Mechanisms of Estrogen Action

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Nilsson, Stefan, Sari Mäkelä, Eckardt Treuter, Michel Tujague, Jane Thomsen, Göran Andersson, Eva Enmark, Katarina Pettersson, Margaret Warner, and Jan-Åke Gustafsson. Mechanisms of Estrogen Action. Physiol Rev 81: 1535–1565, 2001.—Our appreciation of the physiological functions of estrogens and the mechanisms through which estrogens bring about these functions has changed during the past decade. Just as transgenic mice were produced in which estrogen receptors had been inactivated and we thought that we were about to understand the role of estrogen receptors in physiology and pathology, it was found that there was not one but two distinct and functional estrogen receptors, now called ERα and ERβ. Transgenic mice in which each of the receptors or both the
receptors are inactive have revealed a much broader role for estrogens in the body than was previously thought. This decade also saw the description of a male patient who had no functional ERα and whose continued bone growth clearly revealed an important function of estrogen in men. The importance of estrogen in both males and females was also demonstrated in the laboratory in transgenic mice in which the aromatase gene was inactivated. Finally, crystal structures of the estrogen receptors with agonists and antagonists have revealed much about how ligand binding influences receptor conformation and how this conformation influences interaction of the receptor with coactivators or corepressors and hence determines cellular response to ligands.

I. INTRODUCTION

More than 30 years ago Jensen and Jacobsen (156) came to the conclusion, based on the specific binding of estradiol-17β (E2) in the uterus, that the biological effects of estrogen had to be mediated by a receptor protein. For 24 years this protein was extensively studied in several laboratories (57, 120), and in 1986, two groups reported the cloning of this estrogen receptor (ER) (121, 122). Until 1995, it was assumed that there was only one ER and that it was responsible for mediating all of the physiological and pharmacological effects of natural and synthetic estrogens and antiestrogens. However, in 1995, a second ER, ERβ, was cloned from a rat prostate cDNA library (182). The former ER is now called ERα. Since then, several groups have cloned ERβ from various species (93, 242, 352, 355, 358) and have identified several ERβ isoforms (67, 208, 221, 240, 257, 271). The discovery of ERβ has forced a reevaluation of the biology of estrogen and, because of the abundance of ERβ in the male urogenital tract, has refocused attention on the role of estrogen in males.

II. ESTROGEN RECEPTOR TYPES α AND β STRUCTURE AND FUNCTIONAL DOMAINS

ERα and ERβ belong to the steroid/thyroid hormone superfamily of nuclear receptors, members of which share a common structural architecture (99, 118, 123, 166, 218, 363). They are composed of three independent but interacting functional domains: the NH2-terminal or A/B domain, the C or DNA-binding domain, and the D/E/F or ligand-binding domain (Fig. 1). Binding of a ligand to ER triggers conformational changes in the receptor and this leads, via a number of events, to changes in the rate of transcription of estrogen-regulated genes. These events, and the order in which they occur in the overall process, are not completely understood, but they include receptor dimerization, receptor-DNA interaction, recruitment of and interaction with coactivators and other transcription factors, and formation of a preinitiation complex (27, 28, 166, 176, 177, 228, 291, 322).

The N-terminal domain of nuclear receptors encodes a ligand-independent activation function (AF1) (30, 177, 224, 356), a region of the receptor involved in protein-protein interactions (226, 259, 384), and transcriptional activation of target-gene expression. Comparison of the AF1 domains of the two estrogen receptors has revealed that, in ERα, this domain is very active in stimulation of reporter-gene expression from a variety of estrogen response element (ERE)-reporter constructs, in different cell lines (78), but the activity of the AF1 domain of ERβ under the same conditions is negligible. Another striking difference between the two receptors is their distinctive responses to the synthetic antiestrogens tamoxifen, raloxifene, and ICI-164,384. On an ERE-based reporter gene, these ligands are partial E2 agonists with ERα but are pure E2 antagonists with ERβ (23, 223, 227). Dissimilarity in the NH2-terminal regions of ERα and ERβ is one possible explanation for the difference between the two receptors in their response to various ligands. In ERα, two distinct parts of AF1 are required for the agonism of E2 and the partial agonism of tamoxifen, respectively (223). In ERβ, this dual function of AF1 is missing (227). The importance of ERβAF1 in transcriptional activity therefore remains to be clarified.

The DNA binding domain (DBD) contains a two zinc finger structure, which plays an important role in receptor dimerization and in binding of receptors to specific DNA sequences (27, 95, 128a, 128b, 315, 367). The DBDs of ERα and ERβ are highly homologous (93). In particular, the P box, a sequence which is critical for target-DNA recognition and specificity, is identical in the two receptors (371). Thus ERα and ERβ can be expected to bind to various EREs with similar specificity and affinity.

