

# ROLE OF ESTROGEN RECEPTOR BETA IN ESTROGEN ACTION

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■ **Abstract** There was a time when the classification of sex hormones was simple. Androgens were male and estrogens female. What remains true today is that in young adults androgen levels are higher in males and estrogen levels higher in females. More recently we have learned that estrogens are necessary in males for regulation of male sexual behavior, maintenance of the skeleton and the cardiovascular system, and for normal function of the testis and prostate. The importance of androgen in females was never in doubt, it is after all the precursor of estrogen as the substrate for aromatase, the enzyme that produces estrogen. In addition, the tissue distribution of androgen receptors suggests that androgens themselves are important in the ovary, uterus, breast, and brain.

New information promises to clarify some of the complex issues of the physiological roles of estrogen and the contribution of estrogen to the development of neoplastic diseases in humans. The discovery of the second estrogen receptor, the creation of mutant mice defective in both estrogen receptors and in the aromatase gene, the solution of the structures of the ligand-binding domains of estrogen receptor alpha ( $ER\alpha$ ) and estrogen receptor beta ( $ER\beta$ ), the finding of novel routes through which estrogen receptors can modulate transcription, and the identification of a man with a bi-allelic disruptive mutation of the  $ER\alpha$  gene are but some of the milestones. This review focuses on the mechanistic aspects of signal transduction mediated by ERs and on the physiological consequences of deficiency of estrogen or estrogen receptor in the available mouse models.

## MECHANISMS OF ACTION OF ESTROGEN

The existence of a protein responsible for specific binding of  $17\beta$  estradiol ( $E_2$ ) in the uterus was recognized almost 40 years ago (1). The actual cloning of  $ER\alpha$  took place a quarter of a century later (2, 3). During the following decade, this receptor was believed to be the single mediator of the physiological effects of estrogens (reviewed 4). In 1996, however, a novel ER was discovered in the rat prostate (5–7). This receptor was named  $ER\beta$  to distinguish it from the other receptor,  $ER\alpha$ .

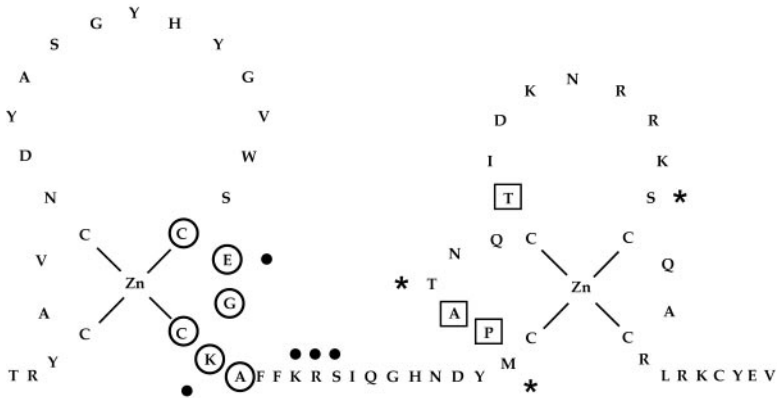
Both ERs belong to the nuclear receptor (NR) gene family of transcription factors, which show an evolutionarily and functionally conserved structure (8). The action of NRs involves binding of the liganded receptor to *cis*-regulatory DNA elements in the promoter region of target genes to influence transcription rate through physical interaction with cofactors and the transcription machinery. Phylogenetic analysis and functional characterization of NRs form the basis for classification into subfamilies: The steroid receptor subgroup (Type 1 receptors) consists of the receptors for glucocorticoids, mineralocorticoids, progesterone, androgens, and estrogens (GR, MR, PR, AR, and ER, respectively) (9). These receptors act principally as homodimers and rely on ligand activation for efficient DNA-binding and transcriptional activity.

NRs have an N-terminal domain of variable length termed the A/B domain. This region has the lowest degree of sequence similarity among NR family members, but in any single family there is generally good conservation of sequence homology between species, suggesting that evolutionary changes are subjected to functional constraints. The A/B domain of NRs usually harbors an activation function (AF-1) that contributes to transcriptional activity. Adjacent to the A/B domain is the DNA-binding or C domain, the most highly conserved region of the NR superfamily. The DNA-binding function consists of two zinc finger motifs coordinated by eight strictly conserved cysteine residues. The DNA-binding domain (DBD) also contains a dimerization interface that mediates cooperativity in DNA binding.

The DBD is linked to the ligand-binding domain (LBD) by the D domain or hinge region. This region is less well characterized and is poorly conserved between different NRs and is involved in association with the molecular chaperone heat shock protein 90 (hsp90). C terminal to the hinge domain is the ligand binding or E/F domain, which displays a high degree of homology among members of the NR superfamily. This multifunctional region is involved in binding of NR agonists and antagonists, dimerization, cofactor binding, and transactivation and, in members of the steroid receptor subgroup of NRs, is involved in a second hsp90 interaction and nuclear localization. Dimerization is important in NR action and is a characteristic of a majority of NRs. The LBD contains a strong dimerization interface that for many receptors functions in the absence of ligand. The transactivation function (AF-2) located in the LBD is in most cases dependent on binding by an agonist ligand to the receptor for proper function.

## DNA BINDING

The DBD is the most conserved region in NRs (reviewed in 10, 11). Nine cysteine residues are invariably conserved. Eight of the residues are coordinated around two  $Zn^{2+}$  ions forming two Zn finger motifs that confer specific DNA-binding capacity. Within the Zn fingers, several subdomains mediate distinct functions. The so-called P-box encompasses six amino acids at the C-terminal base of the first Zn finger and includes the third and fourth cysteines (Figure 1).



**Figure 1** Human ER $\alpha$  zinc finger organization. Circled residues signify the P-box. Residues indicated by a dot make direct basepair contacts with the estrogen responsive element (ERE). Amino acids making direct contacts in the dimerization interface are boxed. Asterisks indicate residues making dimerization contacts via ordered water molecules.

The P-box confers DNA-binding specificity on NRs and is therefore critical in recognition of target genes (see Table 1). The ER P-box (CEGCKA) is most similar to the P-boxes of the estrogen receptor-related receptors (ERRs, CEACKA), and similar to the P-boxes of retinoic acid receptor (RAR), retinoid X receptor (RXR), thyroid hormone receptor (TR), vitamin D receptor (VDR), and others (CEGCKG), reflecting the common recognition of the AGGTCA DNA core sequence displayed by these receptors. In contrast, glucocorticoid receptor (GR), mineralocorticoid receptor (MR), progesterone receptor (PR), and androgen receptor (AR) have deviating P-boxes (CGSCKV) and bind to AGAACA core sequences (10, 11).

