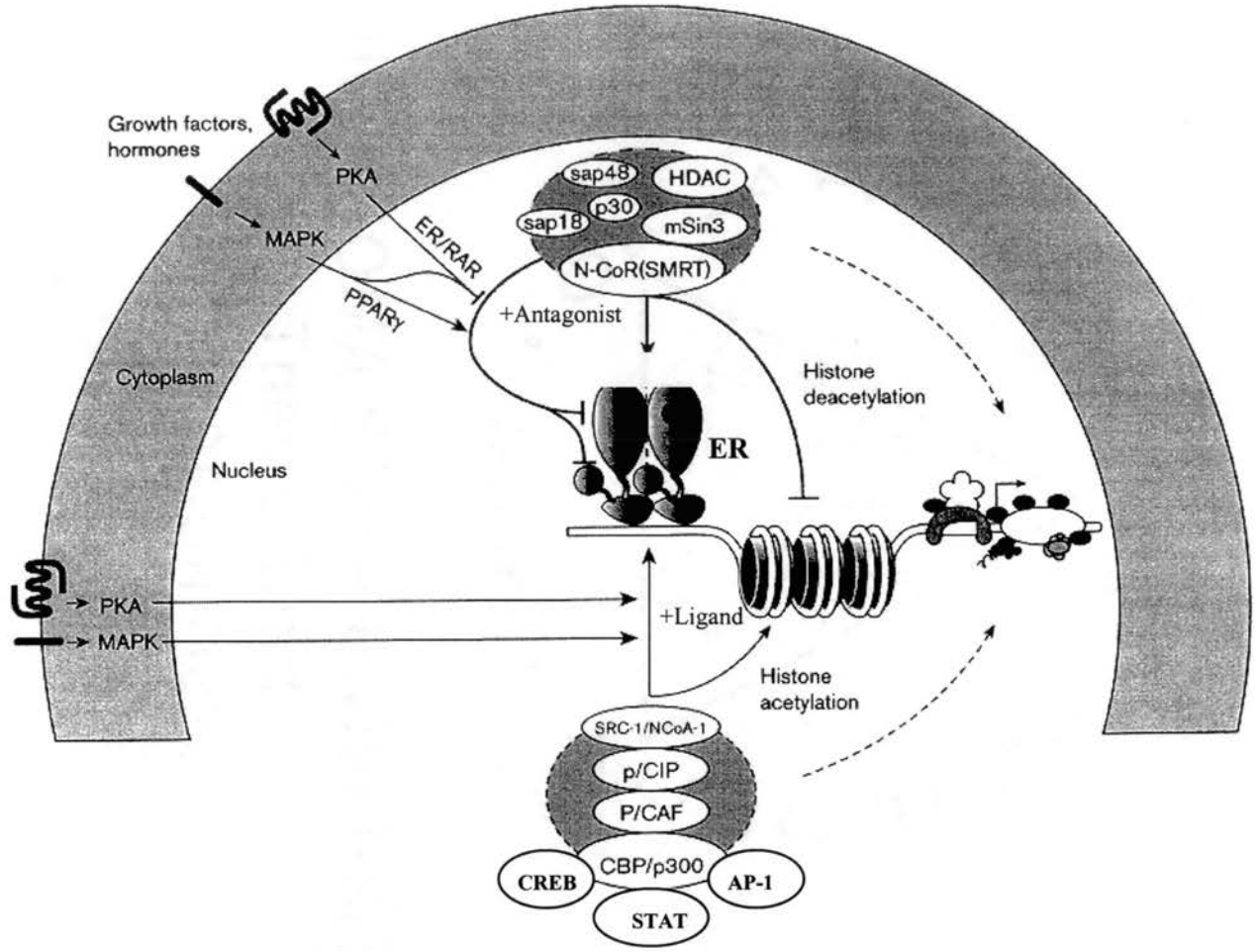

Figure 5. A model for estrogen receptor (ER) functions (adapted from Torchia *et al*, 1998). In the presence of antagonist ER recruit corepressor complex resulting in histone deacetylation. When ER is activated by estrogen or other pathways, they harbor coactivator resulting in histone acetylation and in turn initiate transcriptional activation.

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CHAPTER III

RESPONSES TO STABLE ECTOPIC ESTROGEN RECEPTOR-BETA EXPRESSION IN A RAT FIBROBLAST CELL LINE

Abstract

To examine activity of estrogen receptor beta (ERβ) independently of estrogen receptor alpha (ERα), retrovirus-mediated gene transfer was used to insert rat ERβ into a rat fibroblast cell line (rat-1) that does not ordinarily express ER. Stable expression of ERβ in rat-1 cells was validated and then characterized by RT-PCR analysis to examine the effects of estradiol (E_2) treatment on expression of specific target mRNAs. Results were compared with rat-1 cells and a previously constructed rat-1+ERα cell line. Progesterone receptor mRNA was not detected in rat-1 cells and was induced by E_2 in both rat-1+ERα and rat-1+ERβ cells. Treatment with E_2 resulted in an increased rate of cell proliferation ($P<.05$) in rat-1+ERα cells, but not in rat-1 or rat-1+ERβ cells. Data confirm studies using transient ER expression demonstrating that ERα and ERβ have both discrete and overlapping activity within the same cell type in the presence of the same ligand.

Introduction

Effects of estrogens are mediated by two nuclear receptors, estrogen receptor alpha (ERα) and estrogen receptor beta (ERβ) (Katzenellenbogen, 1996; Kuiper *et al.*, 1996). Comparison of the ERβ with ERα reveals a high degree of conservation in the DNA-binding domain (Kumar *et al.*, 1987) (95%) and ligand-binding domain (60%), suggesting that the two ER forms would bind to DNA and ligand in a similar manner. This has been established (Kuiper *et al.*, 1997; Pace *et al.*, 1997; Paech *et al.*, 1997; Witkowska *et al.*, 1997), although several ligands exhibit significantly different binding characteristics with the two receptors. These include estriol, estradiol-17α, tamoxifen, and the phytoestrogens coumestrol and genistein (Kuiper *et al.*, 1997). The ERα and ERβ diverge significantly in the regions of the receptors involved with transactivation. These are termed transactivation domains 1 and 2 (AF-1 and AF-2) (Berry *et al.*, 1990; Bocquel *et al.*, 1989; Danielian *et al.*, 1992; Kumar *et al.*, 1987; Lees *et al.*, 1989; Tora *et al.*, 1989b) and corresponded to regions of the A/B domain and LBD, respectively (Figure 2 of chapter II). Interactions between the ER and specific co-activator and co-repressor proteins occur at these transactivation domains (Horwitz *et al.*, 1996; Onate *et al.*, 1998; Onate *et al.*, 1995; Shibata *et al.*, 1997; Spencer *et al.*, 1997; Webb *et al.*, 1998). Sequence divergence in these regions between the two ER forms suggests that interactions with co-activator or co-repressor proteins would differ at the enhancer or promoter site during modification of gene expression and, as a result, that ERα and ERβ play different roles in gene regulation. Further complexity lies in the ability of ERα

and ERβ to form heterodimers (Pettersson *et al.*, 1997), which may exhibit activities distinct from either homodimer type.

To examine activities unique to ERβ, we have developed stable, ectopic expression of rat ERβ in a rat embryo fibroblast cell line (rat-1 cells; (Freeman *et al.*, 1970)) using retrovirus-mediated transfer. The rat-1 cells normally do not express either ERα or ERβ and were used previously as a model system for ectopic expression of ERα (Kaneko *et al.*, 1993). Our objectives were to generate stable ERβ expression in a cell type that normally does not express ER, and then to compare the response to estradiol of endogenous gene promoters in the presence and absence of ERβ. In addition, responses were compared in the presence of ERα activity in the previously reported rat-1+ ERα cell line (Kaneko *et al.*, 1993).

Materials and Methods

Cell culture

Experiments were carried out in rat-1 cells (Freeman *et al.*, 1970) maintained in phenol red-free Dulbecco's modified Eagle's medium (DMEM; Gibco-BRL, Gaithersburg, MD) containing 1x antibiotic-antimycotic (Gibco-BRL), 5 mM Hepes (Sigma, St. Louis, MO), and sodium bicarbonate (Sigma). Medium was also supplemented with 10% fetal bovine serum (FBS; Hyclone, Logan, UT), and insulin (0.6 μg/ml; porcine insulin, Gibco-BRL). PT67 retrovirus packaging cells (RetroXpress^{TM} System, Clontech, Palo Alto, CA) were grown under the same

conditions. Cultures were maintained at 37°C in a humidified atmosphere of 95% air, 5% CO_2 and fed every 48 h.

Construction of the retrovirus-mediated insertion vector

Restriction enzymes and other DNA-modification enzymes were purchased from either Promega (Madison, WI) or New England Biolabs (Beverly, MA). The plasmid pLNCX (RetroXpress™ System; Clontech) was linearized by HpaI digestion and treated with EcoRI methylase to protect internal EcoRI sites. Phosphorylated EcoRI linkers (New England Biolabs) were ligated to pLNCX using T4 DNA ligase (2,000,000 units/ml), followed by EcoRI digestion to create sticky ends. The rat ER-β cDNA was excised from pRAT-ER-β plasmid (provided by Dr. G. Kuiper) by EcoRI digestion and inserted into the EcoRI linker sites in pLNCX, resulting in the recombinant plasmid pLNCX-ERβ (see Figure 1 for map). This construct was tested by restriction mapping and by dideoxy chain-termination sequencing (Applied Biosystems, Foster City, CA; Model 373A Automated Sequencer, OSU Recombinant DNA/Protein Resource Facility) to verify the ERβ insert was intact and in the correct orientation.

Establishment of ectopic ERβ expression

The PT67 retrovirus packaging cell line was transfected with pLNCX-ERβ by cationic liposome-mediated transfection (Transfectam®; Promega). The pLNCX plasmid carries the *neo* transposon, which confers resistance to the aminoglycoside G418. After washing with Hanks' Buffered Salt Solution (HBSS, Gibco-BRL) three times, cells were fed with fresh DMEM containing 10% FBS for 24 hours and selected

in the same medium supplemented with G418 (0.5 mg/ml; Gibco-BRL) for 12 days. Following selection, remaining PT67 cells were diluted into 96-well tissue culture plates (Falcon®; Becton Dickinson, Bedford, MA) to propagate clonal lineages. Selected cell lineages were grown to confluence and medium was collected as the source of recombinant retrovirus particles. Media were filtered through 1.0 μm polysulfone membrane filters (Whatman, Clifton, NJ) and resultant cell-free media were stored at -80°C.

To determine retrovirus titer, rat-1 cells were grown in 100×15 mm tissue culture plates (Falcon®; Becton Dickinson) and incubated as described above. Media were supplemented with 1% (v/v), 50% and 100% filtered retrovirus-containing medium for 24 hours. Cells were selected with G418 for 15 days and resistant cell colonies were counted. Viral titer was calculated as follows:

$$G418^r \text{ CFU/ml} = \frac{\text{number of colonies}}{\text{virus volume x replication factor x fraction of infected cells plated}}$$

Following selection, transfected rat-1 cells (hereafter called rat-1+ERβ) were diluted into 96-well tissue culture plates to propagate clonal lineages. Selected cell lineages were grown to confluence in the presence of G418 and tested for integration of the ERβ coding region and stable ERβ expression as described below. All reported estrogen response data was from a single clonal cell lineage of rat-1+ERβ cells.

Southern blot analysis

Genomic DNA was isolated from rat-1 and rat-1+ERβ cells with DNA Isolation Kits (Gentra System, Minneapolis, MN). Southern analysis was performed as described by Sambrook et al (Sambrook *et al.*, 1989). Seventy micrograms of each genomic DNA was digested to completion with EcoRI and loaded onto a 0.8 % agarose-TAE gel [Tris-acetate (40 mM), EDTA (1 mM)]. Resolution was visualized by staining with ethidium bromide. The gel was then denatured and DNA transferred to a nylon membrane (Boehringer Mannheim, Indianapolis, IN) using 10×SSC solution [sodium chloride (1.5 M) plus sodium citrate (0.15 M); pH 7.0] as transfer buffer. The blot was prehybridized in hybridization buffer [$NaPO_4$ (0.5 M), bovine serum albumin (1% w/v), EDTA (1 mM), sodium dodecyl sulfate (SDS; 7% w/v), herring sperm DNA (100 μg/ml)] at 65°C for 2 hr. Hybridization was carried out overnight at 65°C in the presence of radiolabeled probe at a concentration of 2.7×10^6 cpm/ml. The probe was full-length rat ERβ cDNA labeled by random priming in the presence of $\alpha[^{32}P]$-dATP (sp. act. 3000 Ci/mmol; NEN, Boston, MA) and eluted through a Sephadex G-25 spin column (Boehringer Mannheim). Following hybridization, the membrane was washed twice with 2×SSC containing 0.1 % SDS for 30 min at 65°C and twice with 0.2×SSC plus 0.1% SDS for 30 min at 65°C. Hybridization was visualized by autoradiography using Kodak XRP film.

Reporter plasmid constructs

The plasmid pBLCAT2 (Luckow and Schutz, 1987) contains the minimal thymidine kinase promoter from herpes simplex virus (HSV-tk) driving expression of the coding region of the chloramphenicol acetyltransferase (CAT) reporter gene. To

confer estradiol responsiveness, the 15-bp consensus ERE from the Xenopus vitellogenin A2 gene (GGT CAC AGT GAC C) was inserted into the XbaI restriction site, immediately upstream of the HSV-tk, to produce the pERE15 plasmid (Luckow and Schutz, 1987). Both pBLCAT2 and pERE15 are pUC-derived plasmids and thus contain an AP-1 enhancer in the region 5' of the HSV-tk (Kushner *et al.*, 1994). The pBLCAT2 plasmid, hereafter called pAP-1-CAT, was used to examine AP-1 mediated effects upon the HSV-tk promoter. To remove this confounding regulatory element for ERE and control studies, we digested these plasmids with NdeI and EcoO109 as described by Kushner et al (Kushner *et al.*, 1994), followed by religation, to generate pBLCAT2ΔNdeI-EcoO109 and pERE15ΔNdeI-EcoO109, hereafter called pBLCAT and pERE-CAT, respectively. In order to control for variation in transfection efficiency, the plasmid pSV-β-gal (Promega) was used to co-transfect the cells. This plasmid served as marker for relative transfection efficiency through basal expression of the β-galactosidase reporter that was assayed in the same cell extracts used to measure the CAT reporter activity. CAT reporter data was normalized to the β-galactosidase activity to account for the variation in response due to variation in efficiency of reporter transfection and cell numbers.

Transient transfection and hormone treatments

To examine the hormone-response of a transient reporter gene, cells were placed in phenol red-free DMEM supplemented with 10% dextran-charcoal stripped FBS (CS-FBS) for 48 h. Dextran-charcoal stripping was performed as described by Horwitz et al (Horwitz *et al.*, 1976). For tests of functional estrogen response, reporter-plasmid

DNAs were transfected by cationic liposome-mediated transfection (Transfectam®; Promega) into rat-1 and rat-1+ERβ cells. Following a 24 hr recovery period in DMEM supplemented with 10% CS-FBS, cells were refed using medium supplemented with 10% CS-FBS and incubated for 48 hr. Cultures of transfected rat-1 and rat-1+ERβ cells were then treated for 6, 9, or 25 hr in the presence of estradiol-17β (E_2; 1.0 nM; Sigma), and for 25 hr in the presence of ICI 182,780 (ICI; 10 nM; Zeneca Pharmaceuticals, Macclesfield, UK), E_2 plus ICI, or ethanol vehicle. Each treatment was performed in triplicate culture wells. Cell cultures were then assayed in duplicate for a response characterized by increased CAT expression.

Response of endogenous gene promoters to estradiol

To examine alterations in expression of specific endogenous mRNAs by RT-PCR, cells were prepared as described above. Following a 72 hr incubation period in DMEM supplemented with 10% CS-FBS, cells were refed using medium supplemented with 10% CS-FBS. Cultures of rat-1, rat-1+ERα, and rat-1+ERβ cells were treated for 9 hr in the presence of E_2 (either 0.1 nM, 1.0 nM, or 10 nM) or ethanol vehicle and harvested for preparation of total RNA and RT-PCR as described below. Treatments were performed on cell cultures prepared in duplicate, independent experiments. RNA from each treatment well was then subjected to reverse-transcription in duplicate, from which each target cDNA was amplified by PCR as described.

CAT assay

Chloramphenicol acetyltransferase enzyme activity was assayed in duplicate in cell lysates by incubating in the presence of [^{3}H]chloramphenicol and n-butyryl-coenzyme A, then measuring the acetylated [^{3}H]chloramphenicol present in the organic (xylene) phase following two-phase partitioning (CAT Enzyme Assay System Kit; Promega). The radioactivity present in the acetylated chloramphenicol was determined by scintillation spectroscopy. Enzyme activity was compared to cells not transfected as a negative control; [^{3}H]chloramphenicol was also measured in the xylene phase following extraction in the absence of cell lysates to determine background radioactivity following two-phase partitioning.

Control assays for β-galactosidase activity were carried out using the same cell extracts as were used for the CAT assays. The β-galactosidase enzyme assay system (Promega) was based on spectrophotometric detection of the cleavage product of the substrate o-nitrophenyl-β-D-galactopyranoside.