The COOH-terminal, E/F-, or ligand-binding domain (LBD) mediates ligand binding, receptor dimerization, nuclear translocation, and transactivation of target gene.
Amino acid residues that line the surface of the ligand binding cavity, or that interact directly with bound ligands, span the LBD from helix 3 to helix 12 (44, 274, 356). The LBD also harbors activation function 2 (AF2), which is a complex region whose structure and function are governed by the binding of ligands (83, 84, 102, 133, 216, 321). Crystallographic studies with the LBDs of ERα and ERβ revealed that the AF2 interaction surface is composed of amino acids in helix 3, 4, 5, and 12 and that the position of helix 12 is altered by binding of ligands. When the ERα LBD is complexed with the agonists, E2 or diethylstilbestrol (DES), helix 12 is positioned over the ligand-binding pocket and forms the surface for recruitment and interaction of coactivators (44, 321, 398). In contrast, in the ERα- and ERβ-LBD complexes with raloxifene (44, 274) or the ERα-LBD 4-OH-tamoxifen complex (321), helix 12 is displaced from its agonist position over the ligand-binding cavity and instead occupies the hydrophobic groove formed by helix 3, 4, and 5. In this position, helix 12 foils the coactivator interaction surface (Fig. 2). It is evident that different ligands induce different receptor conformations (223, 264) and that the positioning of helix 12 is the key event that permits discrimination between estrogen agonists (E2 and DES) and antagonists (raloxifene and 4-OH-tamoxifen).

The LBDs of ERα and ERβ share a high degree of homology in their primary amino acid sequence and are also very similar in their tertiary architecture. It is, therefore, not surprising that the majority of compounds tested so far bind to ERα and ERβ with similar affinities (183) or have similar potencies in activation of ERE-mediated reporter gene expression (23). The phytoestrogen genistein is one naturally occurring ligand that has an ~30-fold higher affinity for ERβ. When genistein is bound to ERβ, helix 12 does not adopt an agonist conformation but has, instead, a position more similar to that seen with an antagonist. This result is unexpected because the molecular shape and volume of genistein and E2 are very similar (274) and because genistein is a partial (60–70% of E2) agonist with ERβ on an ERE-driven reporter gene (23). There is no explanation at present for the conformation of helix 12 in the ERβ-genistein structure. It may well be that there are subtle conformational differences between the two ER subtypes that are not discerned by comparing their primary amino acid sequences or crystallographic tertiary structures. Agonists induce conformations of ER that promote and stabilize ER-coactivator interaction (357) while ER-coactivator interaction reciprocally stabilizes ER-ligand interaction, markedly slowing the rate of dissociation of bound agonist from both ERα and ERβ in vitro (115). Clearly, further structural studies with ER-ligand complexes are required to fully understand the subtle interrelationship between ligand binding and helix 12 orientation.

### III. ESTROGEN RECEPTOR SPLICE VARIANTS

Several splice variants of ERβ have been described. Some have extended NH₂ termini, and others have truncations and/or insertions at the COOH terminus and in the LBD. A human ERβ cDNA encoding a protein of 530 amino acids was identified in 1998 (256). It was longer than the original rat ERβ clone, and this difference was due to an NH₂-terminal extension, composed of 45 amino acids. Later, a rodent ERβ isoform that was 64 amino acid residues longer than the original rat ERβ clone (ERβ-485)
was reported (208). In addition to extensions of the NH₂ terminus, three groups have reported cloning of ERβ-503, an isoform with an in-frame insertion of 18 amino acids in the LBD (67, 128, 271). This isoform is a splice variant, and the insert is in the junction between exons 5 and 6 (93). In contrast to the NH₂-terminally extended ERβ isoforms, the affinity of ERβ-503 for E2 and other known ER ligands is lower than for ERα and for ERβ-485. In one report, ERβ-503 acted as a dominant negative regulator by suppressing E2-dependent ERα- and ERβ-mediated activation of gene transcription, but did not bind E2 (221). In other reports, ERβ-503 did exhibit E2-dependent transcriptional activation of a reporter gene, but the concentration of E2 needed was 100- to 1,000-fold higher than that for ERα (271, 128). All ERβ isoforms, 503, 485, and 530, bind to consensus ERE and heterodimerize with each other and with ERα (128, 271).