Although the P-box is not the sole determinant of DNA sequence recognition, the specificity of DNA binding can be altered by changing a few amino acids within the P-box. An ER mutant with three amino acid substitutions in the P-box,

**TABLE 1** P-boxes and corresponding DNA recognition sequences of various NRs

P-box	NR	Core sequence
CGSCKV	GR, MR, PR, AR	AGAACA (palindrome)
CEGCKA	ER $\alpha$ , ER $\beta$	AGGTCA (palindrome)
CEACKA	ERR $\alpha$ , ERR $\beta$	AGGTCA (palindrome) TCAAGGTCA
CEGCKG	RAR, RXR, ThR, VDR, PPAR, and others.	AGGTCA (direct repeat)
CEGCKG	SF-1, FTZ-F1	TCAAGGTCA

which transform it into a GR-like P-box (from CEGCKA to CGSCKV), binds to a glucocorticoid responsive element (GRE) instead of an estrogen responsive element (ERE) (12).

Hormone response elements are generally composed of two repeats of core sequences arranged as palindromes, direct repeats, or sometimes as inverted repeats, reflecting the fact that dimerization is important in efficient DNA binding by most NRs. The number of nucleotides separating the two repeats influences the efficiency of DNA binding, with differing requirements displayed by different NRs. A dimerization interface referred to as the D-box is present within the DBD at the N-terminal half of the second Zn finger. Dimerization via the DBDs facilitates cooperative DNA binding, and the interactions of the D-boxes further stabilize NR-DNA contact (10, 11). A small subset of NRs interact with DNA as monomers. These receptors recognize and bind to isolated half sites, generally with a requirement for a specific extended 5' trinucleotide (reviewed in 13). The consensus ERE is composed of two core sequences, organized as palindromes and spaced by three nucleotides.

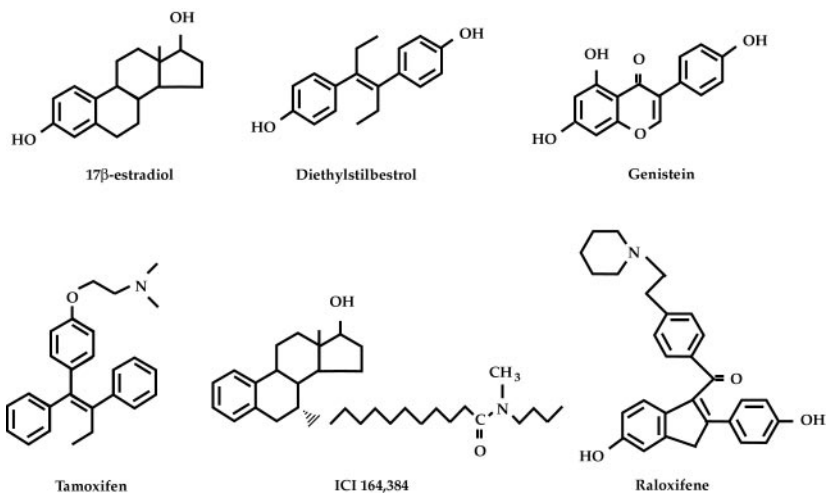
The three-dimensional structures of ER $\alpha$  DBD bound to an ERE and of GR DBD bound to a GRE have been determined (14–17). ER and GR show both similarities and differences in amino acid residues within their respective DBDs that make direct contact with nucleotides of their cognate DNA response elements, and these findings provide a basis for understanding the selective recognition of response elements by NRs. Cooperative interactions between the two DBDs is facilitated by the spacing between the two core elements, which enhances the exposure of the dimerization interfaces of each subunit to one another. Dimerization of the ER $\alpha$  DBDs enhances binding to imperfect EREs, thus contributing to increase the number of sequences with which ER $\alpha$  can interact (18). The D-boxes differ considerably between NRs, perhaps reflecting the different modes of DNA-binding displayed by various NRs.

## COMPARISON OF THE TWO ESTROGEN RECEPTORS

As expected, the DBDs of the two ERs share approximately 97% sequence similarity. In the LBD, the overall amino acid identity is 55%. Regions directly involved in ligand binding and the AF-2 display a higher degree of sequence similarity. ER $\beta$  binds E2 with high affinity, and the ER $\beta$ -E2 complex activates transcription of an ERE-containing reporter construct (5). ER $\beta$  homologs have been cloned from human (19, 20) and mouse (21, 22), and several splicing variants of ER $\beta$  have been described (discussed in more detail below; see References 21, 23–27).

### Ligand-Binding

ER $\alpha$  and ER $\beta$  are similar in those parts of the LBD involved in the actual binding of ligand and have similar binding specificities (29). The major differences in ligand binding between ER $\alpha$  and ER $\beta$  lie in the affinities for various compounds



**Figure 2** Molecular structures of estrogen receptor agonists and antagonists.

and the transcriptional response a given compound is able to elicit. For instance, tamoxifen is a cell- and tissue-specific mixed agonist-antagonist for ER $\alpha$  but is a pure antagonist on ER $\beta$  (30–32). This phenomenon is perhaps not surprising in view of the fact that in ER $\alpha$  it is mediated principally via the N-terminal AF-1, a region where ER $\alpha$  and ER $\beta$  differ. Phytoestrogens such as coumestrol and the isoflavonoid genistein have a generally higher affinity (up to ten times higher) for ER $\beta$  (33). However, despite their higher affinity, the maximal transcriptional stimulation by phytoestrogens achieved with ER $\beta$  is only about half that of ER $\alpha$  (32) (Figure 2).

## ER-LBD Structure

Binding of an agonist to an NR induces a conformational change that is associated with the transition to a transcriptionally active complex. Analysis of the crystal structures of several NR LBDs has revealed a conserved architecture composed of 12 helices termed H1–H12 (reviewed in 34).