Reverse-transcription polymerase chain-reaction (RT-PCR)

RT-PCR was used to detect specific transcripts in RNA obtained from rat-1, rat-1+ERα, and rat-1+ERβ cell cultures. Total RNA was extracted from cultures by guanidinium thiocyanate extraction (Chomczynski and Sacci, 1987). All solutions and glassware used in preparation and analysis of RNA were treated with diethylpyrocarbonate (Sigma). Two micrograms of total RNA was denatured by heating to 95°C and reverse-transcribed in the presence of random hexamers (pdN_6; 100 pmole; Pharmacia, Piscataway, NJ), dATP, dTTP, dCTP, and dGTP (dNTPs; 1 mM; Pharmacia), $MgCl_2$, RNase inhibitor (20 U per reaction; Promega), and reverse

transcriptase (Superscript™, 200 U per reaction; Gibco-BRL) at 37°C for 75 min. The reaction was stopped by heating to 95°C. Aliquots of reverse-transcribed cDNA (1 to 5 μl) were denatured by heating to 95°C and subjected to polymerase chain-reaction in the presence of 75 pmole specific primers (Table 1), $MgCl_2$, dNTPs (1 mM), and Amplitaq™ DNA polymerase (0.5 U per reaction; Perkin-Elmer, Foster City, CA). Products of RT-PCR were resolved on 3% agarose-TAE gels [Tris-acetate (40 mM), EDTA (1 mM)], and visualized by staining with ethidium bromide. Total RNA from each treatment well was reverse-transcribed in duplicate and all PCR amplifications repeated twice from each RT reaction. Specific RT-PCR target mRNAs were amplified by PCR in the same reaction as that for glyceraldehyde-3-phosphate dehydrogenase (G_3PDH) used as a loading control. Representative RT-PCR products from each primer set were excised from agarose and tested by dideoxy chain-termination sequencing (Applied Biosystems, Model 373A Automated Sequencer, OSU Recombinant DNA/Protein Resource Facility). The identity of each product was verified in a sequence homology analysis using the Basic Local Alignment Search Tool (Altschul *et al.*, 1990).

Colorimetric cell proliferation assay

Cell proliferation in response to estradiol was determined by measuring the conversion of the water-soluble tetrazolium salt MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; thiazolyl blue)(Sigma) to an insoluble purple formazan by the action of active mitochondrial dehydrogenase enzymes in living cells. Cells were plated in 96-well dishes (Becton-Dickinson), in DMEM supplemented with 10%

CS-FBS as described above, in three sets of triplicate wells containing $1x10^5$ cells per well. Two sets of triplicate wells were incubated for 72 hr in the presence of E_2 (25 or 50 pM) and the third was incubated with ethanol vehicle. After 72 hr, medium was replaced with DMEM supplemented with 10% CS-FBS and 100 μg MTT and cells were incubated an additional three hours. Medium was then removed and the converted formazan dye was solubilized with 0.1 N HCl dissolved in isopropanol (200 μl per well). Absorbance of the dye at 570 nm was measured (Kinetic Microplate Reader, Molecular Devices Corp., Menlo Park, CA).

Statistical analysis

Duplicate values obtained in the assay of CAT enzyme activity within each culture well were normalized to β-galactosidase activity to correct for variation in transfection efficiency and cell numbers. These values were used to calculate a treatment mean from the triplicate wells within each cell type. Treatment means are expressed as a value relative to the ethanol vehicle control. The relative number, reported as fold induction, was calculated as the ratio of treatment with estradiol or estradiol plus ICI to the vehicle treatment data point, which was set to 1. Standard error was calculated for each averaged point. Data were analyzed with Duncan's test (Steel and Torrie, 1980). Values obtained in the cell proliferation assay were used to calculate a treatment mean from the triplicate wells within each cell type. Treatment means are expressed as a value relative to the ethanol vehicle control. The relative number, reported as cell proliferation, was calculated as the ratio of treatment with estradiol to the vehicle treatment data point, which was set to 1. Standard error was

calculated for each averaged point. Data were analyzed with Duncan's test (Steel and Torrie, 1980).

Results

Stable expression of ERβ

RT-PCR showed ERβ mRNA expression in PT67+ERβ cells but not in PT67 cells (Figure 2) suggesting that PT67+ERβ were able to produce retroviral RNA that carried the ERβ coding region. Southern blot analysis was performed with genomic DNA from rat-1 cells, retrovirus-producing PT-67 cells (PT-67+ERβ) and rat-1+ ERβ cells (Figure 3). Both rat-1 and PT-67 cells contained endogenous ERβ DNA sequences that hybridized with probe. The rat-1+ ERβ cells exhibited a unique hybridization pattern indicating the integration of the ERβ cDNA insert into the genomic DNA.

Southern blot results suggested that ERβ cDNA had integrated into the genome of rat1+ ERβ cells, but did not demonstrate expression of functional receptor. RT-PCR analysis was used to detect ERβ mRNA expression. The results of RT-PCR (Figure 2) indicated that ERβ mRNA was present in rat-1+ ERβ cells, but was absent in rat-1 cells.

Functional estrogen response in rat-1+ERβ cells

To test for the presence of functional ectopic ERβ, transient reporter-gene transfection with pERE-CAT was used to test for a functional estrogen response. In rat-1 cells, there was no effect of E_2 treatment on activation of the reporter gene (not

shown). In rat-1+ERβ cells, after 6 hr exposure to E_2 the reporter gene activity was increased 8.5-fold over control ($P<.05$; Figure 4), indicating functional ERβ activity. Reporter gene activity was maximal after 6 hr, reduced by 9 hr ($P<.05$), and decreased to basal activity after 25 hr exposure. However, in the presence of E_2 plus ICI, reporter gene activity was significantly greater than control or E_2 alone after 25 hr ($P<.05$). In addition, ICI treatment alone resulted in elevated reporter gene activity ($P<.05$) compared to the vehicle control, suggesting that ICI acted as an estrogen agonist when acting at an ERE site in rat1+ ERβ cells.

In contrast, estradiol treatment repressed AP-1 reporter gene expression after 9 hr ($P<.05$; Figure 5) and repression was maintained through 25 hr ($P<.05$). Treatment with ICI did not antagonize the E_2 response under these conditions, and ICI alone for 25 hr had no effect on AP-1 mediated reporter gene activity. We have previously shown that in the presence of ERα this AP-1 reporter plasmid responded to E_2 treatment with an increase in reporter expression in bovine fetal uterine cells, but not in rat-1 cells transiently transfected with either ERα or ERβ [Malayer *et al*, 1999].

Specific endogenous gene expression in response to ERβ expression and E_2 treatment was also examined. RT-PCR analysis was used to determine the effects of ERβ expression and E_2 treatment on expression of mRNA for progesterone receptor (PR), epidermal growth factor receptor (EGF-R), and ERα. Results were compared with rat-1 cells and rat-1+ERα cells under similar conditions. Expression of PR was not detected in rat-1 cells and was induced by E_2 treatment in both rat-1+ERα and rat-1+ERβ cells (Figure 6A). This result in rat-1+ERα cells was similar to findings of Kaneko et al. (Kaneko *et al.*, 1993) that ERα activation resulted in the expression of the

normally silent PR gene in this cell type and suggests an overlapping activity between ERα and ERβ at the PR gene promoter in these cells. Rat-1+ERα cells required a 10-fold higher concentration of E_2 to activate PR expression compared to rat-1+ERβ cells due to the 10-fold higher K_d of the hE0 human ER cDNA (Tora *et al.*, 1989a) used in preparation of the rat-1+ERα cell line. EGF-R mRNA was present in all cell lines and was not affected by E_2 (Figure 6B). This result was also similar to findings of Kaneko et al (Kaneko *et al.*, 1993) that ERα activation exerted no effect on expression of the constitutively-active EGF-R gene in this cell type and also suggested an overlapping activity, or lack of activity, between ERα and ERβ. There was not an increase in expression of endogenous ERα mRNA in rat-1+ERβ cells in response to E_2 (Figure 6C). Note that a very faint product appeared in lanes corresponding to rat-1 and rat-1+ERβ cells. In the rat-1 cell this apparent very low expression level did not result in a functional response to E_2 by a reporter gene (data not shown), this result was extrapolated to the rat-1+ERβ cells. Northern analysis of rat-1+ERα cells showed that ectopic ERα expression did not result in expression of the endogenous ERα gene (Kaneko *et al.*, 1993); mRNA detected by RT-PCR in rat-1+ERα cells in the present study was due to the presence of ectopic ERα gene expression. This result suggests that, in contrast to the PR gene, the ERα gene is silent in rat-1 cells and is not affected by the presence of either the ectopic ERβ or the induced PR.

As a technical control for the PCR amplification and an RNA loading control, the constitutively-expressed G_3PDH mRNA was amplified in the same PCR preparation as products described above. As shown in Figure 6, this product was present at equal concentration in each of the cell lines and was not altered by treatment with E_2.

Cell proliferation in response to estradiol treatment

In response to treatment with E_2 at a concentration of 50 pM, rat-1+ERα cells grew more rapidly over the next 72 hr than vehicle-treated controls ($P<.05$; Figure 7). The rate of cell proliferation for rat-1 cells and rat-1+ERβ cells was not affected by E_2 treatment under the same conditions. Likewise, treatment with E_2 at a concentration of 25 pM did not alter cell numbers compared to vehicle control in any of the three cell lines. As stated above, rat-1+ERα cells required a 10-fold higher concentration of E_2 to activate PR expression compared to rat-1+ERβ cells due to the 10-fold higher K_d of the hE0 human ER cDNA (Tora *et al.*, 1989a) used in preparation of the rat-1+ERα cell line. Thus, the apparent differential sensitivity to E_2 between cell lines containing each ER type, in terms of cell proliferation, is made greater due to the higher K_d of the hE0 mutant receptor.

Discussion

The purpose of this study was to generate a tool to examine separately the activities of ERα and ERβ. Very often both receptors can be found in any particular estrogen target cell, making it difficult to examine characteristics of one independently of the other within the same cellular context. Important exceptions are the studies based on the ERα knockout mouse (Lubahn *et al.*, 1993) in which ERβ has been studied independently of ERα (see for example (Krege *et al* , 1998; Couse *et al*, 1997; Iafrati *et al*, 1997). A number of studies have examined characteristics of ERβ following

bacterial expression of the protein or during transient expression in cell lines that normally do not express ER (Razandi *et al*, 1999; Paech *et al*, 1997; Kuiper *et al*, 1997; Kuiper and Gustafsson, 1997; Pace *et al*, 1997; Witkowska *et al*, 1997).

Stable expression of the receptors to examine the role of ERs has important advantages over transient expression. These include the ability to test endogenous promoter responses in the presence of the receptor in the context of interactions with chromatin assembly processes and other regulatory mechanisms involved in control of gene expression. Other advantages include the ability to examine cell responses to the ligand-bound receptor over long time periods, and the ability to examine alterations in endogenous chromatin configuration in the cell. Another advantage is the offsetting of variable signal strength as a result of variations in transfection efficiency in transient protocols. It was previously shown in rat-1 cells that responses of the endogenous PR promoter differed in the presence of stable expression of ERα versus transiently-expressed receptor (Kaneko *et al.*, 1993).

In the present study we have found that following stable expression of ERβ in rat-1 cells, E_2 treatment resulted in a positive activation of ERE-mediated reporter gene activity. Conversely, in agreement with data of Paech et al. (Paech *et al.*, 1997), treatment with E_2 inhibited transcription of an AP-1 element-mediated reporter gene. We have previously reported (Malayer *et al*, 1999) that in rat-1 cells following transient transfection of either ERβ or ERα, E_2 treatment had no effect on AP-1 element-mediated reporter gene activity, although E_2 treatment did result in increased ERE-mediated reporter activity and ICI182,780 was able to block the E_2-induced increase. In contrast, in the present study treatment with ICI resulted in a positive activation of

ERE-mediated reporter gene activity in cells expressing ERβ and there was no effect of ICI on AP-1 element-mediated transcriptional activation. These data further support a model in which ERβ signals in a manner different from ERα depending upon the type of response element, ligand, and target cell.

The activity of ER is mediated through two transactivation domains in the receptor, TAF-1 at the amino terminal end, and TAF-2, which lies in the ligand-binding domain (Berry *et al.*, 1990; Bocquel *et al.*, 1989; Danielian *et al.*, 1992; Kumar *et al.*, 1987; Lees *et al.*, 1989; Tora *et al.*, 1989b). The transactivation domains are believed to function by binding to other proteins in the cell which act as activators or repressors of transcription. The relative contribution to the ER-protein complex of co-activator or co-repressor proteins determines whether transcriptional activation will occur (Horwitz *et al.*, 1996; Onate *et al.*, 1998; Onate *et al.*, 1995; Shibata *et al.*, 1997; Spencer *et al.*, 1997; Webb *et al.*, 1998). Sequence divergence between ERα and ERβ in TAF-1 and TAF-2 suggests that interactions with co-activator or co-repressor proteins would differ at the enhancer or promoter site during modification of gene expression and, as a result, that ERα and ERβ play different roles in gene regulation. The antiestrogen ICI exhibited agonist activity in the rat-1+ERβ cells at an ERE site, evidence that interactions with co-activator rather than co-repressor proteins was favored in the conformation of the antagonist-bound ERβ.

Estradiol has been reported to induce immediate and transient activation of several proto-oncogenes, including c-jun and c-fos, in uteri of the mouse (Yamashita *et al.*, 1996) and rat (Allen *et al.*, 1997; Webb *et al.*, 1993), and these play important roles in cellular differentiation and proliferation. The AP-1 heterodimer consists of members

of the fos and jun gene families, and transcriptional activity and target gene specificity depends upon the composition of the protein complex (Angel and Karin, 1991). ERα and ERβ act through AP-1 (Paech *et al.*, 1997; Webb *et al.*, 1995) via a protein-protein interaction between the ligand-bound receptor and the DNA-bound AP-1 heterodimer. Repression of AP-1 element-mediated reporter activity by E_2 in the present study suggests that functional fos and jun family proteins were present in the rat-1+ERβ cells. These proteins were possibly expressed in response to the presence of the ectopic ERβ since we observed no AP-1 element-mediated response to E_2 when cells were transiently transfected with ERs (Malayer *et al*, 1999). Glucocorticoid receptor (GR) also binds to AP-1 and there is functional interaction between ER and GR at the AP-1 response element (Uht *et al.*, 1997). Rat-1 cells have been demonstrated to express functional GR (Tverberg and Russo, 1992) and variation between rat-1 and rat-1+ER cells may, in part, be the result of functional integration of the actions of ER and GR at AP-1 response elements.

We have found that both ERα and ERβ act upon the promoter elements of the PR gene with similar result, likely due to common action at an ERE site. While both ER types act to stimulate expression of the normally silent PR gene in rat-1 cells, it is not clear if the mechanism of action is the same. Estradiol treatment resulted in up-regulation of PR expression in the rat uterus (Kraus and Katzenellenbogen, 1993) as well as in several other species, including mouse (Hagihara *et al.*, 1994), sheep (Miller *et al.*, 1977; Spencer and Bazer, 1995), cow (Zelinski *et al.*, 1982), and guinea pig (Pasqualini *et al.*, 1980).

In contrast, expression of the normally silent ERα gene in rat-1 cells was not affected by ERβ expression. Similarly, expression of ectopic ERα in rat-1 cells had no effect on expression of the endogenous ERα gene (Kaneko *et al.*, 1993). Thus, in rat-1 cells there may be no effect of either ER type on the ERα gene at the level of transcription, or the effect may have to be mediated through AP-1 resulting in repression in this cell type due to the combination of factors discussed above.

It has long been known that E_2 treatment would result in increased cell proliferation in a variety of organs in whole animals, organ culture systems, and culture cell lines Mueller *et al*, 1972; Kaye *et al*, 1971; Stormshack *et al*, 1976; Stack and Gorski, 1984; Edwards *et al*, 1980; Aitken and Lippman, 1982). Treatment with E_2 at a concentration of 50 pM resulted in increased cell proliferation in rat-1+ERα but not rat-1+ERβ cells, suggesting that ERα plays a more central role in the mechanism of the E_2-induced increase in cell proliferation. The rat-1+ERα cells used here required a 10-fold higher concentration of E_2 to activate PR expression compared to rat-1+ERβ cells due to the 10-fold higher K_d of the hE0 human ER cDNA (Tora *et al.*, 1989a) used in preparation of the rat-1+ERα cell line. Thus, the apparent differential sensitivity to E_2 between cell lines containing each ER type, in terms of cell proliferation, was even greater due to the 10-fold higher K_d of the hE0 mutant receptor. The hypothesis that ERβ may play a lesser role in cell proliferation responses is supported by studies in the ERα knockout (ERKO) mouse generated by targeted disruption of the ERα gene (Lubahn *et al.*, 1993). In ERKO females, uteri were hypoplastic, and expressed characteristic cell types, i.e., glandular epithelium, luminal epithelium, and stroma, in reduced numbers. Using tissue separation and recombination with normal and ERKO

stroma and epithelia, it was shown that ERα in stroma was required to mediate estrogen-induced cell proliferation in epithelium and stroma (Cooke *et al.*, 1997).

In summary, these data provide evidence that ERα and ERβ have both discrete and overlapping activity within the same cell type in the presence of the same ligand. These data also confirm studies using transient ER and reporter gene expression to show that ERβ exhibited unique activity. These differences likely reflect differential binding of co-activator and co-repressor proteins to the ER in a manner dictated by promoter context. Further studies using stable culture cell lines uniquely expressing either ERα, ERβ, or both, may help to elucidate mechanisms behind the differential activities of these receptor proteins.