Transcripts encoding additional ERβ isoforms with variations at the extreme COOH terminus have been found in human testis cDNA libraries (257, 240). ERβcx (257) is identical to ERβ-530 in exons 1–7, but exon 8 is completely different. The last 61 COOH-terminal amino acids (exon 8), encoding part of helix 11 and helix 12, have been replaced by 26 unique amino acid residues. Due to the exchange of the last exon, ERβcx lacks amino acid residues important for ligand binding and those that constitute the core of the AF2 domain. It is, therefore, not surprising that ERβcx does not bind E2 and has no capacity to activate transcription of an E2-sensitive reporter gene (257). Surprisingly, in view of its intact DBD, ERβcx does not bind to a consensus ERE. Furthermore, ERβcx shows preferential heterodimerization with ERα rather than with ERβ, inhibiting ERα DNA binding. Functionally, the heterodimerization of ERβcx with ERα has a dominant negative effect on ligand-dependent ERα reporter-gene transactivation (257).

Moore et al. (240) have described five ERβ isoforms (ERβ 1–5). ERβ 1 corresponds to the previously described ERβ-530 (256), and the ERβ 2 variant is most likely identical to ERβcx (257). However, ERβ 3–5 are novel splice variants with exchanges of the last exon of ERβ-530 for previously unknown exons. As with ERβcx, neither of the novel COOH-terminal splice variants, ERβ 3–5, can be expected to bind E2 or activate transcription from an ERE-driven reporter, as they all lack amino acids important for ligand binding as well as the core of AF2. In contrast to what was reported for ERβcx (257), two of the COOH-terminal splice variants, ERβ 2 and 3, do bind to a consensus ERE.

Various alternatively spliced forms of ERα have also been described (108–110, 243, 351, 406). To date there is insufficient information as to whether or not all isoforms and splice variants of ERα are expressed as proteins or whether they have any major biological and physiological role. Another source of variability in receptor function, and perhaps also dysfunction, is ERα and -β gene polymorphisms. ERα polymorphisms have been linked to increased litter size in pigs (300, 325), breast cancer susceptibility (4, 313, 411), bone mineral density and osteoporosis (169, 236, 260), hypertension (201), spontaneous abortion (203, 311), and body height (202).

IV. SPECIFIC ESTROGEN RECEPTOR TYPES: α AND β LIGANDS

The discovery of ERβ has revitalized the search for improved tissue-selective estrogen receptor modulators (SERM). Such ligands could provide the benefits of estrogens and avoid unwanted side effects of E2. In the clinical setting, these pharmaceuticals would be used for prevention or treatment of menopausal symptoms, osteoporosis, cardiovascular disease, and breast cancer in women, or other estrogen-related indications affecting both men and women (72, 74, 124, 245, 287). Several large pharmaceutical companies are engaged in the development of ERα- and ERβ-selective SERMs, but none has yet come to market. Katzenellenbogen and co-workers (230, 346) have synthesized ER subtype-specific ligands. The most ERα-selective ligand had a 120-fold higher agonist potency for ERα than for ERβ. Another selective ligand showed full ERα agonism but pure ERβ antagonism (346). The ERβ-selective antagonist was further investigated by the synthesis of a series of analogs with substituents of various sizes in both cis- and trans-configurations (230). All analogs were agonists on ERα, but only those with small substituents were ERβ agonists. As substituent size increased, the agonism on ERβ disappeared, and with larger substituents the ligands were pure ERβ-selective antagonists. The gradual ERβ-selective antagonism by this series of analogs was influenced by both size and shape of the substituent. It was concluded by the authors that less steric perturbation is required to induce an antagonist conformation in ERβ than in ERα. This could be due in part to the observation (274) that the volume of the binding cavity of ERβ is smaller than that of ERα.

Are phytoestrogens natural SERMs? Phytoestrogens are nonsteroidal polyphenolic compounds present in several edible plants. On the basis of their chemical structure, phytoestrogens may be divided into four subclasses: isoflavonoids, flavonoids, coumestans, and mammalian lignans. The major dietary source of isoflavonoids (e.g., genistein, daidzein, formononetin, biochanin A) is soybean. Flavonoids (e.g., chrysin, apigenin, naringenin, kaempferol, quercetin) are more widely distributed in the plant kingdom and are present in several edible plants. Coumestans (e.g., coumestrol) are present in plants not commonly used in human diets, such as alfalfa sprouts. Mammalian lignans (e.g., enterolactone and enterodiol) are not present in human diets as such, but are ingested as...
precursors (plant lignans), which are converted to mammalian lignans by gut microflora. Plant lignans are present in fiber-rich foods, such as flaxseeds and unrefined grain products. Isoflavones and coumestrol interact with ER in vitro, although the activities of individual compounds with similar chemical structures vary remarkably (23, 183). Some flavonoids show modest estrogenic activity, while others are completely inactive (183). Mammalian lignans are very weak estrogens, and concentrations of 1 mM or more are required to show any ERα- or ERβ-mediated activity (303, 308).