The crystal structures of ER $\alpha$  LBD bound to the agonists E2 and diethylstilbestrol (DES) and to the antagonists raloxifene and 4-OH-tamoxifen have been determined (35–37), and the three-dimensional structure of the ER $\beta$  LBD bound to the isoflavonoid genistein and to raloxifene has been described (38). In the ER $\alpha$  LBD bound to E2 or DES, the protein is folded into a three-layered, anti-parallel,  $\alpha$  helical sandwich. A central core layer of three helices is packed between two additional layers of helices creating a molecular scaffold with the ligand-binding cavity in one end. In the agonist-binding configuration, H12 is typically positioned across the ligand-binding cavity in a groove created by H3, H5/6, and H11,

separating the ligand-binding cavity from the outside. This configuration facilitates contact between ER $\alpha$  and the p160 class of coactivators by exposing the coactivator-binding interface of the receptor to the LXXLL motif(s) of the coactivator (36). The hormone-binding pocket of the ERs is relatively large compared with that of several other NRs for which the crystal structure has been solved (39–41). E2 accordingly does not occupy the entire cavity. The large ligand-binding cavity may explain the unique ability of the ERs to bind to a variety of steroids and other compounds. When DES is bound, the conformation of the ER $\alpha$  LBD is similar to that with E2. However, when the anti-estrogens raloxifene or 4-OH-tamoxifen are bound, H12 is translocated to a position that obscures the coactivator interaction site. This conformation appears to prevent coactivator binding and may be a primary mechanism of ER antagonism (35, 36). The overall three-dimensional structure of ER $\beta$  LBD bound to raloxifene is similar to ER $\alpha$  LBD/raloxifene complex (38). When genistein is bound to the ER $\beta$  LBD, H12 does not adopt the typical agonist conformation but is positioned in a manner reminiscent of antagonist binding (38). This arrangement of H12 could account for the fact that genistein is a partial agonist (32).

## COFACTORS AND OTHER INTERACTING PROTEINS:

### The Basis for Cofactor Interaction

A number of NR-interacting proteins have been identified. In addition to factors that coactivate transcriptional initiation by NRs, coregulators and corepressors have been described and are now referred to as cofactors (reviewed in 42, 43). The interaction of cofactors with NRs depends on conserved so-called NR-boxes within the cofactors, composed of the consensus sequence LXXLL (where L is a leucine and X is any other amino acid). One or more of these motifs is present in all NR-interacting cofactors, and mutations in the NR-boxes disrupt NR-cofactor interaction (44).

### Coactivators

Several factors increase ER-mediated transcription (reviewed in 42, 43). One group is referred to as the p160 or SRC (steroid receptor coactivator) class of coactivators. For clarity the nomenclature system is that of McKenna et al (43): SRC-1 (human SRC-1 and mouse NCoA-1), SRC-2 (human TIF2, mouse GRIP1/NCoA-2), and SRC-3 (human RAC3/ACTR/AIB1/TRAM-1/SRC-3 and mouse p/CIP). Three LXXLL/NR box motifs are conserved in all members of the SRC family. The NR-boxes form amphipathic  $\alpha$  helices with the conserved leucine residues forming a hydrophobic surface on one face of the helix. Interestingly, amino acids flanking the NR-boxes appear to impart receptor-specificity in interaction between SRC-1, SRC-2, and different NRs (45–47). SRC-1 coactivates ligand-dependent transactivation of numerous NRs; ER, PR, GR, ThR, RXR, HNF-4 (hepatocyte nuclear factor), and PPAR $\gamma$ . Interaction between ER and SRC-1 is

abolished in the presence of anti-estrogens, possibly because steric interference inhibits coactivator-binding conformation in ER $\alpha$ , as discussed above. Furthermore, SRC-1 mediates functional interactions between AF-1 and AF-2 of ER, PR, and AR via individual domains resulting in AF-1/AF-2 synergy. SRC-3 selectively augments transcriptional activity of ER $\alpha$  over that of ER $\beta$  (48). SRC members also interact with p300/CBP (CBP; CREB binding protein) (CREB; cAMP regulatory element binding protein) (49, 50).

## Cointegrators

p300/CBP was initially characterized as a coactivator required for CREB-dependent activation of cAMP-regulated promoters (51) and was later found to coactivate multiple factors such as NRs, p53, STATs (signal transducer and activator of transcription), and NF- $\kappa$ B (nuclear factor) (43). p300/CBP acts synergistically with ligand-activated ER $\alpha$  in enhancing *in vitro* transcription of chromatin templates (52). p300/CBP contains an N-terminal NR-box that is indispensable for NR interaction. In addition, p300/CBP interacts with coactivators of the SRC family via its C-terminal region. The CBP/SRC-1 complex is rather unstable, but a stable ternary complex is formed in the presence of an NR, containing NR, SRC, and CBP (53, 54). p300/CBP interacts weakly with ER or PR but can synergize with SRC-1 in coactivation of these receptors (55).

## Corepressors

Several factors repress transcriptional activity of NRs. NCoR (nuclear receptor corepressor) and SMRT (silencing mediator for retinoid and thyroid hormone receptors) have been shown to interact with and repress transcriptional activity of Type II receptors (56, 57). NCoR and SMRT interact with the non-ligand bound receptor, which contributes to obstruction of ligand-independent recruitment of coactivators. Upon ligand binding the corepressor is released and interaction with coactivators can occur (58, 59). Binding conventional corepressors does not appear to be an essential feature of the steroid receptor subgroup of NRs. SMRT corepresses the activity of ER and PR only in the presence of their cognate partial agonists tamoxifen and RU486 (60, 61). One explanation for this may be the constitutive DNA-binding character of Type II receptors. Corepressor binding may represent an important regulatory step preventing ligand-independent transactivation. In the case of the steroid receptors, ligand binding precedes DNA binding, and the requirement of additional regulators such as corepressors may be less of a priority. The ability of corepressors to interact only with antagonist-bound ER $\alpha$  and PR may indicate that steroid receptors contain cryptic corepressor-binding sites that are exposed in the antagonist-binding receptor conformation.

Short heterodimer partner (SHP) is an unusual member of the NR family in that it lacks a conventional DBD (62). SHP interacts with several NRs, including RAR and TR, and inhibits transactivation by these receptors. It displays a ligand-dependent interaction with ER $\alpha$  and ER $\beta$  that results in repression of

transcriptional activity (63, 64). SHP interacts with ERs via two NR-box-related motifs that may antagonize binding of p160 coactivators to the ERs. SHP also contains an active repressor mechanism (65).

## HISTONE ACETYLATION

Chromatin accessibility influences transcription regulation. Acetylation of histone tails disrupts higher-order chromatin structure and is associated with increased transcriptional activity. Histone acetylation may facilitate access of transcription factors to promoter elements (reviewed in 66). p300/CBP possesses histone acetyltransferase (HAT) activity and is tightly associated with the RNA polymerase II holoenzyme (67, 68). SRC-1 and SRC-3 also have intrinsic HAT activity located in the C termini (49, 69), whereas no HAT activity has been demonstrated for SRC-2. The HAT activity of p300/CBP is very strong compared with that of the SRC factors. P/CAF (p300/CBP associated factor) is a histone acetyltransferase that associates with p300/CBP, SRC-1, or SRC-3 in concert with RAR, TR, or ER (70). The HAT activity of coactivators and/or their ability to act as bridging factors recruiting other proteins with HAT activity may account for their capacity to enhance transcriptional activity of NRs. Conversely, histone deacetylase complexes specifically interact with DNA-binding repressor proteins such as Mad or the corepressors NCoR and SMRT. The capacity of these factors to repress transcription correlates with their ability to interact with the histone deacetylase complexes (71–73).