Table 1. Primers and Conditions used in RT-PCR Assay

mRNA species	$MgCl_2$ (μM)	Temperature (cycles)	Primer nucleotide sequence
PR	4	95°C, 1'; 58°C, 1'; 72°C, 1' (40)	5'-CCCACAGGAGTTTGTCAAGCTC-3' 5'-TAACTTCAGACATCATTTCCGG-3'
ER-β	3	95°C, 1'; 56°C, 1'; 72°C, 1' (40)	5'-TTCCCGGCAGCACCAGTAACC-3' 5'-TCCCTCTTTGCGTTTGGACTA-3'
EGF-R	6	95°C, 1'; 60°C, 1'; 72°C, 1' (30)	5'-CAGCGCTACCTTGTCATTCA-3' 5'-AAGTCCTGCTGGTAGTCAG-3'

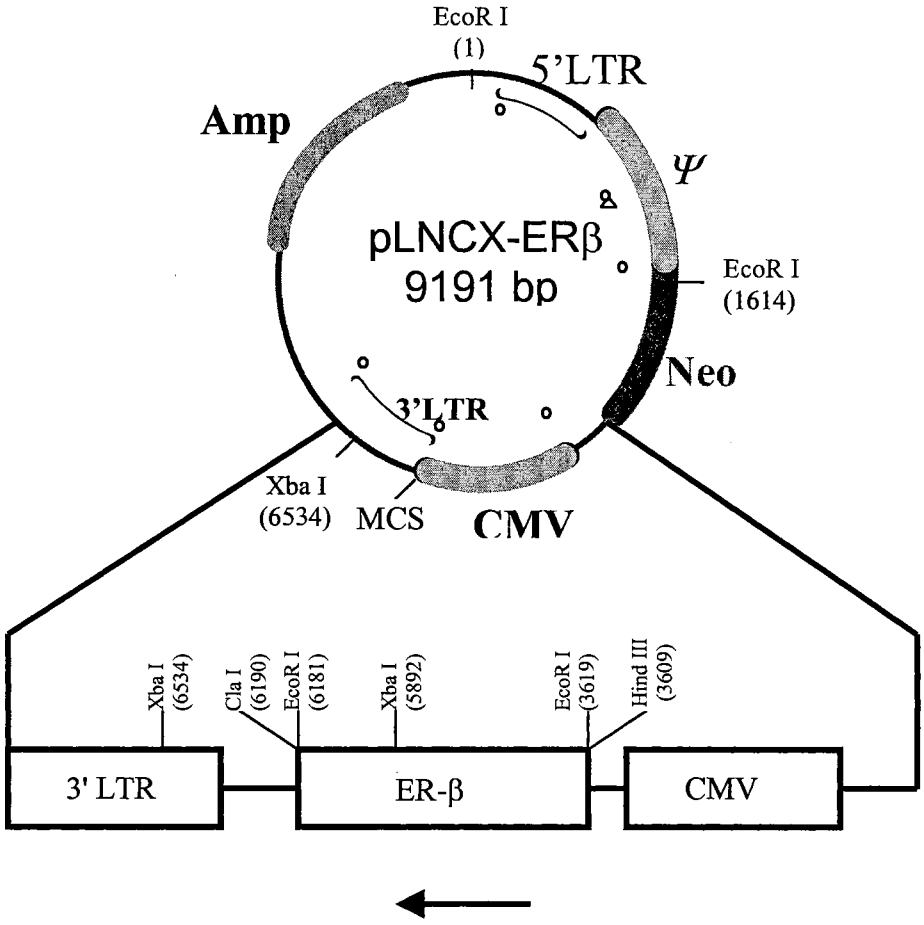

Figure 1. The plasmid pLNCX-ERβ was generated from pLNCX (RetroXpress™ System; Clontech) by insertion of the rat ER-β cDNA (provided by Dr. G. Kuiper). This construct was characterized by restriction mapping and by dideoxy chain-termination sequencing to verify the ERβ insert was intact and in the correct orientation. The 5'LTR retroviral sequence drives expression of the packaging sequence (ψ) and the *neo* selection marker. The human cytomegalovirus immediate-early gene promoter drives expression of the rat ERβ cDNA.

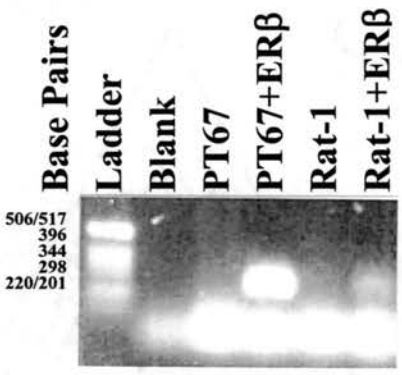

Figure 2. RT-PCR followed by agarose gel electrophoresis was used to detect the presence of ERβ mRNA in PT67 and rat-1 cells. The packaging cell line, PT67, used to generate retrovirus particles did not express detectable ERβ mRNA; following transfection with the plasmid pLNCX-ERβ, proviral RNA was detected in the PT67+ ERβ cells. Rat-1 cells, likewise, did not express detectable ERβ. Following retroviral infection as described in the text, rat-1+ERβ cells did exhibit ERβ mRNA detectable by RT-PCR.

Figure 3. Southern blot analysis was used to detect the presence of ERβ cDNA in PT67+ERβ and rat-1 cells. The packaging cell line, PT67, used to generate retrovirus particles is a rat-derived cell and did exhibit detectable endogenous ERβ DNA sequence; following transfection with the plasmid pLNCX-ERβ, proviral DNA elements were also detected in the PT67+ERβ cells. Rat-1 cells, likewise, exhibited hybridizable endogenous ERβ DNA sequence. Following retroviral infection as described in the text, there was detection in rat-1+ERβ cells of an inserted DNA element of approximately 3200 base pairs.

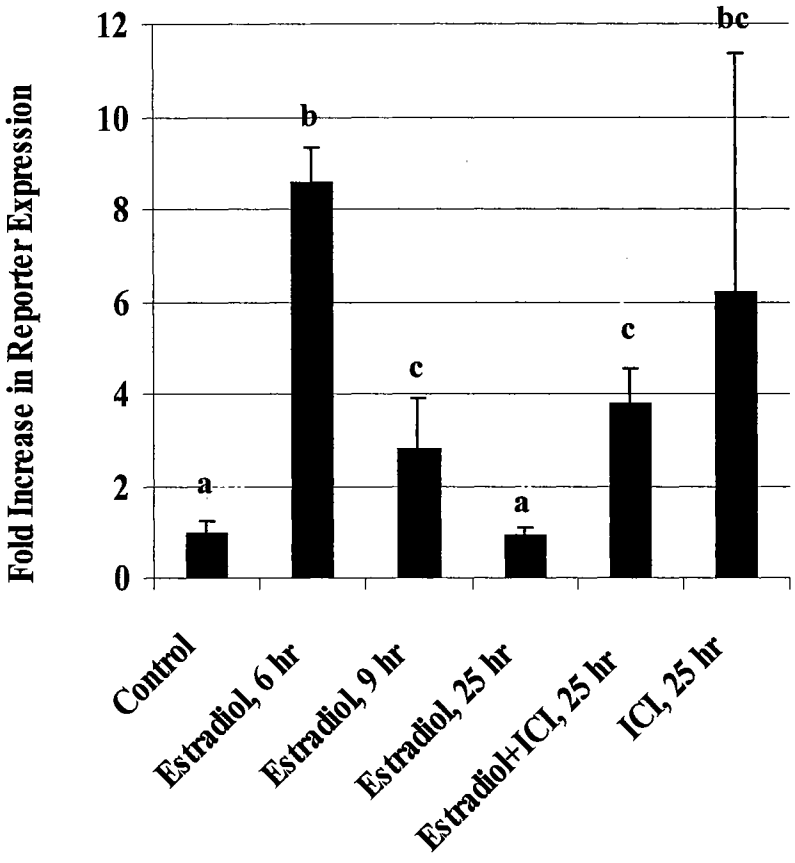

Figure 4. ERE-CAT reporter gene expression following treatment with vehicle control, estradiol, estradiol plus antiestrogen ICI 182,780 (ICI), or ICI alone. Means ± standard error with different superscripts ^{a,b,c} were significantly different (P<.05). In rat-1+ERβ cells, after 6 hr exposure to estradiol the reporter gene activity was increased 8.5-fold over control (P<.05), indicating functional ERβ activity. Reporter gene activity was maximal after 6 hr, reduced by 9 hr (P<.05), and decreased to basal activity after 25 hr exposure. Estradiol plus ICI treatment resulted in greater reporter gene activity after 25 hr compared to the vehicle control or estradiol alone (P<.05). Treatment with ICI alone resulted in elevated reporter gene activity after 25 hr (P<.05) compared to vehicle control, suggesting that ICI acted as an estrogen agonist in rat1+ ERβ cells when acting at an ERE site.

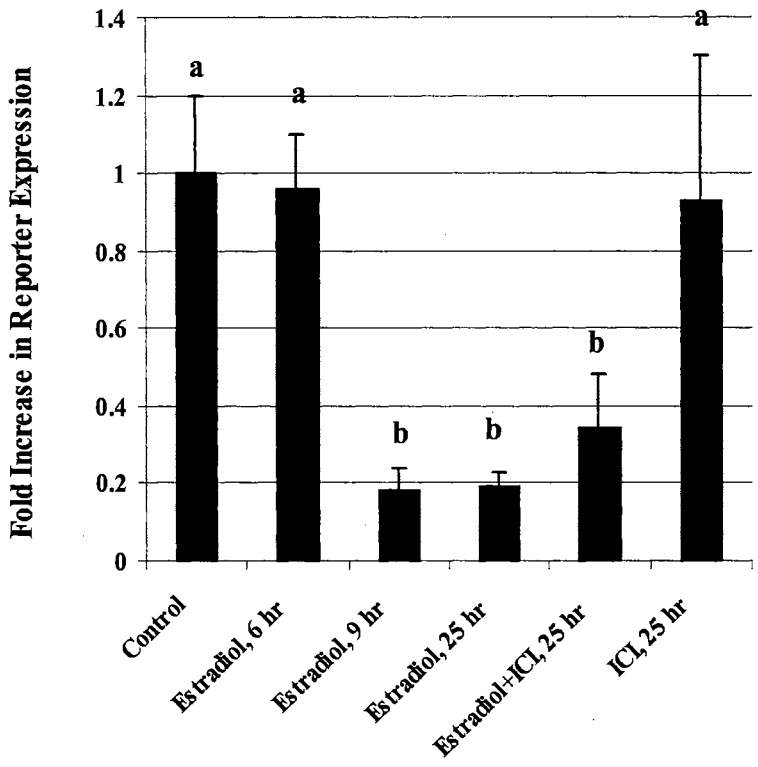

Figure 5. AP-1-CAT reporter gene expression following treatment with vehicle control, estradiol, estradiol plus antiestrogen ICI 182,780 (ICI), or ICI alone. Means ± standard error with different superscripts [a,b,c] were significantly different (P<.05). In rat-1+ERβ cells, the reporter gene activity was 20 percent of control after 9 hr exposure to estradiol (P<.05), indicating repression mediated by ERβ. Reporter gene activity in response to estradiol was repressed through 25 hr (P<.05). Estradiol plus ICI treatment resulted in reporter gene activity after 25 hr similar to estradiol alone (P<.05). Reporter gene activity following treatment with ICI alone was not different from vehicle control at 25 hr.

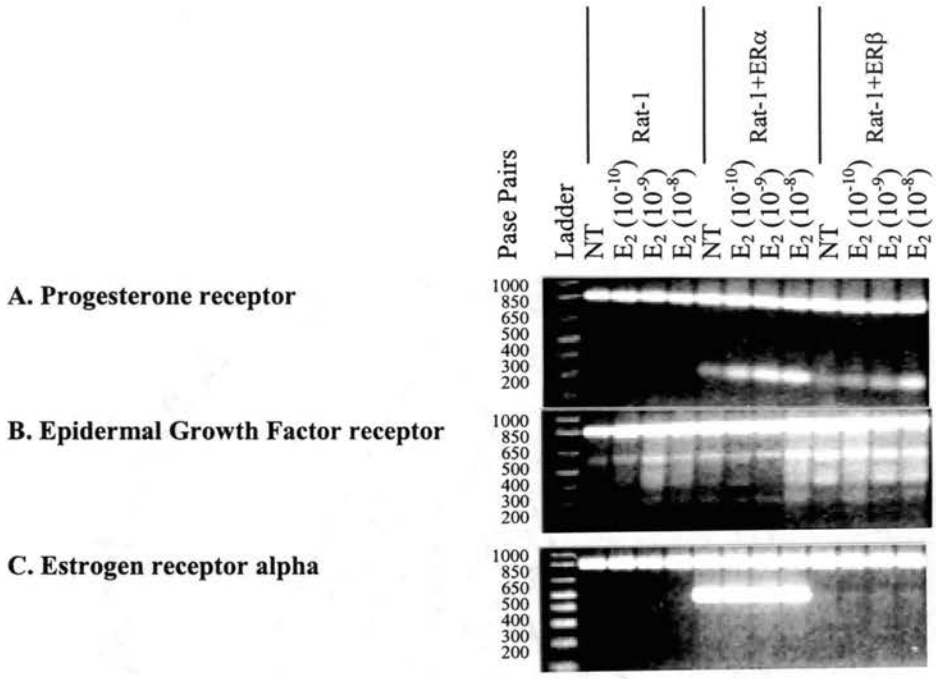

Figure 6. RT-PCR followed by agarose gel electrophoresis was used to examine the effects of estradiol treatment on endogenous promoter activity in rat-1, rat-1+ERβ, and rat-1+ERα cells. Representative results from amplifications of target cDNAs are shown. Treatments were performed on culture wells in duplicate experiments, then RNA from each well was subjected to reverse-transcription in duplicate, and PCR amplification was performed from each RT reaction in duplicate. (A). Expression of PR mRNA (369 base pair product of RT-PCR) was not detected in rat-1 cells and was induced by estradiol treatment in both rat-1+ERα and rat-1+ERβ cells. This result suggests an overlapping activity between ERα and ERβ at the PR gene promoter in these cells. (B). EGF-R mRNA (600 base pair product of RT-PCR) was present in all cell lines and was not affected by estradiol, also suggesting an overlapping activity, or lack thereof, between ERα and ERβ. (C). There was not an increase in expression of endogenous ERα mRNA (477 base pair product of RT-PCR) in rat-1+ERβ cells in response to estradiol. This result suggests that, in contrast to the PR gene, the ERα gene is silent in rat-1 cells and is not affected by the presence of either receptor. As a loading control for the PCR amplification, the constitutively-expressed G_3PDH mRNA (810 base pair product of RT-PCR) was amplified in the same PCR preparation as products described above. This product was present at equal concentration in each of the cell lines and was not altered by treatment with estradiol.

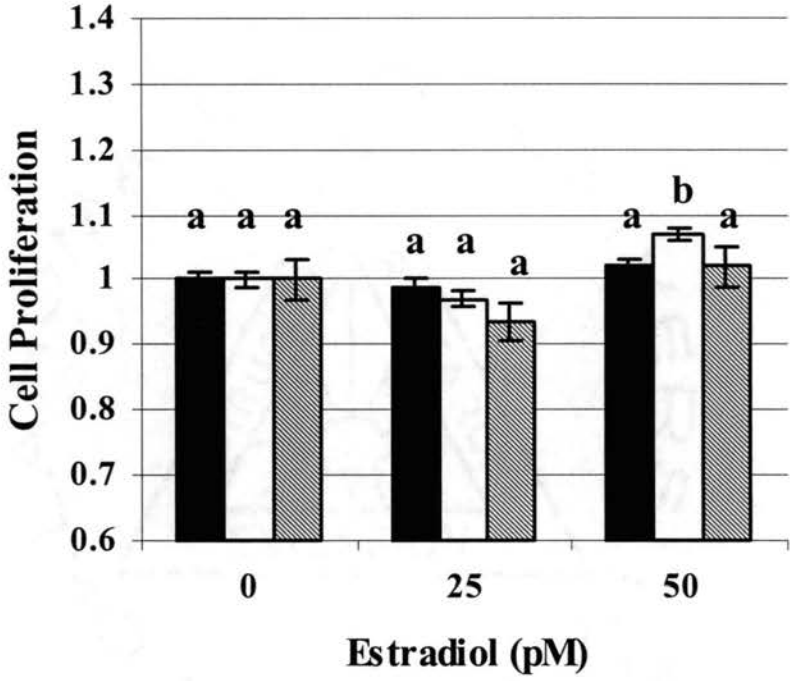

Figure 7. Effect of treatment with estradiol (25 or 50 pM) or vehicle control on cell numbers after 72 hr. Means ± standard error with different superscripts [a,b] were significantly different ($P<.05$). In response to treatment with estradiol at a concentration of 50 pM, rat-1+ERα cells (open bars) grew more rapidly over the first 72 hr than vehicle-treated controls ($P<.05$). The rate of cell proliferation for rat-1 cells (black bars) and rat-1+ERβ cells (hatched bars) was not affected by estradiol treatment under the same conditions. Treatment with estradiol at a concentration of 25 pM did not alter cell numbers after 72 hr for any of the cell lines.