It has been suggested that dietary genistein could play a role in preventing the development of hormone-dependent diseases and conditions, such as breast and prostate cancer, cardiovascular disease, menopausal symptoms, and osteoporosis (reviewed in Ref. 341). This suggestion comes from epidemiological studies, showing an inverse correlation between the intake or serum concentrations of genistein and the risk of estrogen-related diseases and conditions. At present, there is no direct evidence for the beneficial action of genistein in humans. It has also been claimed that genistein would be devoid of the adverse effects typically found with E2, but this has not been shown convincingly either.

There are very little data from in vivo studies to demonstrate the tissue and/or ERβ selectivity of genistein that has been demonstrated in vitro (23). Most studies have been done with high doses of genistein, likely to exert both ERα- and ERβ-mediated activities. Furthermore, careful dose-response studies have not been done. In most in vivo studies, genistein acts as an estrogen and induces effects similar to E2 in female and male experimental animals (308, 340). At low doses, genistein and other isoflavones have been shown to act selectively in the cardiovascular system. In female macaques, isoflavones have favorable effects on the cardiovascular system without affecting the reproductive tract (7). In female rats, genistein was as potent a vasculoprotectant as E2, but unlike E2 showed no uterotrophic activity (216a). At present, it is not known to what extent the vascular responses are mediated by ERs. Both ER subtypes are expressed in vascular tissues, but in addition, there is sufficient evidence to indicate the presence of non-receptor-mediated actions of E2 in the same tissues. Further studies are thus needed to demonstrate that genistein acts in a receptor- or tissue-selective manner in vivo.

V. ESTROGEN RECEPTOR-DNA INTERACTIONS

For several years it was thought that the only mechanism through which estrogens affected transcription of E2-sensitive genes was by direct binding of activated ER to EREs. These estrogen response elements were first observed in the 5’-flanking region of the *Xenopus* vitellogenin A2 gene (168). Today we know that ERα and ERβ can also modulate the expression of genes without directly binding to DNA (Fig. 3). One example is the interaction between ERα and the c-rel subunit of the NFκB complex. This interaction prevents NFκB from binding to and stimulating expression from the interleukin-6 (IL-6) promoter (112). In this way, E2 inhibits expression of the cytokine IL-6 (112, 292). Another example of indirect action on DNA is the physical interaction of ERα with the Sp1 transcription factor (25, 277, 289). ERα enhancement of Sp1 DNA binding is hormone independent (277), and both ERα and ERβ can activate transcription of the retinoic acid receptor α1 (RAR-1) gene, presumably by the formation of an ER-Sp1 complex on GC-rich Sp1 sites in the RAR1 promoter (345, 409). Interestingly, in one study, ERβ activated RAR-1 promoter-reporter constructs in the presence of the estrogen antagonists 4-OH-tamoxifen, raloxifene, and ICI-164,384. E2 did not activate expression of the reporter but blocked the effect of the antagonists (409).

Both ERα and ERβ can interact with the fos/jun transcription factor complex on AP1 sites to stimulate gene expression, however, with opposite effects in the presence of E2 (264, 383, 385). In the presence of ERα, typical agonists such as E2 and DES as well as the antiestrogen tamoxifen function as agonists in the AP1 pathway. Raloxifene is only a partial agonist. In contrast, in the presence of ERβ, tamoxifen and raloxifene behave as fully competent agonists in the AP1 pathway, while E2 acts as an antagonist, inhibiting the activity of both tamoxifen and raloxifene (264). Analysis of the mechanism of ERα-stimulated expression from an AP1 site showed that agonist-bound ERα requires intact AF1 and AF2 functions and that it enhances AP1 activity via interactions with the p160 family of coactivators. In this “agonist” pathway the DBD of ERα is essential. The “antiestrogen” pathway, triggered by antiestrogen-ligated ERβ, enhances AP1 activity in an AF1- and AF2-independent fashion but also requires an intact DBD. The DBD sequesters cointeractors from the API transcription complex, releasing it from suppression and subsequently resulting in API-dependent transcription (385).

The electrophilic/antioxidant response element (EpRE/ARE) is yet another site on DNA where ERα and ERβ behave differently. In MCF-7 cells, antiestrogens but not E2 activate transcription of the quinone reductase gene and increase NAD(P)H:quinone oxidoreductase enzyme activity via an EpRE/ARE (239). Furthermore, E2 inhibits the agonistic effect of the antiestrogens on EpRE/ARE reporter constructs, and in this capacity, ERβ is more efficacious than ERα (238). These findings suggest that antiestrogens are antioxidants and inducers of phase 2 detoxification enzymes, protecting cells from damage by oxygen radicals and other toxic by-products of metabolic processes.
oxidation. Furthermore, it would seem that these protective effects of antiestrogens are mediated by ERβ.