## CHAPERONE COMPLEXES

In the non-active state, members of the steroid receptor subgroup are found in a heterocomplex consisting of heat shock proteins (hsp), immunophilins, and p23. HsP and immunophilins are conserved and ubiquitously expressed proteins (reviewed in 74, 75). Immunophilins bind immuno-suppressant drugs (such as FK506, cyclosporin A, and rapamycin). Three immunophilins of high molecular weight associate with the steroid receptor chaperone complex through a direct binding to hsp90; FKBP50 (FK binding protein), FKBP52 and Cyp40. p23 forms an ATP-dependent complex with hsp90 and stabilizes the interaction between hsp90 and the different target proteins. The mature steroid receptor chaperone complex consists of an hsp90 dimer, one immunophilin, and a p23 monomer. Hsp70 can be coprecipitated with several steroid receptors; its association with the chaperone complex appears transient, and its role is unclear. In ER an additional region of several basic amino acids in the C-terminal part of the DBD, also recognized as a nuclear localization signal (NLS), is required for stable hsp90 interaction (76, 77). No physical interaction has been detected between hsp90 and the ER $\alpha$  NLS region. Interestingly, the immunophilin FKBP52 contains a negatively charged domain that may bind to the basic NLS in ER $\alpha$  and account for the stabilization of the hsp90 complex (75).

## TRANSCRIPTIONAL ACTIVITY: Two Transactivation Functions

Two activation functions, termed AF-1 and AF-2, are present in ER $\alpha$ . The core of AF-2 is located on H12 in the LBD. Ligand-induced conformational changes create the functional AF-2 surface. AF-1 is located in the N-terminal A/B domain and functions autonomously and in the absence of ligands. Different portions of AF-1 in ER $\alpha$  are required for tamoxifen- and estrogen agonism (78, 79). Although AF-1 and AF-2 function autonomously in a cell and promoter context-dependent manner, the intact ER is generally a stronger transactivator than the isolated AF-1 or AF-2 (reviewed in 80). Functional interaction between AF-1 and AF-2 is required for full activity of ER $\alpha$  under conditions where individual action of AF-1 or AF-2 is not supported (81, 82).

### Comparison of Transcriptional Activity of ER $\alpha$ and ER $\beta$

With regard to the ability to activate transcription with constructs that contain ERE (5), ER $\beta$  is weaker than ER $\alpha$  in most cell systems tested (20, 31, 83–85). ER $\beta$  fails to show agonistic response to tamoxifen, which appears to be due to functional differences in the A/B domains in ER $\alpha$  and ER $\beta$  (22, 31, 32). When the A/B domain of ER $\beta$  was exchanged with that of ER $\alpha$ , the resulting chimeric receptor responded agonistically to tamoxifen in HEC-1 cells (human endometrial cancer). Conversely, tamoxifen acted as a pure antagonist of an ER $\alpha$  chimera containing the A/B domain of ER $\beta$ . However, in another cell system (breast cancer cell line MDA-231), which supports the agonistic effect of tamoxifen by wild-type ER $\alpha$ , there was no response with the ER $\beta$  chimera, indicating that cellular context is important in determining transcriptional activity of the ERs and that agonistic response to tamoxifen is also influenced by receptor regions other than AF-1 (86). When the isolated AF-1 and AF-2 of ER $\alpha$  and ER $\beta$ , respectively, were fused to the DBD of the yeast factor Gal4, ER $\beta$  AF-1 was unable to initiate transcription autonomously in co-transfection experiments, in contrast to ER $\alpha$  AF-1. Both full-length ER $\beta$  and ER $\beta$  AF-2 induced transcription from a TATA-containing promoter less efficiently than full-length ER $\alpha$  or ER $\alpha$  AF-2, whereas each AF-2 was equally potent in transcriptional activity on a heterologous thymidine kinase promoter (87). ER $\beta$ , in contrast to ER $\alpha$ , interacts with SRC-1 in the absence of ligand. This interaction results in ligand-independent transcriptional activity (22), is independent of AF-2, and occurs as a consequence of phosphorylation of serine residues in the A/B domain (88). Other laboratories report low or negligible AF-1 activity of ER $\beta$ . These apparent conflicts could be due to cell-specific differences in ER $\beta$  AF-1 activity.

ER $\alpha$  and ER $\beta$  show opposite effects in regulation of AP1 in an AF-1-dependent manner (89). Anti-estrogens induce activity of an AP1 promoter in the presence of ER $\beta$ , whereas E2 blocks transcription. In the presence of ER $\alpha$  the pattern is reversed (90). A similar observation of activation of the RAR $\alpha$  promoter by E2 has

been described (91). Both receptors regulate transcription of the quinone reductase gene in response to anti-estrogens but not E2 (92).