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CHAPTER IV

ESTROGEN RESPONSES IN BOVINE FETAL UTERINE CELLS INVOLVE BOTH ESTROGEN RESPONSE ELEMENT AND ACTIVATOR PROTEIN-1 DIRECTED PATHWAYS

Abstract

Objectives were to examine possible roles of estrogen receptor (ER) in development of the bovine uterine endometrium in the context of ER type, enhancer type, and ligand-independent activation. Expression vectors producing either ERα or ERβ were introduced into fetal uterine cells from day 110-120 of gestation (UBF120 cells) and into rat embryo fibroblasts (Rat-1 cells), neither of which express endogenous ER. Reporter constructs containing either an estrogen response element (ERE) or activator protein-1 (AP-1) response element were co-transfected. These reporters were also transfected into fetal uterine cells from day 180-200 of gestation (UBF180 cells) which express ER. In UBF120 and Rat-1 cells transfected with either ERα or ERβ, treatment with estradiol-17β (E_2) resulted in increased activity of an ERE reporter construct, but not an AP-1 element reporter construct. The antiestrogen ICI 182,780 (ICI) exhibited E_2 antagonist activity with both ERα and ERβ. Thus, all components were present for E_2-dependent transcription from an ERE except ER, however cells

were not competent for E_2-dependent transcription mediated through AP-1. In UBF180 cells, E_2 treatment increased both ERE and AP-1 reporter activity. ICI exhibited E_2 antagonist activity. Treatment with epidermal growth factor resulted in increased ERE reporter activity which was inhibited by ICI, indicative of ligand-independent activation of ER. These data suggest that multiple pathways for ER-mediated gene regulation occur in the developing fetal uterus and that nuclear components necessary for action of both ERα and ERβ are present prior to expression of the receptor.

Introduction

Estrogens are key molecules in development, differentiation, and growth whose actions are mediated by specific cellular receptor proteins localized in the nucleus of the cell; for reviews, see (Beato, 1989; Evans, 1988; Gorski, 1986; O'Malley, 1990; Ribeiro *et al.*, 1995). We have previously shown that estrogen receptor (ER) expression in the uterus of the bovine fetus is first detected in the sixth month of pregnancy (Malayer and Woods, 1998a). It is hypothesized that ER expression at that time is essential for uterine growth and initiation of events in mucosal reorganization associated with uterine gland development.

The presence of ERα (Toft and Gorski, 1966) and ERβ (Kuiper *et al.*, 1996) have now been described in various organ systems, including the bovine uterus (Malayer *et al.*, 1998; Rosenfeld *et al.*, 1998), and have been shown to exert a variety of physiologically important activities. The global nature of estrogen action in growth and development has been demonstrated by examples of naturally occurring ER mutations

and through use of transgenic animal technology in mice (Lubahn *et al.*, 1993; Smith *et al.*, 1994). Evidence suggests that ERβ has a unique set of activities apart from ERα (Kuiper *et al.*, 1997; Paech *et al.*, 1997), and that ERα and ERβ may interact as heterodimers (Pettersson *et al.*, 1997). Studies in various tissues have shown that ER expression is the only factor lacking in estrogen-nonresponsive cells to bring about estrogen-dependent target gene expression.

It has been established in certain estrogen target cells that ligand-bound ER has the capacity to act through different types of DNA elements. The classical estrogen response element (ERE) consists of a palindromic sequence of GGTCA in an inverted repeat separated by a three base "spacer" (GGT CAC AGT GAC C), although most estrogen-responsive genes contain ERE sequences that diverge to varying degrees from this consensus sequence. In addition, ER mediates transcription from the activator protein-1 (AP-1) enhancer sequence (TGA GTC A) (Gaub *et al.*, 1990; Philips *et al.*, 1993; Umayahara *et al.*, 1994). This "AP-1-directed" mechanism involves the ER in protein-protein interactions partially independent of the ER DNA-binding activity, requires the receptor to be ligand-bound, and involves the transcription factors c-fos and *c-jun* which bind the DNA as a dimer at the AP-1 site and mediate the ER action (Webb *et al.*, 1995). In addition, it has been established that the receptor may be activated in the absence of estrogen through post-translational phosphorylation of specific serine residues, resulting in so-called ligand-independent activation (Aronica and Katzenellenbogen, 1993; Katzenellenbogen, 1996).

Our objective was to test the hypothesis that cells of the bovine fetal uterus contain components necessary for ERE and AP-1 mediated transactivation, as well as

for ligand-independent activation of ER. In addition, we wished to test whether these components were expressed in the cell prior to ER expression, or coincident with ER expression. To test these questions, cell cultures were prepared from animals early in the second trimester of pregnancy (days 110 to 120), prior to ER expression, and at the end of the second trimester (days 180 to 200) after ER expression commenced. Cells collected prior to ER expression were transfected with ERα or ERβ expression vectors and reporter gene constructs containing ERE or AP-1 enhancer elements; cells collected after ER expression were transfected with the same reporter constructs to compare activation of endogenous receptor. These data should contribute to understanding of processes related to uterine growth and maturation involving the ER.

Materials and Methods

Tissue collection and sample preparation

In order to obtain fetal bovine tissues, entire uteri were removed from pregnant, crossbred beef cows at a commercial abattoir. The gestational age (GA) of each fetus was estimated by fetal crown-rump measurement (Rexroad *et al.*, 1974). Fetal uteri were dissected free of adipose and connective tissue, placed in sterile tubes containing phenol red-free Dulbecco's modified Eagle's medium (DMEM; Gibco-BRL, Gaithersburg, MD) containing 1x antibiotic-antimycotic (ABAM; Gibco-BRL), Hepes (5 mM; Sigma, St Louis, MO), and sodium bicarbonate (Sigma) and transported to the laboratory.

Fetal reproductive tract tissues were used to prepare primary cell cultures as previously described (Malayer and Woods, 1998b). The cultures were mixed

populations of epithelial and stromal cells that equally expressed both vimentin and cytokeratin, as determined by immunostaining [23]. Cells used in these experiments represent pooled samples from three individuals in each of the age ranges described. These cells from the "uterus of the bovine fetus" (UBF cells) were plated in 6-well culture plates (Becton-Dickinson Labware, Franklin Lakes, NJ) on collagen matrices (Type I collagen, from calf skin, 10 $\mu g/cm^2$; Sigma). Cells were maintained in phenol red-free DMEM containing 1x ABAM, 5 mM Hepes, and sodium bicarbonate supplemented with 10% fetal bovine serum (FBS; Hyclone, Logan, UT), and insulin (0.6 μg/ml; porcine insulin; Gibco-BRL). Cultures were maintained at 37°C in a humidified atmosphere of 95% air, 5% CO_2 and fed every 48 h. Following one to two passages in culture, cells were frozen for use in experiments described below.

A series of control experiments was carried out in a rat embryo fibroblast cell line (Rat-1 cells; (Freeman *et al.*, 1970)) to determine that transient transfection of gene constructs, described below, resulted in functional expression and hormone response. These cells were maintained in phenol red-free DMEM containing 1x ABAM, 5 mM Hepes, and sodium bicarbonate supplemented with 10% FBS, and insulin (0.6 μg/ml). Cultures were maintained at 37°C in a humidified atmosphere of 95% air, 5% CO_2 and fed every 48 h.

Reverse-transcription polymerase chain-reaction (RT-PCR)

RT-PCR was used to detect specific transcripts in RNA obtained from individual uterine tissue samples. Total RNA was extracted from cultures by guanidinium thiocyanate extraction (Chomczynski and Sacci, 1987). All solutions and

glassware used in preparation and analysis of RNA were treated with diethylpyrocarbonate (Sigma). Two micrograms of total RNA was denatured by heating to 95°C and reverse-transcribed in the presence of random hexamers (pdN_6; 100 pmole; Pharmacia, Piscataway, NJ), dATP, dTTP, dCTP, and dGTP (dNTPs; 1 mM; Pharmacia), $MgCl_2$, RNase inhibitor (20 U per reaction; Promega, Madison, WI), and reverse transcriptase (Superscript™, 200 U per reaction; Gibco-BRL) at 37°C for 75 min. The reaction was stopped by heating to 95°C. Aliquots of reverse-transcribed cDNA (1 to 5 μl) were denatured by heating to 95°C and subjected to polymerase chain-reaction in the presence of 75 pmole specific primers, $MgCl_2$, dNTPs (1 mM), and Amplitaq™ DNA polymerase (0.5 U per reaction; Perkin-Elmer, Foster City, CA). Conditions used for PCR were: 95°C, 1 min.; 56°C, 1 min.; 72°C, 1 min.; 40 cycles. Products of RT-PCR were resolved on 3% agarose-TAE gels [Tris-acetate (40 mM), EDTA (1 mM)], and visualized by staining with ethidium bromide. ERβ products (primers 5'-TTC CCG GCA GCA CCA GTA ACC-3'; 5'-TCC CTC TTT GCG TTT GGA CTA-3'; (Kuiper *et al.*, 1996)) were expected to be 262-base pairs (bp). Representative RT-PCR products were excised from agarose and tested by dideoxy chain-termination sequencing (Applied Biosystems, Foster City, CA; Model 373A Automated Sequencer, OSU Recombinant DNA/Protein Resource Facility). The identity of the product was verified in a sequence homology analysis using the Basic Local Alignment Search Tool (BLAST; (Altschul *et al.*, 1990).

Plasmid constructs

The plasmid pBLCAT2 (Luckow and Schutz, 1987) contains the minimal thymidine kinase promoter from herpes simplex virus (HSV-tk) driving expression of the coding region of the chloramphenicol acetyltransferase (CAT) reporter gene. To confer estradiol responsiveness, the 15-bp consensus ERE from the *Xenopus vitellogenin A2* gene (GGT CAC AGT GAC C) was inserted into the *XbaI* restriction site, immediately upstream of the HSV-tk, to produce the pERE15 plasmid (Luckow and Schutz, 1987). Both pBLCAT2 and pERE15 are pUC-derived plasmids and thus contain an AP-1 enhancer in the region 5' of the HSV-tk (Kushner *et al.*, 1994). To remove this confounding regulatory element, we digested these plasmids with *NdeI* and *EcoO109* as described by Kushner et al [28], followed by religation, to generate pBLCAT2ΔNdeI-EcoO109 and pERE15ΔNdeI-EcoO109, hereafter called pBLCAT and pERE-CAT, respectively. The unmodified pBLCAT2 plasmid was used directly to compare effects of the presence of an AP-1 element, for clarity this plasmid was renamed pAP-1-CAT. This resulted in a series of three CAT reporter plasmids (Figure 1) containing: 1) no enhancer element (pBLCAT, control); 2) an ERE alone (pERE-CAT); or 3) an AP-1 element alone (pAP-1-CAT). The plasmid, pBLCAT3 (Luckow and Schutz, 1987), was also digested with *NdeI* and *EcoO109* to remove the AP-1 element, then religated to produce a negative control plasmid with no promoter or enhancer elements. In order to control for variation in transfection efficiency, the plasmid pSV-β-gal (Promega) was used to co-transfect the cells. This plasmid served as marker for relative transfection efficiency through basal expression of the β-galactosidase reporter that was assayed in the same cell extracts used to measure the CAT reporter activity. CAT reporter data was normalized to the β-galactosidase activity

to account for the variation in response due to variation in efficiency of reporter transfection and cell numbers.

To confer ER expression on Rat-1 cells and cells from day 110-120 of gestation (UBF120 cells), the cDNAs for human ERα (HEG0; (Tora *et al.*, 1989)) and rat ERβ (Kuiper *et al.*, 1996) were inserted into the expression plasmid pBKCMV (Stratagene, La Jolla, CA) to produce pBKCMV-ERα and pBKCMV-ERβ, respectively (Figure 1). These plasmids contain the human cytomegalovirus (CMV) immediate early promoter driving expression of the inserted ER cDNA. The 3' end of the cDNA is adjacent to the SV40 3' splice site and polyadenylation signal. Both plasmids were analyzed by restriction fragment analysis and DNA sequence analysis to verify insertion of full-length cDNA.

Transient transfection and hormone treatments

To examine hormone-response, cells were placed in phenol red-free DMEM supplemented with 10% dextran-charcoal stripped FBS (CS-FBS) for 48 h. Dextran-charcoal stripping was performed as described by Horwitz et al (Horwitz *et al.*, 1976). Plasmid DNAs were co-transfected by cationic liposome-mediated transfection (Transfectam®; Promega) into Rat-1, UBF120 (0.5 μg of each plasmid DNA; 1.5 μg total DNA per culture well), and UBF180 cells (0.5 μg of each plasmid DNA; 1.0 μg total DNA per culture well). The transfection reagent and DNAs were prepared according to manufacturer's recommendations and incubated with the cells for 16 h. Following a 24 h recovery period in DMEM supplemented with 10% CS-FBS, cells were refed using medium supplemented with 10% CS-FBS and incubated for 48 h.

Culture wells of transfected Rat-1 cells were then treated in duplicate for 12 h in the presence of estradiol-17β (E_2; 1.0 nM; Sigma), E_2 plus the antiestrogen ICI 182,780 (10 nM; Zeneca Pharmaceuticals, Macclesfield, UK), or ethanol vehicle. Each culture well was then assayed in duplicate for a response characterized by increased CAT expression. The entire experiment was then repeated; reported results are based on three to six replicates of each experiment as described in the figure legends. Similarly, cultures of transfected fetal bovine cells collected at 110-120 days gestational age (UBF120 cells) or at 180-200 days gestational age (UBF180 cells) were treated in duplicate wells for 12 h in the presence of E_2 (1.0 nM), E_2 plus ICI 182,780 (10 nM), or ethanol vehicle and each well assayed in duplicate for a response characterized by increased CAT expression. Reported results are based on three replicates of each experiment. To test for ligand-independent ER activation in fetal uterine cells, cultures of transfected UBF180 cells were treated in duplicate wells for 2 h in the presence of epidermal growth factor (EGF; Promega; 1.0 μM), EGF plus ICI 182,780 (10 nM), or ethanol vehicle. Each well was then assayed in duplicate for a response characterized by increased CAT expression. Reported results are based on three replicates of the experiment. All cultures of UBF cells were used during passages two through four. Based on the rate of expansion of a fixed number of cells to confluency in 60 mm^2 culture wells (Becton-Dickinson Labware) each passage was estimated to contain five doublings of the cell population, or five generation times.

CAT Assay

Chloramphenicol acetyltransferase enzyme activity was assayed in duplicate in cell lysates by incubating in the presence of [^{3}H]chloramphenicol and n-butyryl-coenzyme A, then measuring the acetylated [^{3}H]chloramphenicol present in the organic (xylene) phase following two-phase partitioning (CAT Enzyme Assay System Kit; Promega). The radioactivity present in the acetylated chloramphenicol was determined by scintillation spectroscopy. Enzyme activity was compared to cells not transfected as a negative control; [^{3}H]chloramphenicol was also measured in the xylene phase following extraction in the absence of cell lysates to determine background radioactivity following two-phase partitioning.

Control assays for β-galactosidase activity were carried out using the same cell extracts as were used for the CAT assays. The β-galactosidase enzyme assay system (Promega) was based on spectrophotometric detection of the cleavage product of the substrate o-nitrophenyl-β-D-galactopyranoside.

Statistical analysis

Values obtained in the assay of CAT enzyme activity were normalized to β-galactosidase activity to correct for variation in transfection efficiency and cell numbers. The resulting ratios were used to calculate means within each cell type representing duplicate sampling of cell cultures prepared and subjected to each treatment in duplicate within each experiment. Data were collected from similar experiments replicated from three to six times and were expressed in arbitrary units relative to controls to allow statistical analysis of data from separate experiments. The relative number, given as fold induction, was calculated as the ratio of treatment with

hormone or hormone plus ICI 182,780 to the vehicle treatment data point. Overall treatment means were calculated based on data from three to six separate experiments as described in each figure legend. Standard error was calculated for each averaged point except the vehicle control, which was set to 1. Fold induction data were subjected to one-way analysis of variance using the data analysis package of Microsoft Excel 97 to test effects of hormone treatment on each enhancer-element type. Comparisons were made of the effects of E_2 or EGF treatment versus control, of E_2 or EGF plus ICI versus control, and of E_2 or EGF treatment versus E_2 or EGF plus ICI.

Results

As determined by RT-PCR, ERβ mRNA was present in fetal uterine cells collected at both 120 and 180 days of gestation (Figure 2). We previously have shown that ERα mRNA was present in the fetal uterus during this same time frame, but ligand binding and a functional hormone response was present only after day 160 of pregnancy (Malayer and Woods, 1998a). In addition, ERα mRNA and a functional hormone response were present in UBF180 cells under these conditions (Malayer and Woods, 1998b), but there was no estrogen response in UBF120 cells. Rat-1 cells do not exhibit estrogen ligand binding (Kaneko *et al.*, 1993).

When Rat-1 cells were transiently-transfected with ERα, treatment with E_2 for 12 hours resulted in a 2-fold increase in CAT reporter activation ($P<0.001$) when an ERE enhancer element was present (pERE-CAT; Figure 3). Treatment with E_2 plus ICI 182,780 effectively antagonized this estradiol-induced rise. There was no effect of E_2 on CAT reporter activation when an AP-1 enhancer element was present (pAP-1-CAT).

Similarly, there was no effect in the absence of an enhancer element (pBLCAT), or in the absence of the promoter (pBLCAT3).

When Rat-1 cells were transiently-transfected with ERβ, treatment with E_2 for 12 hours resulted in a 1.7-fold increase in CAT reporter activation ($P<0.06$) when an ERE enhancer element was present (pERE-CAT; Figure 4). Following treatment with E_2 plus ICI 182,780, mean CAT reporter activity was not different from control, but also not different from the E_2 treatment mean. There was no effect of E_2 treatment on CAT reporter activation when an AP-1 enhancer element was present (pAP-1-CAT). Similarly, there was no effect in the absence of an enhancer element (pBLCAT), or in the absence of the promoter (pBLCAT3).