Another potentially important pathway of estrogen action is constituted by the very rapid, so-called, nongenomic effects of certain ligands (232). There is evidence that some of the rapid effects of nuclear receptor ligands require the presence of the receptor protein (237, 266, 293). In endothelial cells, E2-mediated membrane effects lead to sequential activation of ras, raf, mitogen-activated protein kinase kinase (MEK), and subsequently activation of mitogen-activated protein kinase (MAPK) (71). It has been suggested that this leads to activation of endothelial nitric oxide synthase (eNOS) and release of nitric oxide (NO). In neurons, membrane effects of estrogen lead to stimulation of src, ras, MEK, and MAPK, resulting in neuroprotection, and in osteoblasts the membrane effects of estrogen may be involved in control of apoptosis, cell proliferation, and differentiation (71).

VI. PHOSPHORYLATION OF ESTROGEN RECEPTORS

During the past 15 years, the dogma of the strict requirement of hormones for activation of steroid receptors has been challenged. Evidence for ligand-independent activation of these receptors through alternative signaling pathways has emerged (reviewed in Refs. 55, 91, 386, 387). In most instances, the mechanisms underlying this activation involve phosphorylation of the receptors (318, 386). The phosphorylation sites on native ERα from calf, mouse, and rat uterus, and from human MCF-7 cells, have been extensively studied by analysis of the phosphoamino acid content of the receptor and/or its binding to anti-phosphotyrosine antibodies (21, 22, 233). There are still several controversial issues such as whether both tyrosine and serine are phosphorylated and which phosphorylations are ligand dependent. Some investigators report phosphorylation on tyrosine (3, 22, 231, 233) while others detect serine as the only phosphorylated amino acid (13, 51, 85, 191). A single tyrosine phosphorylation site in human ERα at position 537 has been identified in vitro (12) and in vivo (11, 12, 14). Whether or not this phosphorylation is E2 dependent is still a point of contention (14).

Phosphorylation of ERα on serine residues has been studied with recombinant human or mouse ERα expressed in COS-1 cells in the presence of E2 (190, 200). The vast majority of phosphoserine residues were

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**FIG. 3.** Model representing the various modes through which estrogen receptors can modulate transcription of genes.

In the first panel is depicted the classical interaction of the activated receptor with estrogen response elements (EREs) on DNA. In the other three panels are representations of the indirect effects of estrogen receptors on transcription interactions. This occurs through protein-protein interactions with the Sp1, AP1, and NFκB proteins.
A. Ligand Binding and ER Phosphorylation

Even though ERα is phosphorylated in the absence of E2 in different cell lines (12, 200), enhanced phosphorylation of the receptor occurs in response to physiological doses of E2 (3, 16, 85, 200). E2 treatment of MCF-7 cells has also been reported to result in the dephosphorylation of a single unspecified site in ERα (11). The enzymes responsible for E2-dependent phosphorylation of ER seem to be diverse. They include an E2-dependent tyrosine kinase, which has been purified from calf uterus (52), and a casein kinase II in MCF-7 cells, which phosphorylates Ser-167 (11). In addition, serines other than Ser-167 are E2-stimulated phosphorylation sites (3, 158, 159, 200) that do not have casein kinase II binding sites in their environment (157a), and this suggests other novel and (so far) unspecified kinases.

B. Non-estrogen-dependent Activation of ER

In the absence of E2, other signaling pathways can modulate ER through phosphorylation. These include 1) regulators of the general cellular phosphorylation state, such as protein kinase A (PKA) (16, 46, 153) or protein kinase C (PKC) (152, 267, 189, 200); 2) extracellular signals such as peptide growth factors, cytokines, or neurotransmitters (56); and 3) cell cycle regulators. PEPtide growth factors represent a large class of ER activators. Epidermal growth factor (EGF) can mimic the effects of E2 in the mouse reproductive tract, and pretreatment of mice with the pure anti-estrogen ICI-164,384 greatly diminishes the uterine response to EGF (151). In line with this cross-talk between ERα and EGF, mice in which ERα has been inactivated (ERKO mice) lack uterine E2-like response to EGF even though the EGF signaling pathway is not disrupted (82). Other growth factors which activate ERα signaling include insulin (249, 267, 268), insulin-like growth factor I (IGF-I) (16, 152, 215, 249), and transforming growth factor (TGF)-β (152).