## Dimerization

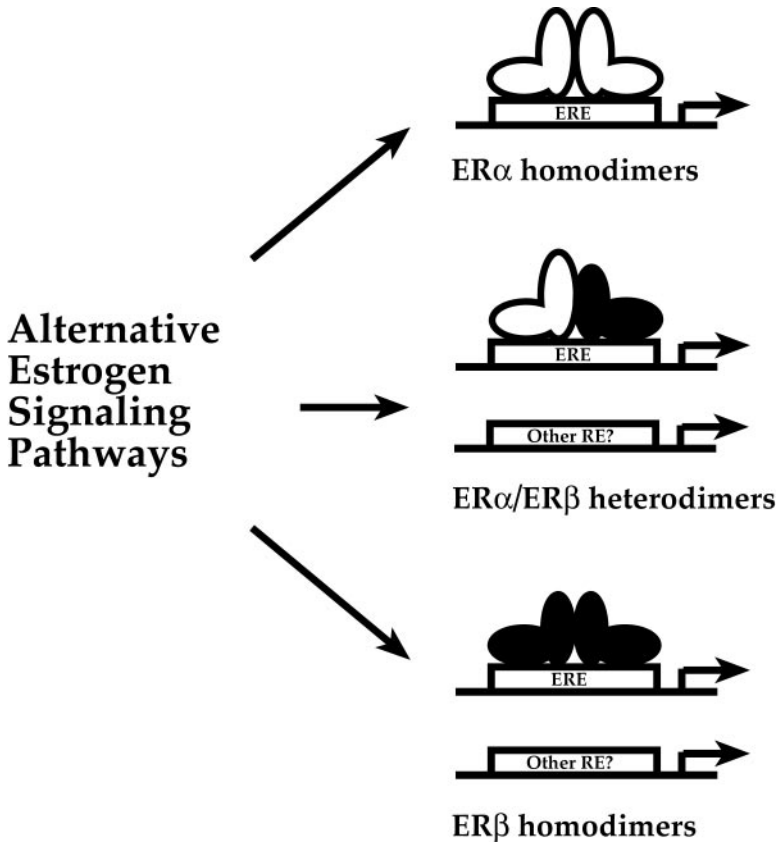
Transcriptional activity of ERs depends on receptor dimerization. Both receptor subtypes dimerize independently of ligand in solution and on DNA *in vitro*, but whether dimerization is ligand independent *in vivo* is unclear. Heterodimerization between the glucocorticoid and mineralocorticoid receptors contributes to tissue-specific actions of glucocorticoids (93). ER $\alpha$  and ER $\beta$  have almost identical DBDs, and the parts of the LBDs involved in dimerization also share a significant degree of similarity. ER $\alpha$  and ER $\beta$  form functional heterodimeric complexes both *in vitro* and in cells (27, 83, 85, 94). ER $\alpha$ /ER $\beta$  heterodimers bind to ERE sequences with a specificity and affinity similar to those of the respective homodimers, and ER $\alpha$  and ER $\beta$  are co-expressed in the same cells in mouse mammary gland and in rat cardiac myocytes and fibroblasts, suggesting that heterodimerization probably occurs *in vivo* (Figure 3) (95–97).

The physiological role of ER heterodimeric complexes remains to be determined. Creation of mutations in the DBD of ER $\alpha$  or ER $\beta$  resulted in receptors with altered DNA-binding specificity (from ERE to GRE). Co-transfection experiments, performed with a reporter construct containing a composite response element consisting of one ERE half site and one GRE half site, and the mutated ER $\alpha$ , together with wild-type ER $\beta$  or mutated ER $\beta$  with wild-type ER $\alpha$ , permitted measurement of transcriptional activity exclusively induced by  $\alpha/\beta$  heterodimers (98). Transcriptional activity of heterodimeric complexes was found to be dependent on a functionally intact AF-2 within both receptor subunits, supporting earlier findings with ER $\alpha$  and ER $\beta$  dominant-negative F domain mutants (99). The complex initiated transcription when only one ER subunit was competent to bind ligand, albeit to a lower extent than if both subunits interacted with ligand. In addition, subtype-specific AF-1 functions were retained within the context of the heterodimeric complex (98). Co-expression of ER $\alpha$  with ER $\beta$  modulates responses to tamoxifen and low levels of E2 in an ER $\beta$  dose-dependent manner, probably owing to formation of heterodimeric complexes (100).

## ER $\beta$ ISOFORMS

Analysis of ER $\beta$  transcripts revealed additional open reading frames upstream of the first reported sequence, with two putative translation start sites. Translation from these upstream ATGs gives rise to proteins containing 548 or 530 amino acids, respectively, in addition to the 485 amino acid ER $\beta$  first reported. These open reading frames are present in the human, mouse, and rat ER $\beta$  (27, 101).

Another variant, termed ER $\beta$ 2, contains 54 extra nucleotides within the reading frame, which causes an 18 amino acid insertion in the LBD of ER $\beta$ 1 (21, 23, 24, 26). ER $\beta$ 2 shows severely impaired E2-binding ability and consequently initiates



**Figure 3** Putative pathways by ER $\alpha$ /ER $\beta$  homo- and heterodimeric complexes.

transcription of reporter constructs poorly in transient transfection experiments. ER $\beta$ 2 binds to ERE sequences in vitro and can form heterodimeric complexes with both ER $\beta$  and ER $\alpha$ . Co-expression in cells of ER $\beta$ 2 with ER $\alpha$  or ER $\beta$  results in loss of transcriptional activity in an ER $\beta$ 2 dose-dependent fashion, indicating that ER $\beta$ 2 functions as a dominant-negative partner of both ER $\alpha$  and ER $\beta$ 1. Another splice variant in human tissue, ER $\beta$ cx, has an alternative C terminus and is unable to bind either ligand or DNA. It selectively heterodimerizes with ER $\alpha$  and inhibits its transcriptional activity (102). In addition, several other ER $\beta$  isoforms have varying C-terminal ends (103).

### Phosphorylation and Ligand-Independent Activity

The transition of NRs from silent into transcriptionally active states is more complex than initially proposed. Activation of ER appears to be a multistep process

relying on a number of events, including dimerization, the binding of ligand, phosphorylation, interaction with cofactors, and DNA binding. Phosphorylation of ERs occurs as part of both the ligand-induced activity and ligand-independent transcriptional activation (reviewed in 104, 105).

## Phosphorylation of the LBD

A conserved tyrosine residue in the LBD in both ER subtypes of all species, located in position 537 in human ER  $\alpha$  (Tyr 541 in mouse ER $\alpha$ ), appears to be important in the regulation of ER $\alpha$  transcriptional activity. Tyr 537 is phosphorylated by the Src family of tyrosine kinases in the absence of E2 (106). Mutation in human ER $\alpha$  of Tyr 537 to Ser or Ala produces a constitutively active receptor, whereas Lys, Phe, and Glu substitutions retain E2-dependent transcriptional activity (107). Peptide profiles of proteolytic digests of an ER $\alpha$  mutant, in which Tyr 537 has been replaced by Ser, show that this mutant appears to be in a ligand-bound conformation in the absence of E2 (108). Mouse ER $\alpha$  with Tyr 541 replaced by Asp, Glu, or Ala, but not Phe, also displays ligand-independent activity (109). Mouse and human ER $\alpha$  with cognate tyrosine substitutions show E2-independent interaction with SRC-1. SRC-1 furthermore enhances ligand-independent transcriptional activity of these mutant receptors. Interestingly, SRC-1 peptides stabilize the H12 position in the agonist-bound ER $\alpha$  and slow the dissociation rate of the agonist (36, 110). The three-dimensional structure of ER $\alpha$  shows that Tyr 537 is located at the C-terminal end of the loop preceding H12. Interestingly, in the crystal structure of ER $\alpha$  LBD bound to 4-OH-tamoxifen, Tyr 537 contacts residues in H3 and H4, which were not observed in the ER $\alpha$  DES three-dimensional structure. These contacts appear to stabilize the N-terminal turn of H12, albeit not in the agonist position observed in the DES-bound structure (36). Mutation of the corresponding tyrosine to asparagine in ER $\beta$  LBD also results in a receptor that is active in the absence of ligand (30).