When UBF120 cells were transiently-transfected with ERα, treatment with E_2 for 12 hours resulted in a 2.5-fold increase in CAT reporter activation ($P<0.05$) when an ERE enhancer element was present (pERE-CAT; Figure 5, left). Treatment with E_2 plus ICI 182,780 antagonized this estradiol-induced rise, although activation remained elevated compared to control values. There was no effect of E_2 treatment on CAT reporter activation when an AP-1 enhancer element was present (pAP-1-CAT; Figure 5, right). When UBF120 cells were transiently-transfected with ERβ, treatment with E_2 for 12 hours resulted in a 1.8-fold increase in CAT reporter activation ($P<0.05$) when an ERE enhancer element was present (pERE-CAT; Figure 5, left). Treatment with E_2 plus ICI 182,780 antagonized this E_2-induced rise; similarly, activation remained elevated compared to control values. There was no effect of E_2 treatment on CAT reporter activation when an AP-1 enhancer element was present (pAP-1-CAT; Figure 5, right).

Exposure of UBF180 cells to E_2 for 12 hours resulted in a 4.7-fold increase in CAT reporter activation ($P<0.01$) when an ERE enhancer element was present (pERE-CAT; Figure 6). When an AP-1 enhancer element was present (pAP-1-CAT), E_2 treatment resulted in a 1.7-fold rise ($P<0.05$) in CAT reporter activation. Similar to Rat-1 cells, there was no effect in the absence of an enhancer element (pBLCAT), or in the absence of the promoter (pBLCAT3).

Exposure of UBF180 cells to EGF for 2 hours resulted in a 1.75-fold increase in CAT reporter activation ($P<0.01$) when an ERE enhancer element was present (pERE-CAT; Figure 7). Treatment with EGF plus ICI 182,780 effectively antagonized this EGF-induced rise, suggesting reporter gene activation mediated through the ER.

Discussion

In Rat-1 cells and in cells from the bovine fetal uterus at day 110-120 of gestation, following transfection with either ERα or ERβ, E_2 treatment resulted in increased reporter gene activity in the presence of an ERE, but not in the presence of an AP-1 response element. These data suggest that in both of these cell types, all components were present for estrogen-dependent gene activation from an ERE except ER. The cells were not competent for estrogen-dependent gene activation mediated through AP-1, suggesting that components of the AP-1 heterodimer were lacking. The antiestrogen ICI 182,780 acted as an estrogen antagonist in Rat-1 cells.

Cells from the bovine fetal uterus at day 180-200 of gestation express endogenous ER (Malayer and Woods, 1998a). In these cells, E_2 treatment resulted in increased reporter gene activity in the presence of an ERE or an AP-1 response element.

This suggests that in UBF180 cells all components were present for estrogen-dependent gene activation from an estrogen response element including the ER. The cells were also competent for estrogen-dependent gene activation mediated through AP-1, suggesting that components of the AP-1 heterodimer begin to be expressed between 120 and 180 days of gestational age, possibly in response to expression of the ER itself. Further, treatment with EGF stimulated gene activation from an ERE, demonstrating the phenomenon of ligand-independent activation of the ER. It is noteworthy that ICI 182,780 exhibited mixed agonist-antagonist activity in the presence of estradiol in some experiments. At the time points reported in this study, antagonist activity was consistent. At longer incubation times up to 24 hours, agonist activity was often observed. In general, agonist activity of ICI plus estradiol treatment was coincident with reduction of estradiol stimulation back to baseline values when estradiol alone was used, suggesting a differential time-course for estradiol action in the presence and absence of ICI.

The ERβ was first identified in the rat (Kuiper *et al.*, 1996), and homologs in the mouse (Tremblay *et al.*, 1997) and human (Mosselman *et al.*, 1996) have been cloned; there is recent evidence for expression of an ERβ in the bovine uterus (Figure 2) and (Malayer *et al.*, 1998; Rosenfeld *et al.*, 1998). Sequence alignment analysis shows an expected high degree of homology in ERβ sequence among the bovine, rat, mouse, and human. Comparison of the ERβ with ERα sequence revealed a high degree of conservation in the C-domain (95%) and E-domain (60%), suggesting that the two ER forms would bind to DNA and ligand in a similar manner. This has been established (Kuiper *et al.*, 1997; Paech *et al.*, 1997), although several ligands exhibit significantly

different binding characteristics with the two receptors, including: estriol, estradiol-17β, tamoxifen, and the phytoestrogens coumestrol and genistein (Kuiper *et al.*, 1997). The ERα and ERβ sequences diverge significantly in the regions of the receptors involved with transactivation (TAF-1 and TAF-2). Specific interactions between the ER and these co-activator proteins occur at the amino-terminal transactivator functional domain, TAF-1, and the C-terminal transactivator domain, TAF-2 (Horwitz *et al.*, 1996; O'Malley, 1990; Onate *et al.*, 1998; Onate *et al.*, 1995; Shibata *et al.*, 1997; Spencer *et al.*, 1997). These interactions are critical to ER transactivation, and steroid receptors may compete for binding to these co-activation factors (Meyer *et al.*, 1989). This divergence suggests that interactions with co-activator proteins would differ at the enhancer or promoter site during modification of gene expression and, as a result, that ERα and ERβ play different roles in gene regulation. If this is true, then both sets of interacting proteins were already present in UBF120 and Rat-1 cells suggesting a low degree of specialization. Further complexity arises from the findings that ERα and ERβ may operate together as heterodimers (Pettersson *et al.*, 1997). Additionally, more than one ERβ transcript may be present in target tissues (Chu and Fuller, 1997; Maruyama *et al.*, 1998; Moore *et al.*, 1998).

ERα and ERβ act through AP-1 (Paech *et al.*, 1997; Webb *et al.*, 1995) via a protein-protein interaction between the ligand-bound receptor and the DNA-bound AP-1 heterodimer. The AP-1 heterodimer consists of members of the fos and jun gene families. In general, fos family members combine with jun proteins to form heterodimers. Jun family members can also form heterodimers or homodimers with other jun proteins. Transcriptional activity and target gene specificity depend upon the

composition of the protein complex (Angel and Karin, 1991). Estrogen induced immediate and transient activation of several proto-oncogenes, including *c-jun* and *c-fos*, in uteri of the mouse (Yamashita *et al.*, 1996) and rat (Allen *et al.*, 1997; Webb *et al.*, 1993), and these play important roles in cellular differentiation and proliferation. Activation of *c-jun* was limited to stromal and myometrial cells, while activation of *c-fos* was specific to luminal and glandular epithelium in both the mouse (Yamashita *et al.*, 1996) and rat (Nephew *et al.*, 1995; Papa *et al.*, 1991). It is reasonable to suggest that an early response to ER expression at the end of the sixth month of pregnancy in the bovine fetal uterus is cell type-specific activation of *c-fos* and *c-jun*.

The ER undergoes phosphorylation and dephosphorylation as part of the process of transition from a hormone-bound to hormone-unbound state. This process involves specific kinase and phosphatase enzymes and was mediated by phosphorylation at a tyrosine residue (Auricchio *et al.*, 1987; Migliaccio *et al.*, 1991; Migliaccio *et al.*, 1989). The receptor also contains several other target sites for protein kinases and appeared to be phosphorylated at specific serine residues in the presence of activated protein kinase A or protein kinase C signal transduction pathways (Le Goff *et al.*, 1994). In the rat uterus, stimulation of ER mediated transcription and serine phosphorylation on the receptor were caused by estrogen, by cyclic 3'-5' adenosine monophosphate (cAMP), and by insulin-like growth factor-1 (IGF-1) (Aronica and Katzenellenbogen, 1993). Phosphorylation of ER in the absence of estrogen resulting in ligand-free activation of the receptor was likely mediated through mitogen-activated protein kinase (Kato *et al.*, 1995). IGF-1 was present in the bovine fetal blood circulation in increasing concentration throughout the last third of gestation (Holland *et*

al., 1997). The bovine fetal uterus contains mRNA for EGF-R (Malayer and Woods, 1998a), and EGF-stimulation of ER-mediated transcriptional activity was present in UBF180 cells. These data suggest a role for ligand-independent activation of ER activity mediated by post-translational modification of the receptor protein in late pregnancy in the developing bovine uterus.

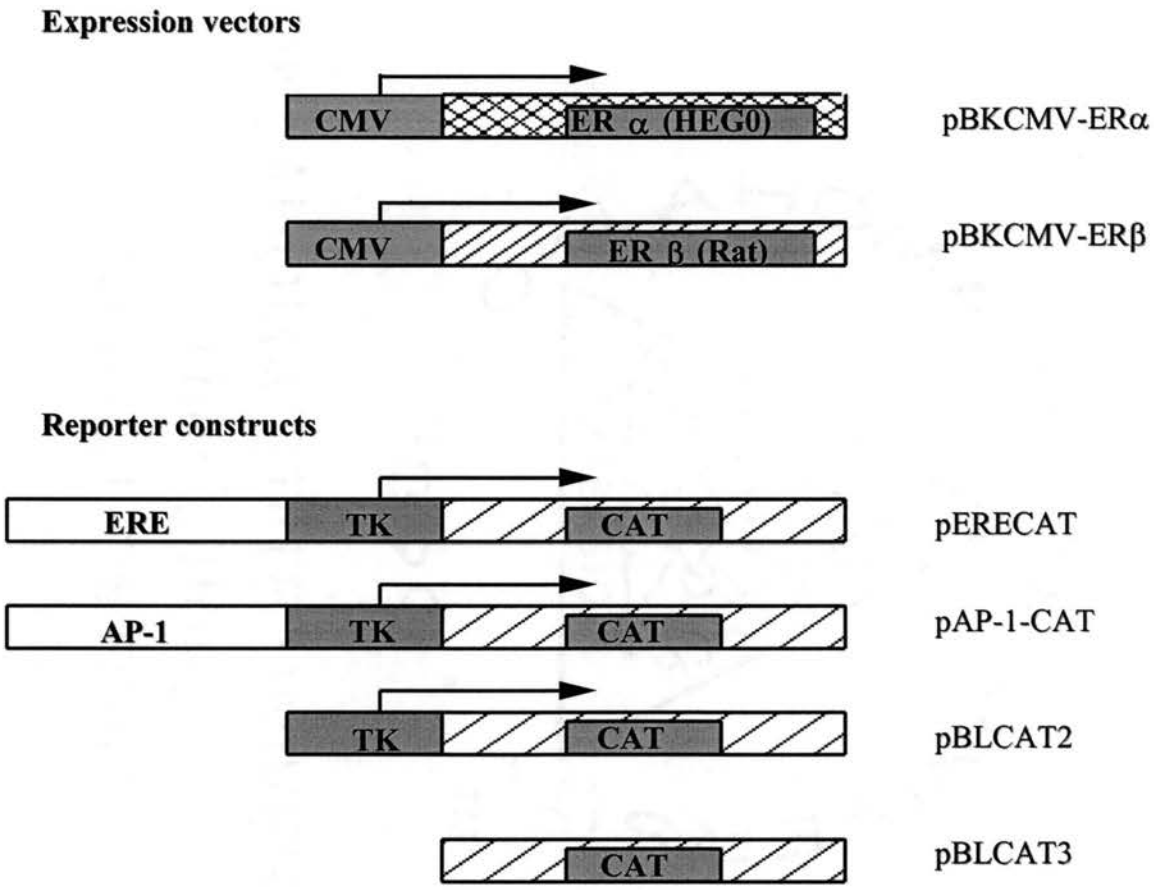

Figure 1. Structure of the expression vectors and CAT reporter constructs used in the study. In the pBKCMV vector, expression of ERα or ERβ was driven by the CMV immediate-early promoter. Reporter constructs were developed from the pBLCAT2 plasmid and contained ERE or AP-1 enhancer elements in the presence of the HSV-tk promoter.

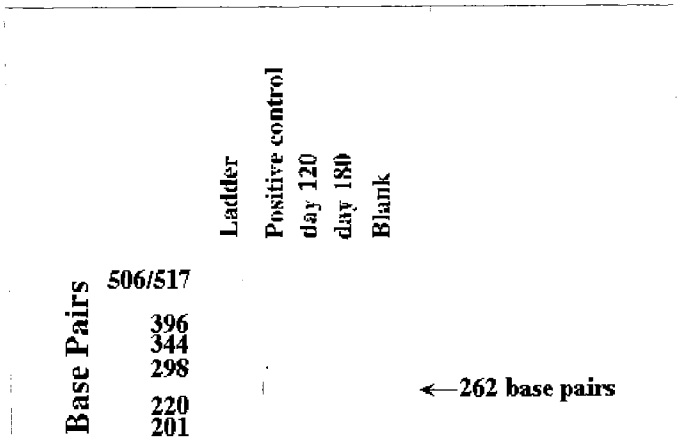

Figure 2. Agarose gel electrophoresis of RT-PCR of ERβ mRNA in fetal uterine cells collected at 120 and 180 days of gestation. The 262-bp product of RT-PCR was isolated and sequenced for identification. No product was observed in the blank control reaction. The positive control was the same 262-bp fragment of bovine ERβ previously subcloned into the Bluescript cloning vector (Stratagene) and verified by sequence analysis.

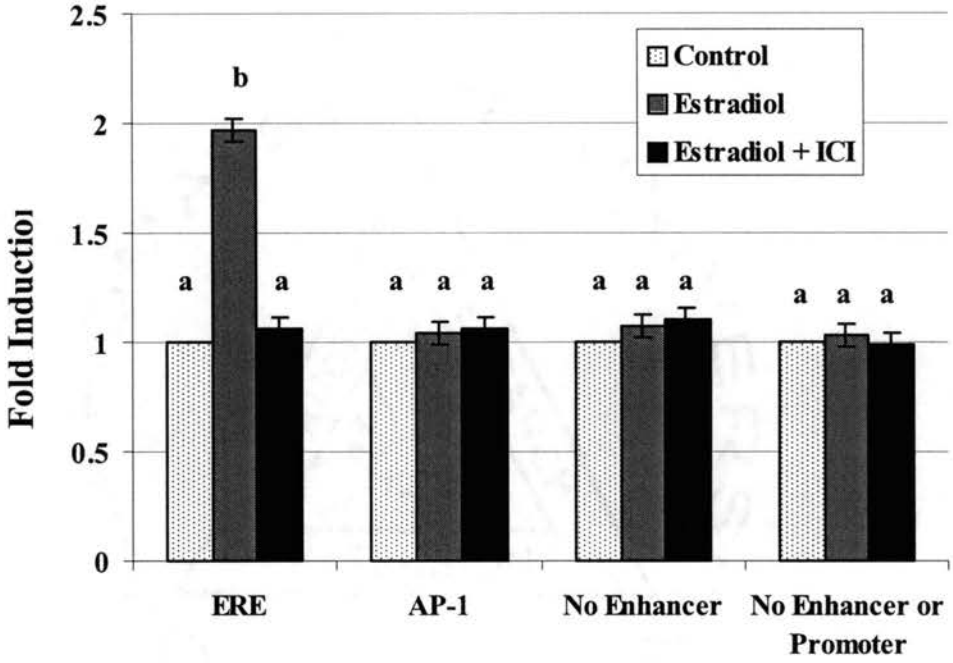

Figure 3. Mean values ± standard error of acetylated [^{3}H]chloramphenicol expressed as fold-increase over control in Rat-1 cells. Cells were transiently-transfected with a vector expressing ERα and a reporter plasmid having an ERE (pERE-CAT), an AP-1 element (pAP-1-CAT), no enhancer (pBLCAT), or no promoter or enhancer (pBLCAT3). Cultures were incubated for 12 hours with vehicle (stippled bars), E_2 (hatched bars), or E_2 plus ICI 182,780 (black bars). Each mean represents duplicate assay of cell cultures prepared in duplicate in each experiment, replicated 3 to 6 times. Means within each reporter plasmid classification with different superscripts[a,b] were different ($P<0.001$).

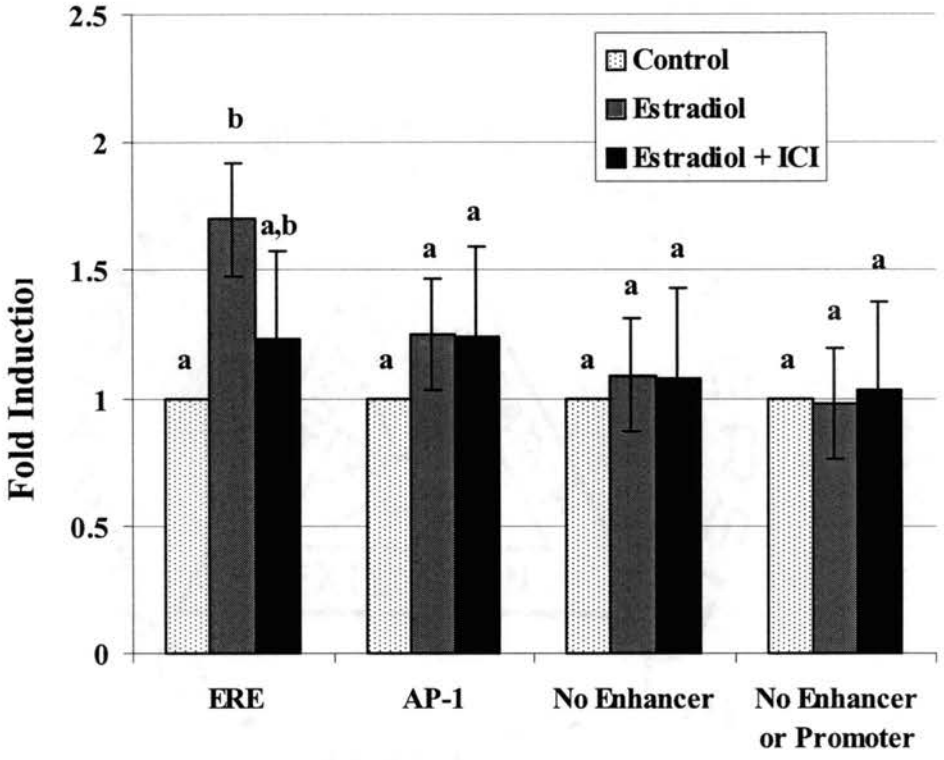

Figure 4. Mean values ± standard error of acetylated [^{3}H]chloramphenicol expressed as fold-increase over control in Rat-1 cells. Cells were transiently-transfected with a vector expressing ERβ and a reporter plasmid having an ERE (pERE-CAT), an AP-1 element (pAP-1-CAT), no enhancer (pBLCAT), or no promoter or enhancer (pBLCAT3). Cultures were incubated for 12 hours with vehicle (stippled bars), E_2 (hatched bars), or E_2 plus ICI 182,780 (black bars). Each mean represents duplicate assay of cell cultures prepared in duplicate in each experiment, replicated 3 times. Means within each reporter plasmid classification with different superscripts[a,b] were different ($P<0.06$).