In all of these cross-couplings between growth factors and ERα, the mediators are the guanine nucleotide binding protein p21<sup>ras</sup> and the MAPK (46), p21<sup>ras</sup> functions as an intermediate between the membrane-associated growth factor receptor tyrosine kinase and MAPK phosphorylation cascades. The target site of these kinases on ERα has been mapped to the AF-1 domain. In most studies, this target has been further localized to Ser-118 in hERα, which corresponds to the consensus phosphorylation site for MAPK (46, 165). However, it appears that there are alternative pathways of cross-coupling between growth factors and ER. For example, in the SK-N-BE neuronal cell line, the target for the insulin activation of ERα has been mapped to the AF-2 region (267). This observation suggests that different mediators of the growth factor signal, downstream of p21<sup>ras</sup>, might be involved. In line with this hypothesis, EGF activation of ER in endothelial cells of the pulmonary vein is independent of MAPK and does not involve the same phosphorylation site in ERα (164). Furthermore, the 90-kDa ribosomal S6 kinase, pp90<sup>rasL</sup>, a serine/threonine protein kinase which is phosphorylated by MAPK upon EGF stimulation, phosphorylates Ser-167 in hERα (158).

Other extracellular signals that can modulate ER activity are heregulin (273), interleukin-2 (IL-2), and dopamine (279, 334). Dopamine is, so far, the only neurotransmitter identified as an ER activator. Dopamine activation of ER is distinct from activation by EGF, since a mutant hERα, which is no longer activated by dopamine, can still be activated by EGF (15, 114). The mechanisms involved in ER activation by IL-2 or by dopamine remain elusive, and it has not yet been shown whether phosphorylation of the receptor is involved. Phosphorylation is clearly involved in heregulin activation of ER. In breast cancer cells, activation, by heregulin, of the heregulin receptor, HER-2 (a tyrosine kinase), leads to direct and rapid phosphorylation of ER on tyrosine residues, followed by transcription of the progesterone receptor gene. Heregulin promotes hormone-independent growth in human breast cancer cells, and overexpression of HER-2 results in the development of E2-independent growth, which is insensitive to both E2 and tamoxifen.

C. Cyclins as Activators of ER

Cyclins are positive regulatory subunits of cyclin-dependent kinases (CDKs), a class of serine/threonine kinases that are major determinants in cell cycle progres-
D. Outcome of Phosphorylation on ER Function

All of the steps in transcriptional activation of ER-dependent genes, i.e., ligand binding, ER dimerization, DNA binding, and the interaction with coactivators, appear to be influenced by phosphorylation of ER. Ligand binding of ERα is regulated by phosphorylation of Tyr-537 (10, 231, 233). The dimerization function of ERα appears to be enhanced by phosphorylation at Ser-236 in the DBD (62) and its DNA binding capacity by phosphorylation at Ser-167 (11, 51, 85). MAPK-mediated phosphorylation at Ser-118 in ERα potentiates the interaction with the coactivator p68 (92).

E. Phosphorylation of ERβ

In contrast to the numerous studies related to ERα phosphorylation, only one group has so far investigated ERβ phosphorylation (357). This study demonstrated phosphorylation of recombinant ERβ when it was expressed in human embryonic kidney cells (293 T). The target sites for EGF-induced phosphorylation of ERβ are two serine residues located at positions 106 and 124 in consensus sequences for MAPK. Despite a poor overall homology between ERα and ERβ in the A/B domain, Ser-106 is part of a motif shared with ERα and other steroid receptors and corresponds to Ser-118 in human ERα. Phosphorylation at Ser-106 and -124 enhanced the interaction with the coactivator SRC-1 (357).

VII. TRANSCRIPTIONAL COFACTORS: COACTIVATORS, NEGATIVE COREGULATORS, AND COREPRESSORS

Current models of eukaryotic gene regulation suggest the existence of distinct gene activity states i.e., repressed/silenced, basal/ground state, and activated states (342, 343). Estrogen signaling is usually associated with gene activation, i.e., the switch between basal and activated state. As cellular environments change, ERs can associate with distinct subsets of cofactors depending on binding affinities and relative abundance of these factors (66, 125). Coactivators turn on target gene transcription, while negative coregulators and corepressors inhibit gene activation and possibly also turn off activated target genes. These proteins exist in multiple complexes, possess multiple enzymatic activities, and (in a simplified view) bridge ERs, to either chromatin components such as histones, to components of the basal transcription machinery, or to both. During the past 5 years, protein-protein interaction screenings and biochemical approaches have led to identification of a large number of cofactors which may act at different functional levels (for current review, see Refs. 107, 142, 229, 396, 397, 400). The majority of these cofactors bind to the LBD, which (as described above) plays a central role not only in binding of ligands but also in transforming the ligand signal. So far, only very few receptor-specific cofactors have been identified, and the various nuclear receptors appear to utilize similar cofactors.