Because phosphorylation of Tyr 537 in ER $\alpha$  occurs in the absence of E2, without inducing ligand-independent transcriptional activity, the phosphorylation status of Tyr 537 may regulate the ability of ER $\alpha$  to bind E2. Substitutions of this residue with a charged or an alanine residue result in a constitutively active receptor, probably the result of conformational changes within the ER $\alpha$  LBD that mimic ligand activation. This notion is supported by the observation that anti-estrogens inhibit the ligand-independent activity of both ER subtypes with tyrosine to asparagine substitutions, probably through disruption of the agonist-like conformation of these mutant receptors (30).

## EGF-Induced Phosphorylation

The fact that growth factors and protein kinase A (PKA) activators induce transcriptional activity of ER $\alpha$  in the absence of E2 challenged the view of ER as a strictly ligand-dependent transcription factor. Peptide growth factors, PKA-activating

agents, neurotransmitters, and cyclins can induce or enhance ER-mediated transcriptional activity (reviewed in 104). Phosphorylation appears to be important in ligand-independent activation of ER $\alpha$  in all of these pathways.

The N terminus of ER $\alpha$  contains several conserved serine residues within AF-1 that are targets for phosphorylation. Phosphorylation of Ser 118 of human ER $\alpha$  is induced by EGF and is dependent on the Ras-MAPK (mitogen-activated protein kinase) pathway (111, 112). Phosphorylation of Ser 118 via MAPK occurs in the absence of E2 and results in ligand-independent activity of ER $\alpha$ . E2 also induces phosphorylation of Ser 118, but this appears to be independent of MAPK (113), indicating that alternative signal transduction pathways can act on the same residue, depending on the E2 status of the cell. Serine residues in ER $\beta$  are also phosphorylated via the MAPK pathway. Co-expression of a dominant form of H-Ras (an integral factor in MAPK signaling) enhances E2-induced activity of mouse ER $\beta$ , indicating that alternative signal transduction pathways can act on the same residue, depending on the E2 status of the cell (22). In addition, phosphorylation of Ser 106 and Ser 124 in ER $\beta$  (numbered assuming 548 amino acids in mER $\beta$ ) by MAPK results in ligand-independent recruitment of SRC-1 and concomitant increase in transcriptional activity (88).

The physiological relevance of EGF-activation of ER $\alpha$  is supported by the observation that EGF imitates the effects of E2 in the murine female reproductive tract and in breast epithelial cells (114, 115). Mice deficient in ER $\alpha$  (ERKO) lack a uterotrophic response to EGF, demonstrating the involvement of ER $\alpha$  in mediating EGF action in vivo (116). EGF alone can increase the expression of PR in the mammary gland of sexually mature mice in an ER-dependent manner, mimicking the effects of E2. Inhibition of EGF action in the mammary gland blocks E2-induced expression of PR and development of the terminal end buds (117 and references therein). Another phosphorylation target in the A/B domain of ER is Ser 167, which is phosphorylated by casein kinase II upon binding of E2 in vitro (118). This residue is also phosphorylated by pp90rsk1 (90-kDa ribosomal S6 kinase, a Ser/Thr protein kinase) (119).

## cAMP-Dependent Phosphorylation

In addition to ligand-independent activation through phosphorylation of serine residues in the AF-1 of ER $\alpha$ , other compounds induce transcriptional activity in the absence of ligand but in a manner depending on AF-2 rather than AF-1 (120). cAMP activates PKA by inducing the release of the regulatory element from the catalytic subunit. Agents that increase cellular content of cAMP (forskolin, okadaic acid, and cholera toxin) evoke ligand-independent transcriptional activity of ER $\alpha$  and also synergize with E2-mediated activation (121). The partial agonistic response to tamoxifen increases with increased cellular cAMP (122). Activation via cAMP signaling pathways requires ER $\alpha$  AF-2 and appears to be dependent on PKA and thus represents a pathway distinct from activation via peptide growth

factors (123). However, no specific phosphorylation targets for PKA have been determined within the AF-2 (124). PKA phosphorylates Ser 236 in the DBD of ER $\alpha$ , which enhances receptor dimerization.

## Cyclins

Cyclins and their kinases, cyclin-dependent kinases (cdks) regulate cell cycle progression, and defects in the regulation of cyclins or cdks play an important role in the development and progression of cancer. Interestingly, E2 increases the protein levels of G1 regulatory cyclins A, B1, D1, D3, and E in mammary carcinoma female 7 (MCF7) cells (125). Cyclin D1, in addition, induces transcriptional activity of unliganded ER (126, 127), which is interesting because cyclin D1 is frequently expressed abnormally in breast tumors. Cyclin D1 activation of ER $\alpha$  occurs independently of the cognate cdk and direct phosphorylation of ER $\alpha$ ; cyclin D1 acts as a bridging factor between unliganded ER $\alpha$  and SRC-1 (128). E2 also increases cyclin D1 expression in an ER-dependent manner in breast cancer cell lines, which may be of relevance in explaining the mitogenic effects of E2 on breast tumors (129, 130). Ser 104 and Ser 106 in human ER $\alpha$  are phosphorylated by the cyclin A-cdk2 complex, enhancing transcriptional activity of ER $\alpha$  in both the absence and presence of ligand, which may be of consequence for cellular proliferation (131, 132).

## THE PHYSIOLOGICAL IMPORTANCE OF ESTROGENS AND THE ROLE OF HERS

In addition to their role in reproduction, estrogens influence a number of systems in females, including the mammary gland, the cardiovascular system, and bone. E2 is synthesized in the granulosa cells of the mature ovary through aromatization of testosterone supplied by the thecal cells. The rate-limiting enzyme in E2 synthesis is aromatase, a member of the P450 family. Synthesis of estrogens also occurs in adipose tissue, skeletal muscle, skin, hair follicles, and bone. As illustrated by the increased risk of fractures in post-menopausal women and the decreased risk with hormone replacement therapy (reviewed 133), estrogens are important for maintenance of female bone mass.