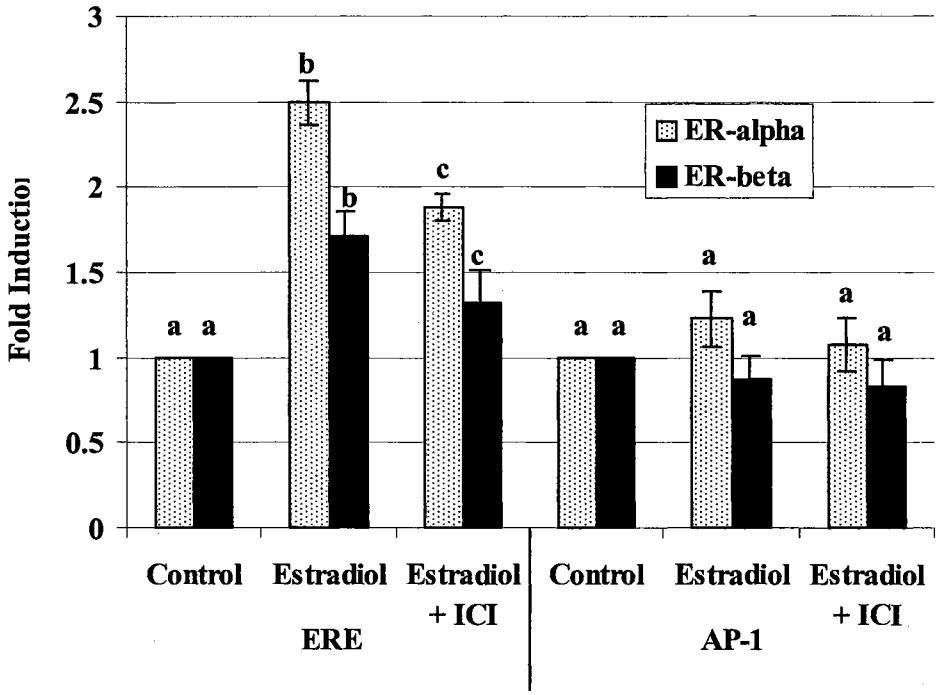

Figure 5. Mean values ± standard error of acetylated [^{3}H]chloramphenicol expressed as fold-increase over control in UBF120 cells. Cells were transiently-transfected with a vector expressing ERα (stippled bars) or ERβ (black bars) and a reporter plasmid having an ERE (pERE-CAT; left), or an AP-1 element (pAP-1-CAT, right). Cultures were incubated for 12 hours with vehicle (control), E_2 , or E_2 plus ICI 182,780. Each mean represents duplicate assay of cell cultures prepared in duplicate in each experiment, replicated 3 times. Means within each treatment classification with different superscripts^{a,b,c} were different ($P<0.05$).

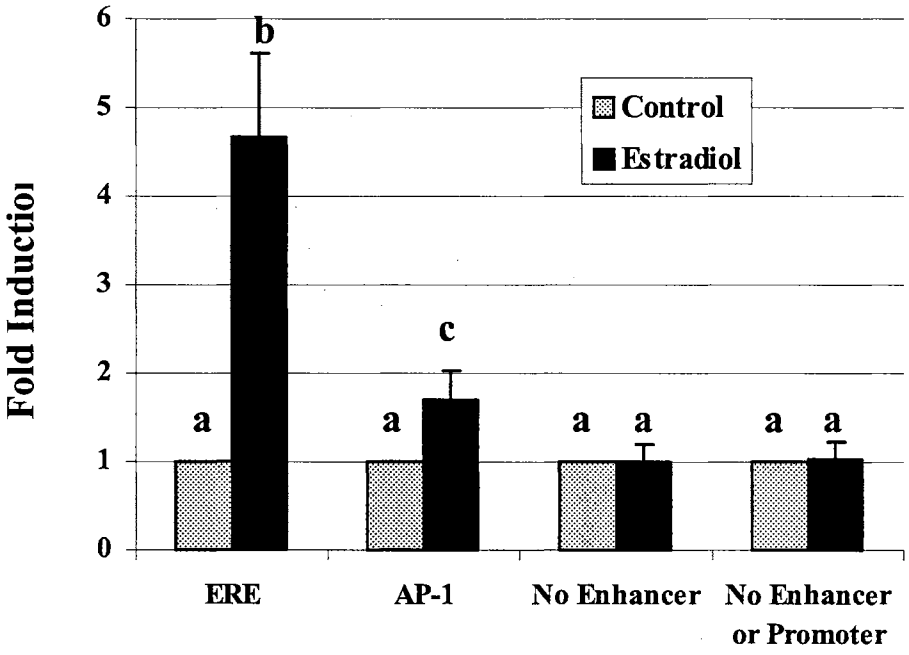

Figure 6. Mean values ± standard error of acetylated [^{3}H]chloramphenicol expressed as fold-increase over control in UBF180 cells. Cells were transiently-transfected with a reporter plasmid having an ERE (pERE-CAT), an AP-1 element (pAP-1-CAT), no enhancer (pBLCAT), or no promoter or enhancer (pBLCAT3). Cultures were incubated for 12 hours with vehicle (stippled bars) or E_2 (black bars). Each mean represents duplicate assay of cell cultures prepared in duplicate in each experiment, replicated 3 times. Means with different superscripts^{a,b,c} were different ($P<0.05$).

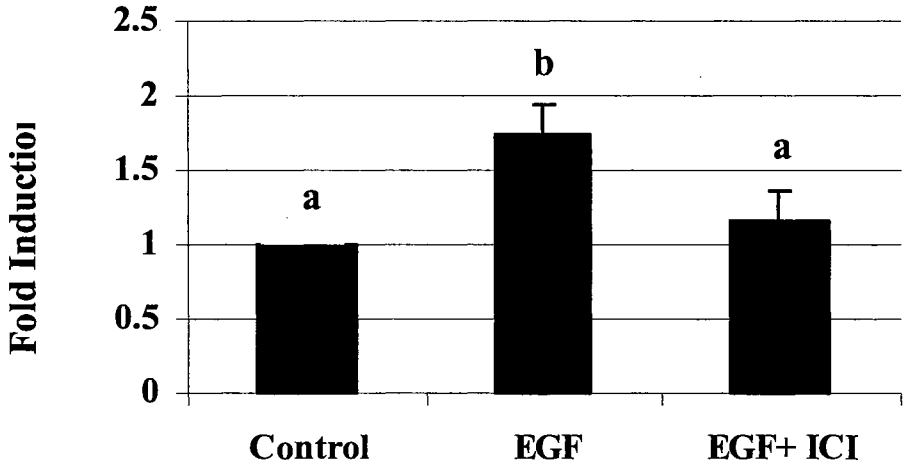

Figure 7. Mean values ± standard error of acetylated [^{3}H]chloramphenicol expressed as fold-increase over control in UBF180 cells. Cells were transiently-transfected with a reporter plasmid having an ERE (pERE-CAT). Cultures were incubated for 2 hours with vehicle (control), EGF, EGF plus ICI 182,780. Each mean represents duplicate assay of cell cultures prepared in duplicate in each experiment, replicated 3 times. Means with different superscripts^{a,b} were different ($P<0.05$).

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CHAPTER V

EFFECTS OF INSULIN ON ESTROGEN ACTION IN A RAT FIBROBLAST CELL LINE IN WHICH THERE IS STABLE ECTOPIC EXPRESSION OF ESTROGEN RECEPTOR BETA

Abstract

The objective of these experiments was to examine interactions between the insulin-signaling pathway and estrogen receptor (ER) mediated signal transduction. Rat-1 fibroblast cells expressing either ERα (rat-1+ERα) or ERβ (rat-1+ERβ) were grown in phenol red-free Dulbecco's modified Eagle's medium in the presence or absence of supplemental insulin. Cells were transfected with reporter plasmids to examine both ERE- (pERE-CAT) and AP-1- (pAP-1-CAT) mediated ER activity, and treated with 17-β estradiol (E_2) or ethanol vehicle (control). In rat-1+ERα cells transfected with pERE-CAT, reporter activity was increased 10-fold over control following E_2 treatment in the presence of insulin, while reporter activity was 14-fold higher following E_2 treatment than control in the absence of insulin (insulin×E_2, $P < .01$). In contrast, in rat-1+ERβ cells transfected with pERE-CAT plasmid, reporter activity was elevated 1.4-fold upon E_2 treatment in the presence of insulin but decreased 0.5-fold in the absence of insulin (insulin×E_2, $P < .01$). In rat-1+ ERα cells transfected with pAP-1-CAT, after E_2

stimulation reporter activity was increased 1.6- and 2.3-fold over control in the presence and absence of insulin, respectively (insulin×E_2, $P < .01$). In rat-1+ ERβ cells transfected with pAP-1-CAT, E_2 induced transcriptional activation was suppressed by 0.6 fold in the presence of insulin, while in the absence of insulin reporter activity was elevated 1.2-fold (insulin×E_2, $p < .01$). We conclude that insulin exhibited both positive and negative interactions with ER depending on the ER subtype and ER subtype-coupled pathway. Thus, the relative concentration of insulin may elicit different transcriptional activation in response to estrogen stimulation in target cells.

Introduction

Estrogen receptors (ER) are members of the superfamily of nuclear hormone receptors that operate as ligand induced transcription factors. Numerous reports have shown that ER interact with other transcription factors, including AP-1 and Sp1 proteins (Gaub *et al.*, 1990; Paech *et al.*, 1997; Rishi *et al.*, 1995; Webb *et al.*, 1995; Wu-Peng *et al.*, 1992) (Sun *et al.*, 1998; Wang *et al.*, 1998). Numbers of the steroid receptor superfamily members also interact with specific coactivator and corepressor proteins (Horwitz *et al.*, 1996). These interactions result in complex combinatorial control of gene transcription involving the estrogen-signaling pathway. Further, ER can be activated in the absence of ligand by several growth factors, as well as insulin in a process mediated by phosphorylation of the receptor (Aronica and Katzenellenbogen, 1993; Bunone *et al.*, 1996; El-Tanani and Green, 1997; Ignar-Trowbridge *et al.*, 1996; Ignar-Trowbridge *et al.*, 1993; Patrone *et al.*, 1996; Smith *et al.*, 1993).

It has been observed that acute withdrawal of insulin significantly reduced sexual receptivity and retention of nuclear ER in brain tissues of experimental diabetic female rats following ovariectomy and treatment with estradiol-benzoate (Gentry *et al.*, 1977; Siegel and Wade, 1979). These data suggested that diminished estrogen binding by target sites in the brain might be responsible for reproductive dysfunction in diabetes. This hypothesis was supported by work of Dudley *et al* (Dudley *et al.*, 1981a; Dudley *et al.*, 1981b) showing that chronic insulin deprivation significantly reduced estradiol binding in the hypothalamus and pituitary of rats. This result suggested that insulin played a role in maintaining ER function in target organs. Moreover, Katzenellenbogen and Norman (1990) reported that treatment with insulin and estradiol resulted in a synergistic effect on expression of the progesterone receptor (PR), a well known estrogen target gene, in MCF-7 cells. Most of this work predates identification of ERβ (Kuiper *et al*, 1996). It is unclear whether ERα or ERβ, or both mediated this apparent cross talk with insulin. The purpose of this study was to examine the role of ER subtype and response element type in the interaction between estrogen and insulin in rat fibroblast cell lines.

Materials and methods

Cell culture

Experiments were carried out in rat-1 (Freeman *et al.*, 1970), rat-1+ERα cells (Kaneko *et al.*, 1993) and rat-1+ERβ (Cheng and Malayer, 1999) cells. Cell cultures were maintained at 37° C in a humidified atmosphere of 95% air, 5% CO_2. Cells were grown in phenol red-free Dulbecco's modified Eagle's medium (DMEM; Gibco-BRL,

Gaithersburg), containing 1x antibiotic-antimycotic (Gibco-BRL), 5 mM Hepes (Sigma, St. Louis, MO), and sodium biocarbonate supplemented with 10% charcoal-stripped fetal bovine serum (FBS; Hyclone, Logan, UT). Charcoal stripping was performed as described by Horwitz (Horwitz *et al.*, 1976).

Establishment of ectopic ERβ expression in rat-1 cells

Stable expression of ERβ was established in rat-1 cells using a retrovirus-mediated transfer (Cheng and Malayer, 1999). Briefly, the plasmid pLNCX (RetroXpress™ System; Clontech) was linearized by HpaI digestion and treated with EcoRI methylase to protect internal EcoRI sites. Phosphorylated EcoRI linkers (New England Biolabs) were ligated to pLNCX using T4 DNA ligase (2,000,000 units/ml), followed by EcoRI digestion to create sticky ends. The rat ER-β cDNA was excised from pRAT-ER-β plasmid (provided by Dr. G. Kuiper) by EcoRI digestion and inserted into the EcoRI linker sites in pLNCX, resulting in the recombinant plasmid pLNCX-ERβ. This construct was tested by restriction mapping and by dideoxy chain-termination sequencing (Applied Biosystems, Foster City, CA; Model 373A Automated Sequencer, OSU Recombinant DNA/Protein Resource Facility) to verify the ERβ insert was intact and in the correct orientation.

The PT67 retrovirus packaging cell line was transfected with pLNCX-ERβ by cationic liposome-mediated transfection (Transfectam®; Promega). The pLNCX plasmid carries the *neo* transposon, which confers resistance to the aminoglycoside G418. After washing with Hanks' Buffered Salt Solution (HBSS, Gibco-BRL) three times, cells were fed with fresh DMEM containing 10% FBS for 24 hours and selected in the same

medium supplemented with G418 (0.5 mg/ml; Gibco-BRL) for 12 days. Following selection, remaining PT67 cells were diluted into 96-well tissue culture plates (Falcon®; Becton Dickinson, Bedford, MA) to propagate clonal lineages. Selected cell lineages were grown to confluence and medium was collected as the source of recombinant retrovirus particles. Media were filtered through 1.0 μm polysulfone membrane filters (Whatman, Clifton, NJ) and resultant cell-free media were stored at -80°C.

Rat-1 cells were incubated with retrovirus-containing medium. Following selection, transfected rat-1 cells (rat-1+ERβ) were diluted into 96-well tissue culture plates to propagate clonal lineages. Selected cell lineages were grown to confluence in the presence of G418 and tested for integration of the ERβ coding region and stable ERβ expression as described below. All reported estrogen response data was from a single clonal cell lineage of rat-1+ERβ cells.

Southern blot analysis

Genomic DNA was isolated from rat-1 and rat-1+ERβ cells with DNA Isolation Kits (Gentra System, Minneapolis, MN). Southern analysis was performed as described by Sambrook et al (Sambrook *et al.*, 1989). Seventy micrograms of each genomic DNA was digested to completion with EcoRI and loaded onto a 0.8 % agarose-TAE gel [Tris-acetate (40 mM), EDTA (1 mM)]. Resolution was visualized by staining with ethidium bromide. The gel was then denatured and DNA transferred to a nylon membrane (Boehringer Mannheim, Indianapolis, IN) using 10×SSC solution [sodium chloride (1.5 M) plus sodium citrate (0.15 M); pH 7.0] as transfer buffer. The blot was prehybridized in hybridization buffer [$NaPO_4$ (0.5 M), bovine serum albumin (1% w/v), EDTA (1 mM),

sodium dodecyl sulfate (SDS; 7% w/v), herring sperm DNA (100 μg/ml)] at 65°C for 2 hr. Hybridization was carried out overnight at 65°C in the presence of radiolabeled probe at a concentration of 2.7×10^6 cpm/ml. The probe was full-length rat ERβ cDNA labeled by random priming in the presence of $\alpha[^{32}P]$-dATP (sp. act. 3000 Ci/mmol; NEN, Boston, MA) and eluted through a Sephadex G-25 spin column (Boehringer Mannheim). Following hybridization, the membrane was washed twice with 2×SSC containing 0.1 % SDS for 30 min at 65°C and twice with 0.2×SSC plus 0.1% SDS for 30 min at 65°C. Hybridization was visualized by autoradiography using Kodak XRP film.