A. Cofactors and ER Agonism

Although all ER ligands bind exclusively to the LBD, binding of an agonist triggers AF-2 activity, but binding of antagonists does not. As described above, structural analyses of ER complexed with E2 or raloxifene/tamoxifen show that each class of ligand induces a different LBD conformation (44, 274, 321). These structural rearrangements are critical for ligand-induced transcriptional regulation, since it is the exposed surface of the receptor that recruits coactivators and other coregulatory proteins. Some characteristic AF-2 cofactors with relevance for agonist-bound ERs are illustrated in Figure 4. They can be categorized in several subgroups.

B. The p160/SRC Coactivator Family

This well-characterized coactivator family consists of three related members (reviewed in Ref. 228): SRC-1, the first identified nuclear receptor coactivator and founding member of the family (also p160–1, N-CoA1); SRC-2 (also TIF-2, GRIP1, N-CoA2); and SRC-3 (also P/CIP, ACTR, AIB1, RAC3, TRAM1). Critical for the function of these coactivators is the central nuclear receptor-interaction domain consisting of three equally spaced conserved LXXLL motifs, also called NR-box or LCD/LXD (reviewed in Ref. 400). These motifs represent the primary docking sites to the AF-2 domain and exist in many different cofactors (199). With regard to ERs, it has been found that, in the case of SRC-1 and -2, the second motif (NR-box 2) has the highest affinity for the agonist-bound ERα.
(i.e., the AF-2 domain), while in SRC-3, the first motif serves as primary docking site (66, 344). Recent ERα costructures with a single NR-box 2 peptide have visualized the interactions of the NR-box with the AF-2 domain (322). It is hoped that, in the future, costructural analysis of the entire SRC interaction domain including all three NR-boxes with dimeric ERs will be possible. An additional COOH-terminal region in SRC-1 has been implicated in binding to the NH₂-terminal ERα AF-1 domain (140, 214, 384). This offers an interesting explanation for the collaboration of receptor NH₂ and COOH termini in activation, which is exhibited by ERα but not ERβ.

All SRC coactivators contain two separate transcription activation domains, AD-1 and AD-2. AD-1 is involved in recruitment of CBP/p300 coactivators and acetyltransferases and AD-2 in recruitment of a second protein-modifying enzyme called coactivator-associated arginine methyltransferase (CARM1) (61). The COOH termini of SRC-1 and SRC-3 exhibit intrinsic lysine acetyltransferase activity. Together with evidence for the existence of histone acetyltransferase (HAT) complexes consisting of SRC, CBP/p300, and/or PCAF/GCN5 (400), these features strongly indicate that SRC coactivators function primarily by recruiting chromatin (i.e., histone)-modifying enzymatic activities to ligand-activated nuclear receptors and, thereby, to hormone-regulated target genes. Additionally, acetylation of lysine residues adjacent to the core of NR-box1 in SRC-3 abolishes the interactions with the AF-2 domain of ERα and may represent one important mechanism for attenuation and feedback regulation in estrogen-regulated gene expression (64). A role for SRC coactivators in ER-mediated gene expression and, possibly in carcinogenesis, is suggested by the discovery of frequent AIB1 gene amplification in ER-positive breast and ovarian cancer cells (8) as well as by the phenotype of SRC-1 knock-out mice (399).

C. CBP/p300: Coactivators and SRC-Associated Acetyltransferases

CBP/p300 are viewed as general coactivators and cointegrators involved in multiple signaling pathways. They are ubiquitously expressed, possess acetyltransferase activity, and contain docking sites for ERs, via NH₂-terminal NR-boxes. Several studies have highlighted
their involvement in ER activation (127, 176), but it is yet unclear whether they bind directly to ERs under physiological conditions, i.e., in the presence of other competing coactivators. Instead, affinity considerations (407) and the existence of multiple interactions between CBP/p300 and other acetyltransferases argue for a scenario in which SRC coactivators are required for the recruitment of CBP/p300 to receptors. In support of a critical role of CBP/p300 in histone acetylation, studies utilizing receptors other than ERs have shown that CBP HAT activity was required for efficient target gene activation in vivo (175). Possibly, the requirement for multiple ER-associated HATs may also reflect the partially different substrate specificity (histones and nonhistone targets) of these enzymes. The role of CBP/p300 on ERβ functions has not yet been studied.