Estrogens also exert a protective effect on the cardiovascular system, possibly accounting for the low incidence of heart disease in women of reproductive age. After menopause the risk of cardiovascular disease increases and at 60 years of age or older the sexes converge in incidence of heart disease. Hormone replacement therapy has a beneficial effect on the cardiovascular system in post-menopausal women (reviewed in 134). In adult men endogenous estrogens are produced by the adrenals, testes, and adipose tissue. The role of estrogen production and ERs in males has historically been unclear, but testicular responses to exogenous estrogens in men includes an increase in Leydig cell number, atrophy of seminiferous tubules, and hyperplasia of the rete testis (reviewed in 135). Steroidogenesis and

proper development of Leydig cells are impaired in rats treated with exogenous E2 (reviewed in 136). ERs are present in all fetal reproductive organs in male mice. Furthermore, ER $\alpha$  is present in seminal vesicles, epididymis, and nonhyperplastic prostate in men.

## EXPRESSION PATTERN OF ER $\beta$ IN THE BODY

ER $\beta$  was originally cloned from a rat prostate cDNA library and is expressed in parts of the rat prostate (reviewed in 137). ER $\beta$  is also expressed in human prostate (138). In the testis ER $\beta$  is present in Sertoli cells at all developmental stages, in fetal Leydig cells, peritubular cells, and gonocytes. In pubertal rats, ER $\beta$  is expressed in spermatogonia and pachytene spermatocytes (139). ER $\beta$  in the ovaries is mainly found in the granulosa cells of primary, secondary, and mature follicles (5,140–144). The expression of ER $\beta$  in rat ovaries fluctuates during the estrus cycle; lowest levels occur in the estrus phase (140). ER $\alpha$  is not detected in granulosa cells but is present in theca cells, interstitial gland cells, and germinal epithelium (142). In the adult rat uterus ER $\beta$  is found only in the glandular epithelial cells, whereas ER $\alpha$  is present in both luminal and glandular epithelial cells and in the stroma (140, 142). Both ER subtypes are expressed in rat pituitary (145, 146), and ER $\beta$  is present in multiple human tissues (138).

## AROMATASE AND ER DEFICIENCY

During the 1990s, two men and five women with mutations in the CYP19 (aromatase) gene were reported to have negligible aromatase activity (reviewed in 147). The men were born with normal genital development and had normal onset of puberty, but linear growth persisted into adulthood. Examination of the skeleton showed unfused epiphyseal plates, delayed bone age, and osteoporosis. The women had ambiguous external genitalia at birth. Internal reproductive organs were normal, but at puberty several effects of the lack of sufficient E2 included failure of breast growth, amenorrhea, enlargement of the clitoris (androgenization), absence of a growth spurt, delayed bone age, and later multicystic ovaries as well as unfused epiphyses. Treatment with estrogens and progesterone resulted in regression of ovarian cysts, restored ovarian function, and normal pubertal development. Levels of testosterone, FSH, and LH in serum were elevated in both sexes, confirming the long-held concept of a role for estrogens in regulation of gonadotropin secretion in both males and females.

The generation of mutant mice lacking functional aromatase (ArKO) has been described by two groups (148, 149). Mice homozygous for the mutation are viable with normal genital development. However, at the time of puberty female mice display atrophic but histologically otherwise normal uteri; complete lack of corpora lutea in the ovaries, which in addition show hypertrophied stroma; and an increased number of granulosa cells and follicles arrested before ovulation.

Mammary glands remain in a prepubertal stage. FSH, LH, and testosterone levels are elevated. Treatment with E2 resulted in normal response of uteri of mutant mice compared with wild-type mice.

Male mutant mice showed an age-related disruption of spermatogenesis with increased germ cell apoptosis, Leydig cell hypertrophy and hyperplasia, and normal numbers of Sertoli cells and germ cells (150). In addition, ArKO male mice showed impaired sexual behavior, with reduced mounting frequency and increased latency between mounts; however, young males were able to sire litters (148, 149). Mice of both sexes showed increased internal fat. The normal fetal development of aromatase-deficient mice and humans is attributed to the transfer of maternal estrogens during pregnancy. The question still remains as to whether estrogen signaling is required for embryonic development (reviewed in 151).

At the time of the creation of ER $\alpha$  KO (ERKO) [ER $\alpha$  (-/-) and ER $\beta$  (+/+)] mice, only one ER had been identified, and ERKO mice were thought to be completely devoid of estrogen signaling. Not long afterward a man was identified with a bi-allelic disruptive mutation of the ER $\alpha$  gene. This patient was externally normal with normal genitalia but suffered from osteoporosis and was still growing at the age of 28 because the epiphyseal plates were unfused (152). Both male and female ERKO mice are infertile. Females do not ovulate. The ovaries are hyperemic and lack corpora lutea. LH levels are elevated, and the ovarian phenotype of females can be successfully rescued by prepubertal treatment with a gonadotropin-releasing hormone antagonist (153). The uteri are hypoplastic and have very reduced response to E2 treatment. The mammary glands of ERKO females appear to exhibit normal prepubertal development, but no pubertal growth (154). Ductal growth of the mammary gland during puberty normally occurs in response to ovarian synthesis of E2, and ER $\alpha$  appears to be indispensable in mediating the growth-stimulation.

In ERKO males the testes are normal at birth and develop normally to puberty when they begin to degenerate. By 5 months of age the testes are atrophic. Luminal fluid appears to accumulate in the seminiferous tubules, rete testes, and efferent ductules owing to a lack of reabsorption in the efferent ductules that results in reduced sperm concentrations in the epididymis (155). Sperm from ERKO mice have severely impaired motility and fertilization ability indicating a critical role for ER $\alpha$  in the maturation process of spermatozoa. ERKO mice, in addition, show compromised sexual behavior at several levels. The males appear normal with regard to interest in receptive females and mounting attempts; however, latency between mounts is prolonged, number of intromissions is reduced, and ejaculations do not occur. ERKO males, furthermore, display less offensive aggressive behavior and an increased rate of pup-killing compared with heterozygous or wild-type mice (156). Comparison with ArKO male mice (which are exposed to maternal estrogens during the gestation period) and which show less severe impairment of sexual behavior, indicates that development of appropriate sexual behavior may be dependent on estrogen signaling via ER $\alpha$  during early brain development or, alternatively, that maintenance of sexual behavior in the adult mouse involves other signals than those mediated by ER $\alpha$ . ERKO females show increased aggressive

behavior toward other females, reject mating advances by males, and exhibit impaired parental behavior including a higher degree of infanticide (84, 157, 158).