Reverse-transcription polymerase chain-reaction (RT-PCR)

RT-PCR was used to detect specific transcripts in RNA obtained from rat-1, rat-1+ER , and rat-1+ERβ cell cultures. Total RNA was extracted from cultures by guanidinium thiocyanate extraction (Chomczynski and Sacci, 1987). All solutions and glassware used in preparation and analysis of RNA were treated with diethylpyrocarbonate (Sigma). Two micrograms of total RNA was denatured by heating to 95°C and reverse-transcribed in the presence of random hexamers (pdN_6; 100 pmole; Pharmacia, Piscataway, NJ), dATP, dTTP, dCTP, and dGTP (dNTPs; 1 mM; Pharmacia), $MgCl_2$, RNase inhibitor (20 U per reaction; Promega), and reverse transcriptase (Superscript™, 200 U per reaction; Gibco-BRL) at 37°C for 75 min. The reaction was stopped by heating to 95°C. Aliquots of reverse-transcribed cDNA (1 to 5 μl) were denatured by heating to 95°C and subjected to polymerase chain-reaction in the presence of 75 pmole specific primers , $MgCl_2$, dNTPs (1 mM), and Amplitaq™ DNA polymerase (0.5 U per reaction; Perkin-Elmer, Foster City, CA). Products of RT-PCR were resolved

on 3% agarose-TAE gels [Tris-acetate (40 mM), EDTA (1 mM)], and visualized by staining with ethidium bromide. Total RNA from each type of cells was reverse-transcribed in duplicate and PCR amplifications repeated twice from each RT reaction. Specific RT-PCR target mRNAs were amplified by PCR in the same reaction as that for glyceraldehyde-3-phosphate dehydrogenase (G_3PDH) used as a loading control. Representative RT-PCR products from each primer set were excised from agarose and tested by dideoxy chain-termination sequencing (Applied Biosystems, Model 373A Automated Sequencer, OSU Recombinant DNA/Protein Resource Facility). The identity of each product was verified in a sequence homology analysis using the Basic Local Alignment Search Tool (Altschul *et al.*, 1990).

Reporter plasmid construction

The plasmid pBLCAT2 (Luckow and Schutz, 1987) contains the minimal thymidine kinase promoter from herpes simplex virus (HSV-tk) driving expression of the coding region of the chloramphenicol acetyltransferase (CAT) reporter gene. To confer estradiol responsiveness, the 15-bp consensus ERE from the *Xenopus vitellogenin A2* gene (GGT CAC AGT GAC C) was inserted into the *XbaI* restriction site, immediately upstream of the HSV-tk, to produce the pERE15 plasmid (Luckow and Schutz, 1987). Both pBLCAT2 and pERE15 are pUC-derived plasmids and thus contain an AP-1 enhancer in the region 5' of the HSV-tk (Kushner *et al.*, 1994) The pBLCAT2 plasmid, hereafter called pAP-1-CAT, was used to examine AP-1 mediated effect upon the HSV-tk promoter. To remove this confounding regulatory element, we digested pERE15 with

NdeI and *EcoO109* as described by Kushner et al (1994) followed by religation, to generate pERE15ΔNdeI-EcoO109, hereafter called pERE-CAT.

Transient transfection and hormone treatment

Cells were plated on 100 mm dishes at 2×10^6 cells per dish. Medium was either supplemented with insulin (1.2μg/ml, Clonetics, San Diego, CA) or left unsupplemented. Cells were transfected with the pERE-CAT plasmid or the pAP-1-CAT plasmid (8μg DNA/dish) by liposome-mediated transfection reagent (FuGENE; Boehringer Mannheim, Indianapolis, IN) for 24 hours. After a 24 hour recovery period, cells were divided into eight 35-mm dishes and treated with either estradiol (1 nM) or vehicle. Reported results are for 12 hour treatments in rat-1+ERα cells, and 9 hours in rat-1+ERβ cells. Data represent four culture wells per treatment used to determine receptor gene activity as described below.

CAT assay

Chloramphenicol acetyltransferase enzyme activity was assayed in duplicate in cell lysates by incubating in the presence of [^{3}H]chloramphenicol and n-butyryl-coenzyme A, then measuring the acetylated [^{3}H]chloramphenicol present in the organic (xylene) phase following two-phase partitioning (CAT Enzyme Assay System Kit; Promega). The radioactivity present in the acetylated chloramphenicol was determined by scintillation spectroscopy. Enzyme activity was compared to cells not transfected as a negative control; [^{3}H]chloramphenicol was also measured in the xylene phase following extraction in the absence of cell lysates to determine background radioactivity following

two-phase partitioning. Duplicate measurements conducted on each culture well and these values were used to calculate treatment means.

Statistical analysis

Duplicate values obtained in the assay of CAT enzyme activity within each culture well were used to calculate treatment means. Treatment means are expressed as a value relative to the ethanol vehicle control. The relative number, reported as fold induction, was calculated as the ratio of treatment with estradiol to the vehicle treatment data point, which was set to 1. Standard error was calculated for each averaged point. Analysis were done using the SAS/GLM procedure (SAS, 1996). Appropriate means comparisons were made using the least squares means (LSMEANS).

Results

Stable expression of ERβ

RT-PCR showed ERβ mRNA expression in PT67+ERβ cells but not in PT67 cells suggesting that PT67+ERβ were able to produce retroviral RNA that carried the ERβ coding region (Cheng and Malayer, 1999). Southern blot analysis was performed with genomic DNA from rat-1 cells, retrovirus-producing PT-67 cells (PT-67+ERβ) and rat-1+ ERβ cells. Both rat-1 and PT-67 cells contained endogenous ERβ DNA sequences that hybridized with probe. The rat-1+ ERβ cells exhibited a unique hybridization pattern indicating the integration of the ERβ cDNA insert into the genomic DNA. Southern blot results suggested that ERβ cDNA had integrated into the genome of rat1+ ERβ cells.

RT-PCR analysis was used to detect ERβ mRNA expression and results indicated that ERβ mRNA was present in rat-1+ ERβ cells, but was absent in rat-1 cells (Cheng and Malayer, 1999). Transient reporter-gene transfection with pERE-CAT was used to test for a functional estrogen response in rat-1+ERβ cells. After exposure to E_2 the reporter gene activity was significantly increased over control ($P < .05$), indicating functional ERβ activity in the cells (Cheng and Malayer, 1999).

Insulin effects in rat-1+ERα cells

Estradiol treatment increased reporter activity in rat-1+ ERα cells transfected with the pERE-CAT plasmid in both the presence and absence of insulin. Reporter activity was increased 10-fold over control ($P < .01$) following estradiol treatment in the presence of insulin while reporter activity was 14-fold higher than control ($P < .01$) in the absence of insulin (Figure 1). There was a significant negative interaction ($P < .01$) between insulin and estrogen in rat-1+ ERα cells transfected with the pERE-CAT because the estradiol-stimulated reporter activity was not as great ($P < .01$) in the presence of insulin.

In rat-1+ ERα cells transfected with the pAP-1-CAT, estradiol treatment also stimulated reporter activity. After estradiol stimulation, reporter activity was increased 1.6- and 2.3-fold over control ($P < .01$) in the presence and absence of insulin, respectively (Figure 2). A significant negative interaction between insulin and estradiol was also observed in rat-1+ ERα cells transfected with the pAP-1-CAT ($P < .01$) because, again, estradiol-stimulated reporter activity was not as great ($P < .01$) in the presence of insulin.

Insulin effects in rat-1+ERβ cells

In rat-1+ ERβ transfected with the pERE-CAT plasmid, reporter activity was elevated 1.4-fold upon estradiol treatment in the presence of insulin, but decreased 0.5-fold in the absence of insulin (E_2 × insulin, $P < .01$) (Figure 3).

In rat-1+ ERβ cells transfected with the pAP-1-CAT plasmid, the presence of insulin suppressed estrogen-induced transcription activation by 0.6-fold compared to control, while in the absence of insulin reporter activity was elevated 1.2-fold (E_2 × insulin, $P < .01$) (Figure 4).

Discussion

Factors present in serum, including insulin, contribute stimulatory signals critical to cell growth and proliferation. Serum components modify responses to steroids in cultured rat uterine cells through modification of receptor ligand-binding activity due, at least in part, to alteration of receptor protein levels (Greco and Gorski, 1989; Kassis *et al.*, 1984). There was a synergistic relationship between insulin and estradiol in activation of the progesterone receptor (PR) gene in MCF-7 cells (Katzenellenbogen and Norman, 1990). Lack of insulin associated with diabetes in rats resulted in infertility and alterations in estrogen binding characteristics in the pituitary and hypothalamus (Dudley *et al.*, 1981a; Dudley *et al.*, 1981b). Our data have shown that there is an interaction between signaling activities of estrogen and insulin in rat-1 fibroblasts. Further, effects of this interaction are variable and depend upon the combinatorial effects of several factors, including ER type and promoter context. Of particular interest is the finding that

in the absence of supplemental insulin, estradiol treatment resulted in transcriptional repression in the context of ERβ and the AP-1 response element. Based upon tissue distribution of the ERβ (Shughrue *et al.*, 1997), we suggest that observed alterations in estrogen action in the hypothalamus and pituitary in insulin-dependent diabetes (Dudley *et al.*, 1981a; Dudley *et al.*, 1981b) may be due to the interaction of estradiol and insulin signaling upon the ERβ.

The activity of ER is mediated through two transactivation domains in the receptor, TAF-1 at the amino terminal end, and TAF-2, which lies in the ligand-binding domain (Berry *et al.*, 1990; Bocquel *et al.*, 1989; Danielian *et al.*, 1992; Kumar *et al.*, 1987; Lees *et al.*, 1989; Tora *et al.*, 1989). These transactivation domains function by binding to specific proteins in the cell, which act as activators or repressors of transcription. The relative contribution to the ER-protein complex of co-activator or co-repressor proteins determines whether transcriptional activation will occur (Horwitz *et al.*, 1996; Onate *et al.*, 1998; Onate *et al.*, 1995; Shibata *et al.*, 1997; Spencer *et al.*, 1997; Webb *et al.*, 1998). Sequence divergence between ERα and ERβ in these regions, particularly in the TAF-1 domain, suggests that interactions with co-activator or co-repressor proteins would differ at the enhancer or promoter site during modification of gene expression and, as a result, that ERα and ERβ play different roles in gene regulation. In addition, promoter context plays a crucial role in estrogen action. ER may interact with several general transcription factors, including Sp1 (Dubik and Shiu, 1992; Wu-Peng *et al.*, 1992) and AP-1 (Gaub *et al.*, 1987; Philips *et al.*, 1993). ERα and ERβ act through AP-1 (Paech *et al.*, 1997; Webb *et al.*, 1995) via a protein-protein interaction between the ligand-bound receptor and the DNA-bound AP-1 heterodimer. The AP-1

heterodimer consists of members of the fos and jun gene families. In general, fos family members combine with jun proteins to form heterodimers. Jun family members can also form heterodimers or homodimers with other jun proteins. Transcriptional activity and target gene specificity depend upon the composition of the protein complex (Angel and Karin, 1991).

There is considerable evidence that ER can be activated in the absence of its cognate ligand. It was originally found that 8-bromo-3'-5' cyclic adenosine monophosphate (8-Br-cAMP) was able to activate PR-mediated gene expression in the absence of progesterone (Denner *et al.*, 1990). Phosphorylation of PR was found to be essential for its activation by dopamine in the absence of progesterone (Power *et al.*, 1991). Shortly thereafter, it was demonstrated that ER dependent gene expression could be activated by several chemical agents including 8-Br-cAMP, CT/IBMX (CT plus IBMX; CT, cholera toxin; IBMX, isobutylmethylxanthine) (Aronica and Katzenellenbogen, 1993) and 12-O-tetradecanoylphorbol-13-acetate (TPA) (Ignar-Trowbridge *et al.*, 1996). The neurotransmitter dopamine, and a number of growth factors such as IGF-1, EGF, TGF-α and insulin (Bunone *et al.*, 1996; El-Tanani and Green, 1997; Ignar-Trowbridge *et al.*, 1993; Patrone *et al.*, 1996; Smith *et al.*, 1993) were all found to activate ER in the absence of estrogenic ligand.

Ligand-independent activation of ER is accompanied by the phosphorylation of the ER protein. As an activator of protein kinase A (PKA), 8-Br-cAMP increased the phosphorylation of endogenous ER in rat uterine cells (Aronica and Katzenellenbogen, 1993). Similarly, the PKA activator CT (Aronica and Katzenellenbogen, 1993) and the protein kinase C (PKC) activator TPA (Ignar-Trowbridge *et al.*, 1996) both elevated the

phosphorylation of ligand-free ER. Moreover, the growth factors, IGF-1 and EGF, were also shown to induce hyperphosphorylation of ER (Aronica and Katzenellenbogen, 1993; Ignar-Trowbridge *et al.*, 1996). These data suggested that ER-mediated gene expression could be activated by multiple extracellular stimuli, and that phosphorylation of ER was crucial for ligand-independent activation of ER by chemicals and growth factors.

Ligand-independent activation of ER may involve phosphorylation in different domains or residues, depending on the particular signaling pathway impinging upon the receptor. Serine^{118} located in the AF-1 domain has been identified as a site responsible for ER activation in ligand-independent pathways. Mutation of serine^{118} to alanine was found to completely abolish the ability of EGF to activate ER (Ignar-Trowbridge *et al.*, 1996). In addition, phosphorylation of human ER resulting from TPA was also found to occur at serine^{118} (Joel *et al.*, 1995). Mutation studies revealed that activation of ER occurred in specific domains under the influence of different stimuli. The AF-1 domain was essential for activation of ER by IGF-1 and EGF (El-Tanani and Green, 1997; Ignar-Trowbridge *et al.*, 1996; Ignar-Trowbridge *et al.*, 1993). AF-2 was not necessary for EGF-mediated ER activation (Bunone *et al.*, 1996). In contrast, ligand-independent activation of ER induced by CT/IBMX was dependent on AF-2 and independent of AF-1 (El-Tanani and Green, 1997). Similarly, activation of ligand-free ER by dopamine was shown to require the AF-2 domain (Smith *et al.*, 1993).

The second largest family of cell surface receptors is the receptor tyrosine kinase (RTK) family, which feature an intracellular domain possessing tyrosine kinase activity, and includes the insulin (IR) and epidermal growth factor receptors (EGFR) (Kahn and

White, 1988). Ligand binding to the receptor elicits a conformational change resulting in the dimerization and autophosphorylation of the IR (Treadway *et al.*, 1992). These phosphorylated tyrosine residues serve as docking sites for SH2 domain proteins, which contain a binding pocket for phosphotyrosine (Pawson and Gish, 1992). The receptor may then recruit both insulin receptor substrate (IRS) and Grb2 proteins (White and Kahn, 1994). In addition, IRS and Grb2 are both able to dock other SH2 domain proteins that possess specific effector activity. For instance, IRS can recruit phosphotidylinositol-3' kinase (PI-3) resulting in accelerated glucose uptake (Saltiel, 1996) and Grb-2 can dock with the mammalian homologue of Drosophila SOS (son of sevenless) which converts Ras into its active form (GTP-bound form) (Myers and White, 1996). Ras then activates Raf protein, also called mitogen-activated protein (MAP) kinase kinase kinase (MAPKKK). Once MAPKKK has been activated, it then phosphorylates MAP kinase kinase (MAPKK). Finally, MAPKK phosphorylates and activates MAP kinase (MAPK) (Elion, 1998). There are three major subfamilies of MAPK: ERKs, which activate transcription factors such as Elk and Ets, JNKs, which activate transcription factors such as C-jun (Elion, 1998), and FRKs, which activate Fos. Phosphorylation of ER may involve interaction with a specific MAPK (Kato *et al*, 1995). Among stimuli for ligand-independent ER activation, insulin is somewhat unique since it appears to require both AF-1 and AF-2 domains of ER to fully activate the receptor. Deletion of AF-2 completely abolished the ability of insulin to activate ER, while deletion of AF-1 significantly reduced the ability of insulin to activate ER (Patrone *et al.*, 1996).

Taken together, the action of ER to modulate gene expression may exhibit combinatorial diversity based on the protein-protein interactions dictated by structural

differences between the two receptor types, the promoter context, and the effects of peptide factors acting upon RTKs to stimulate hyperphosphorylation of nuclear ER. Metabolic status may modify action of gonadal steroids through the interaction with insulin of ERα and ERβ in various estrogen target tissues, including the hypothalamus and pituitary, to integrate nutritional status with reproductive activity.

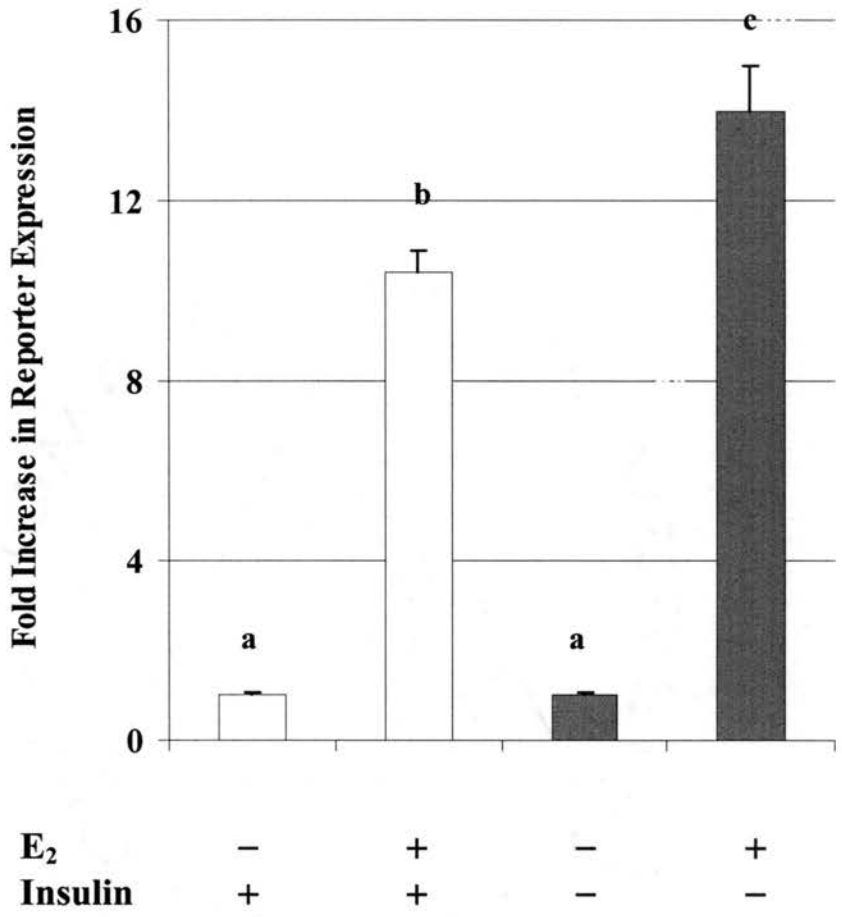

Figure 1. Mean values ± standard error of acetylated [^{3}H]chloramphenicol expressed as fold-increase over control in rat-1+ERα cells. Cells were transiently-transfected with a reporter plasmid having an ERE (pERE-CAT). Cultures were incubated with E_2 or vehicle in the presence (open bars) and absence (shaded bars) of supplemental insulin. Means ± standard error with different superscripts ^{a,b,c} were significantly different ($P<.01$). There was a significant interaction between E_2 and the presence of supplemental insulin ($P<.01$) on gene activation mediated via the ERα-ERE pathway.

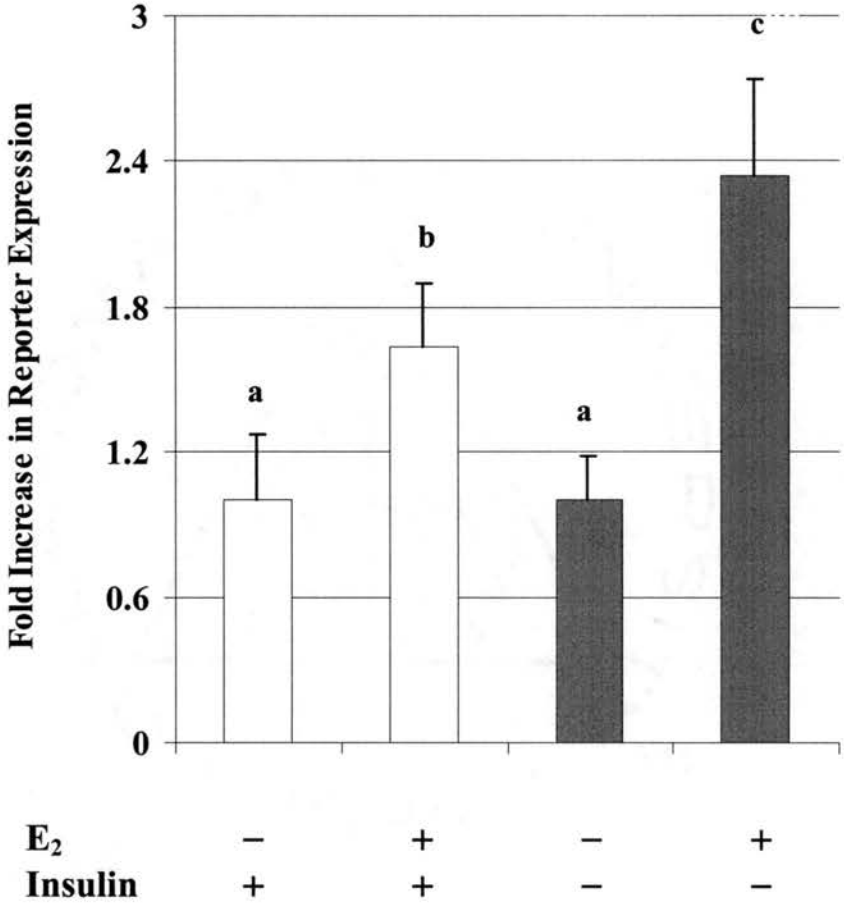

Figure 2. Mean values ± standard error of acetylated [^{3}H]chloramphenicol expressed as fold-increase over control in rat-1+ERα cells. Cells were transiently-transfected with a reporter plasmid having an AP-1 site (pAP-1-CAT). Cultures were incubated with E_2 or vehicle in the presence (open bars) and absence (shaded bars) of supplemental insulin. Means ± standard error with different superscripts ^{a,b,c} were significantly different ($P<.01$). There was a significant interaction between E_2 and the presence of supplemental insulin ($P<.01$) on gene activation mediated via the ERα-AP-1 pathway.

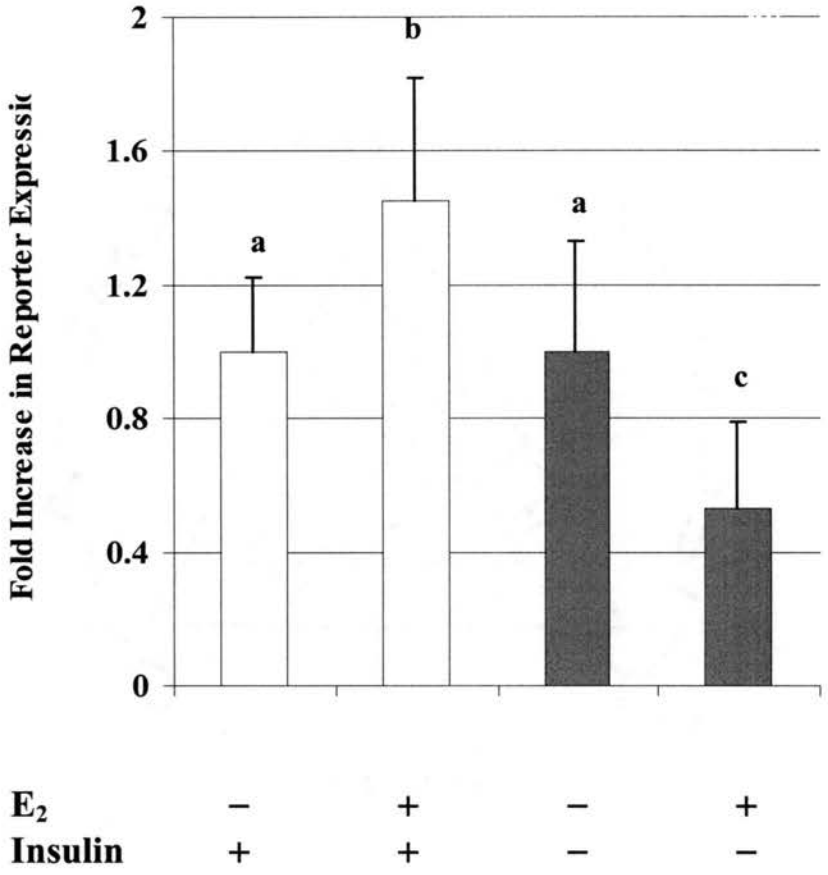

Figure 3. Mean values ± standard error of acetylated [^{3}H]chloramphenicol expressed as fold-increase over control in rat-1+ERβ cells. Cells were transiently-transfected with a reporter plasmid having an ERE (pERE-CAT). Cultures were incubated with E_2 or vehicle in the presence (open bars) and absence (shaded bars) of supplemental insulin. Means ± standard error with different superscripts ^{a,b,c} were significantly different ($P<.01$). There was a significant interaction between E_2 and the presence of supplemental insulin ($P<.01$) on gene activation mediated via the ERβ-ERE pathway.

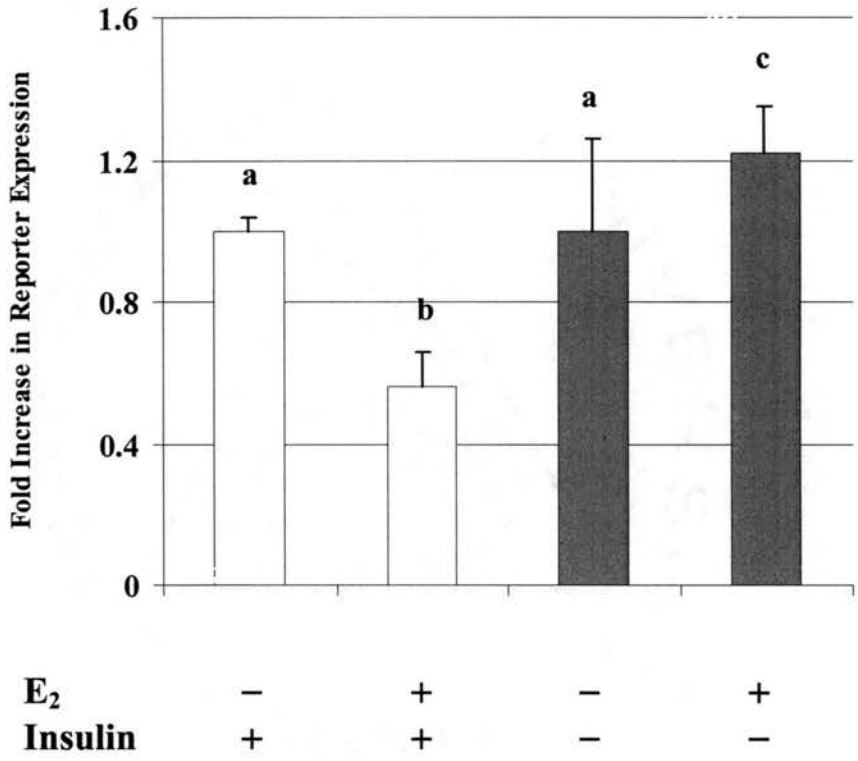

Figure 4. Mean values ± standard error of acetylated [^{3}H]chloramphenicol expressed as fold-increase over control in rat-1+ERβ cells. Cells were transiently-transfected with a reporter plasmid having an AP-1 site (pAP-1-CAT). Cultures were incubated with E_2 or vehicle in the presence (open bars) and absence (shaded bars) of supplemental insulin. Means ± standard error with different superscripts ^{a,b,c} were significantly different ($P<.01$). There was a significant interaction between E_2 and the presence of supplemental insulin ($P<.01$) on gene activation mediated via the ERβ-AP-1 pathway.

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CHAPTER VI

SUMMARY AND CONCLUSIONS

In the past decade, there have been four major areas in which new discoveries have resulted in significant changes in our understanding of the biology of steroid receptors, including the ER. This work is based largely on one of these, i.e., the discovery of a second form of the ER, termed ERβ. The overall purpose of these studies was to develop a cell culture system to characterize unique properties of two forms of the ER, i.e., ERα and ERβ. One approach to examine unique activities of each form of the receptor is ectopic expression of the receptor cDNA in a cell line that ordinarily does not express ER. This serves the dual purpose of providing a model for activities of each form of the receptor independently of the other, and of providing a model for development in which a naïve cell first begins to respond to ER expression. An important advantage of stable expression of the ER is that we may observe effects of the ER on endogenous gene expression in the cell. Further, because cell lineages were developed, all cells in the cultures were identical with respect to ER expression and so signal-to-noise ratio in response assays was superior to a system utilizing transient receptor expression.

Interaction with the preinitiation complex is a necessary but not a sufficient condition for ER (as well as other steroid receptors) to modulate transcriptional activation. To achieve efficient hormone-mediated transcription control, ER requires other cofactors that are termed coactivators. The identification and characterization of

these "adaptor" or "bridging" proteins such as SRC-1 and the p300 family of proteins is another of these recent developments in the understanding of how steroid receptors operate. The basal transcription factors and coactivators are both essential to the processes that specify and stabilize the basal transcription machinery (Goodrich *et al*, 1993). Since several steroid receptors are identified as transcriptional repressors in the absence of hormone, there are also proteins recognized as corepressors, which interact with the steroid receptor AF-2 domain to mediate transcriptional repression. One of these is NCoR (nuclear receptor co-repressor) and the other is SMRT (silencing mediator for RXR and TR). Although ER has not been identified as functioning as a transcriptional repressor in the absence of estrogen, the presence of corepressor NCoR or SMRT does change ER agonist/antagonist activity. This suggests that the relative amounts of coactivators and corepressors can affect ER activity in the presence of certain ligands and provides insight into tissue-specific or cell type-specific responses to estrogen. The agonist/antagonist activity of the ligand depends on what coactivators or corepressors are available to be recruited in the particular target cell or tissue.

To date, at least ten coactivators and three corepressors have been identified (Horwitz *et al*, 1996; Glass *et al*, 1997; Shibata, 1997). SRC/NCoA (nuclear receptor co-activators) were initially recognized as 160 kDa proteins (p160). So far, the p160 family is known to include three members: SRC-1/NCoA-1, TIF2/GRIP1/NCoA-2, and p/CIP/ACTR/AIB1/RAC3/TRAM-1. CBP and related p300 proteins (CBP/p300) have roles as coactivators and more importantly, serve as a platform for transcription factors and coactivators to form a coactivator complex (Shibata *et al*, 1997). Generally, CBP/p300 binds to pCAF (CBP/p300 associated factor) that harbors p/CIP (CBP/p300

co-integrator associated protein). Other coactivators, such as SRC-1/NCoA-1 bind to p/CIP, while CREB, AP-1, and steroid receptors directly bind to CBP/p300. CBP/p300, pCAF p/CIP and SCR-1 all have intrinsic histone acetytransferase (HAT) activity (Torchia *et al*, 1998). NCoR/SMRT interacts with two related corepressor proteins, mSin3A and mSin3B (Schreiber-Agus *et al*, 1995; Ayer *et al*, 1995), that recruit HDACs (histone deacetylases) (Taunton *et al*, 1996; Yang *et al*, 1997) to form a corepressor complex which leads to transcriptional silencing. This variety of proteins suggests considerable combinatorial diversity in the response to a given estrogenic ligand in a target cell.

There is increasing evidence that ER may activate transcription through associating with other transcription factors at sites other than the classical EREs. This association can either be via a pure protein-protein interaction without ER-DNA contact, or, can be via a more complex interaction, such as half-site ERE plus protein-protein interaction. This is another major new area in steroid receptor biology within the past decade. Gaub and his co-workers (1990) observed ER interaction with the Fos-Jun complex in the human ovalbumin promoter. Work of Philips *et al* (1993), Umayahara *et al* (1994), and Webb *et al* (1995) further established an ER interaction with AP-1 in specific transcriptional activation. Webb *et al* (1995) demonstrated that the ER-AP-1 interaction is achieved by ER binding to c-Jun rather than c-Fos. A transient transfection assay revealed that ERα and ERβ exhibited different responses when acting through the classic ERE-mediated pathway and the AP-1-mediated pathway. In HeLa cells, increased ERE-reporter activity was observed following transient expression of both ERα and ERβ after E_2 treatment (Paech *et al*, 1997). However, reporter activity was

stimulated by E_2 following transient expression of ERα in AP-1-mediated activation but not when ERβ was expressed (Paech *et al*, 1997). These data suggested further combinatorial diversity of response to estrogenic ligand based on receptor type and promoter context.

Although ER is classically activated by estrogen-dependent phosphorylation, there is vast evidence that ER can be activated in the absence of a ligand. This phenomenon of ligand-independent activation is another major insight to steroid receptor function in the past decade. It was originally found that 8-Br-cAMP was able to activate PR mediated gene expression in the absence of a ligand (Denner *et al*, 1990). Shortly thereafter, it was demonstrated that ER dependent gene expression can be activated by several chemical agents including 8-Br-cAMP, CT/IBMX (CT plus IBMX; CT, cholera toxin; IBMX, isobutylmethylxanthine) (Aronica and Katzenellenbogen, 1993) and 12-O-tetradecanoylphorbol-13-acetate (TPA) (Ignar-Trowbridge *et al*, 1996). The neurotransmitter dopamine, and a number of growth factors such as IGF-1, EGF, TGF-α and insulin (Smith *et al*, 1993; Ignar-Trowbridge *et al*, 1993; Bunone *et al*, 1996; Patrone *et al*, 1996; El-Tanani *et al*, 1997) were all found to activate ER in the absence of estrogenic ligand. Activation of ER-mediated gene expression can be achieved through multiple signal transduction pathways and phosphorylation of ER is crucial for ligand-independent activation of ER by chemicals and growth factors. The communication observed between ER and insulin is likely mediated by the Ras-Raf-MAPK pathway. Bunone *et al* (1996) found that overexpression of an inactive mutant form of Ras resulted in 40% reduction in activation of ER induced by EGF in SK-Br-3 and HeLa cells. Furthermore, overexpression of an inactive mutant form of MAPKK considerably

reduced the EGF activation of the ER in SK-Br-3 cells and almost completely blocked the EGF effect on ER activation in HeLa cells. Cotransfection of wild type ER with a mutant MAPKK possessing constitutive activity, resulted in the activation of ER in the absence of any stimuli. Therefore, it is highly likely that the EGF-induced ER activation pathway is mediated via a MAPK pathway (Bunone *et al*, 1996).

Overall, data presented have shown the following:

1. We have observed both unique and overlapping activities of ERα and ERβ when they are stably-expressed in a rat fibroblast cell line. A significant difference was observed in PR gene expression in response to ER in different cell types in culture. In rat-1 fibroblasts, expression of either ERα or ERβ was sufficient to activate the silent PR gene, while in bovine fetal cells PR gene expression was not observed in spite of the presence of ER.
2. We have observed differential responses to ligands, including antiestrogens, depending upon whether ERα or ERβ was present and upon the promoter context, i.e., ERE- versus AP-1-directed transcriptional activation. Differential responses were also observed in different cell types and at different stages in development, i.e., UBF120 versus UBF180 cells.
3. We have observed activation of ER through cell-surface receptor pathways. Both EGF and insulin were observed to stimulate or alter ER activity. Further, insulin was observed to communicate with ER in a differential manner depending upon ER type and promoter context.

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