The phenotype of BERKO mice [ER $\beta$  (-/-), ER $\alpha$  (+/+)] is different from that of ERKO mice (159). BERKO mice develop normally and are viable. BERKO females have very reduced fertility. When they do have pups, the litter size is no more than three. Poor reproductive capacity in BERKO females is due to defects in both the ovary and the uterus. Unlike ERKO females, where the ovarian defects are the result of pituitary dysfunction, the defects in BERKO females are in the ovary itself. There is a marked paucity of corpora lutea and an increase in the number of follicles with premature atresia. It appears that follicles are actively recruited into the growth pool but they fail to mature. The oocytes are not released and die within the preantral follicles. By 1.5 years of age no follicles are left in the BERKO ovary. Surprisingly, in the heterozygote females, the ovary also ages much more rapidly than in the wild-type littermates, and breeding becomes very inefficient after a few matings. Uterine dysfunction probably contributes to infertility in BERKO mice because in pregnant BERKOs most of the fetuses are resorbed. Because the uterus is traditionally thought of as ER $\alpha$ -regulated tissue, it was most surprising to find defects in BERKO uteri. In both immature and adult BERKO females expression of the proliferation marker, Ki-67, was increased, and the uterus was hyperresponsive to estradiol, as indicated by the over-expression of VEGF and IGF-I (160).

BERKO males appear to be normal, but develop prostatic hyperplasia as young as 3 months of age. This suggests that estrogens may control prostatic growth via ER $\beta$ . Double-mutant mice lacking both ERs are viable with normal development (161). Phenotypes appear essentially similar to ERKO mice except for the appearance of structures in the ovary that resemble seminiferous tubules of the testis. These structures have not been fully characterized, but the phenomenon has been described as post-natal sex reversal.

## THE MAMMARY GLAND

Until the onset of puberty in humans there is no significant difference in the primitive mammary gland between the sexes. In females, the growth rate of the mammary epithelia accelerates during puberty to eventually fill the mammary fat pad. The functional portion of the human mammary gland is called the terminal ductal lobular unit (TDLU), corresponding to the terminal end buds in mice. After development is completed these structures are the major hormone-sensitive areas of the mammary epithelium in all species (reviewed in 162). Most mammary cancers also originate from these structures because they constitute the major proliferative cell population in the mammary gland. The TDLU responds by DNA synthesis to both estrogen and progesterone during the luteal phase of the human menstrual cycle. This is in contrast to the endometrium, where the bulk of proliferation occurs in response to E2 during the follicular phase, and progesterone secretion during the luteal phase opposes E2-stimulated proliferation (163 and references therein).

During pregnancy the mammary glands of all species form side buds that either branch or form alveoli. This process continues to fill the entire fat pad with alveolar structures. In the post-lactating mammary gland regression takes place with death of most of the functional cells. The early phases of regression are characterized by widespread apoptosis. In post-menopausal women TDLU also regresses. ER $\beta$  appears to be ubiquitously expressed during all developmental stages, whereas ER $\alpha$  expression fluctuates, with an increase around puberty, gradual decline during pregnancy, up-regulation during lactation, and a decrease in the post-lactating gland (97).

The role of estrogen receptors in the proliferative response of the breast to estrogen is not understood. In the normal mammary gland, ER $\alpha$  is not expressed in proliferating epithelial cells, which indicates that the growth stimulatory effect of E2 occurs indirectly (97, 164, 165). In contrast, breast cancers that express ER $\alpha$  respond to estrogen by proliferation, and their growth is blocked by the anti-estrogen tamoxifen. It is thought that ER in the stroma mediates growth-stimulatory effects of estrogen by increasing growth factor secretion (163). Growth factors are the mediators of proliferation in the epithelial cells. This still does not explain why ER $\alpha$ -containing epithelial cells do not divide in response to estrogen in normal mammary gland but do so in breast cancer. It appears that ER $\beta$  might down-regulate growth factor receptors or some key protein involved in proliferative pathways. The respective roles of the two ERs in estrogen-mediated breast growth remains to be delineated.

## ER $\beta$ IN BREAST CANCER

ER $\beta$  is present in breast cancer (166–176), but it is not clear what this means in development, progression, and treatment of breast cancer. Clearly, in the rodent breast ER $\beta$  is the more highly expressed of the two receptors. If this is also true in the human breast, the presence of ER $\beta$  in breast cancer would not be surprising. If ER $\beta$  plays a role in growth repression, and if repression is mediated through EREs, anti-estrogen therapy would inhibit this growth repression. If, on the other hand, ER $\beta$  exerts its growth repressive effects via AP-1 or SP-1 sites, anti-estrogens would limit growth via ER $\beta$ . What is needed are more studies with larger numbers of both normal and malignant breast samples. Such studies would permit an evaluation of the ER $\beta$  content of the breast as disease progresses and its correlation with responsiveness to anti-estrogen treatment.

## SUMMARY AND CONCLUSIONS

The biological effects of estrogens are mediated via two estrogen receptors, ER $\alpha$  and ER $\beta$ , which regulate transcription through direct interaction with specific binding sites on DNA (EREs) in promoter regions of target genes. In addition, ERs are transcriptional regulators at AP-1 and SP-1 sites.

Both ER $\alpha$  and ER $\beta$  bind specifically to estrogen responsive elements (EREs) and activate ERE-containing promoters in response to 17 $\beta$ -estradiol (E2). ER $\beta$

requires approximately five- to tenfold higher concentrations of E2 than ER $\alpha$  for maximal transcriptional activity to occur, and ER $\beta$  is only approximately 30% as efficient as ER $\alpha$  in a variety of reporter systems. The anti-estrogen, tamoxifen, is a mixed agonist/antagonist on ER $\alpha$  but is a pure antagonist on ER $\beta$ . ER $\alpha$  and ER $\beta$  can form functional DNA-binding heterodimeric complexes both in vitro and in cell systems, and in these complexes ER $\beta$  is the dominant partner repressing transcriptional activity at low concentrations of E2 and in the presence of tamoxifen. At AP-1 and SP-1 sites, ER $\alpha$  and ER $\beta$  can have opposite actions. This is true in the presence of both agonists and antagonists. These opposing effects of the two estrogen receptors may be very important in helping the understanding of the good and bad effects of estrogens. How, for example, can estrogen be the cause of breast malignancies and yet phytoestrogens perhaps be the factor responsible for the low incidence of the same malignancy in the Asian population? In conclusion, signal transduction by ERs involves multiple pathways, depending on tissue-selective expression of different receptor subtypes, the nature of the DNA target, concentration of agonistic or antagonistic ligand, and formation of heterodimeric complexes.

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