D. The TRAP/DRIP Coactivator Complex

A large coactivator complex, referred to as TRAP/SMCC/DRIP/ARC complex, may connect ERs directly to the basal transcription machinery (155, 107). The complex was first identified biochemically from HeLa cells using the thyroid-hormone receptor (155 and references therein). The receptor-binding subunit of the entire complex, referred to as TRAP220 (TRIP2/PBP/DRIP205/RB18A), has been isolated independently in several laboratories using two-hybrid screenings. It is not known whether the two complexes act sequentially or independently on nuclear receptors (107, 155). It has been suggested, however, that acetylation of the AF-2 NR-box binding motif could be one possible mechanism for dissociation of the SRC coactivators from ERs, allowing other cofactor complexes such as TRAP/DRIP to bind (64). ERα is less efficient than ERβ in recruiting the TRAP/DRIP complex in vitro (228), and this may indicate differences in the physical interaction of the ER subtypes with coactivator complexes.

E. Unique Coactivators With Possible Relevance for ERs

Additional unique coactivators exist that are structurally distinct from CBP/p300, p160/SRC, or TRAP/DRIP proteins. However, functional connections to ERs have not yet been made for these cofactors. Examples are 1) NSD1, a bifunctional coactivator and corepressor containing a SET domain, a motif found in several chromatin-modifying proteins (146); 2) TIF1, which exhibits or associates with protein kinase activity and probably connects receptors with other chromatin components such as the heterochromatin protein HP1 and the SNF-2 component of the mammalian SWI/SNF-complex (106, 197, 284); 3) the PPARγ coactivator PGC-1, which interacts with ERs probably via its NR-box motif and which exists in a complex with SRC-1 (254, 285, 286); and 4) a novel coactivator called ASC-2/RAP250 (48, 198). This coactivator contains one functional NR-box motif, is widely expressed in many tissues (48), and appears to be overamplified or overexpressed in certain cancers (198). It is currently not known whether ASC-2/RAP250 functions separately or in conjunction with other coactivator complexes.

F. Negative Coregulators and Corepressors

Studies on several additional ER cofactors illustrate that not all AF-2 binding proteins act simply as coactivators. Two AF-2 interacting proteins, RIP140 and SHP (short heterodimerization partner) (see Fig. 5), exhibit negative coregulatory functions, because they can antagonize SRC-1 coactivators in vivo and compete for AF-2 binding in vitro (53, 161, 359, 360). Therefore, these coactivators may belong to a separate category of coregulators that are distinct from conventional corepressors. SHP is a remarkable and unusual orphan receptor that has a recognizable LBD but lacks a DBD (161, 162, 316). Both SHP and its closest relative DAX-1 interact with several nuclear receptors and inhibit their transcriptional activity (79, 316, 317). SHP binds directly to the AF-2 domain of ERs via two separate NR-box motifs and thus functions in the same way as AF-2 cofactors (161, 162). SHP mRNA is widely expressed in rat tissues including certain estrogen target tissues, and subcellular localization studies demonstrate that SHP is a nuclear protein. Under some circumstances ER may associate with corepressors utilizing SHP or DAX-1 as bridging proteins. This may recruit corepressors/deacetylases to ER target genes and thus antagonize acetyltransferase/coactivator complexes.

G. Corepressors

The related “conventional” corepressors N-CoR (nuclear receptor corepressor) and SMRT (silencing mediator of retinoid and thyroid receptors), associate with ERα in the presence of antagonistic ligands (reviewed in Ref. 228), and it has been suggested that they may play a role in regulating ER activity in tumors treated with antiestrogens (335). There is evidence that histone deacetylase activity recruited by corepressor complexes such as N-CoR/SAP30/SIN3/HDAC2 is required for the transcriptional repression of tamoxifen-bound ERα (188, 194, 335). Lavinsky et al. (194) have observed that acquisition of tamoxifen resistance in MCF-7 cells growing in athymic nude mice (a model of human breast cancer) is accompanied by a decrease in corepressor levels. They suggest that loss of corepressors may be one mechanism of tamoxifen resistance.

Because direct binding of antagonist-bound ER to corepressors has not been demonstrated, indirect recruitment mechanisms are also possible. In view of the in-
sights gained from the structure of antagonist-bound ERs (44, 274, 321) and from corepressor interactions with retinoic acid, thyroid, and ecdysone receptors (405, 145, 362, 405), it is likely that ER antagonists, via the realignment of helix 12 and possibly the F-domain (251), expose as yet unidentified corepressor binding epitopes.

In a yeast two-hybrid screen with a dominant negative ERα as bait, a novel corepressor was recently identified (238). This factor also repressed the agonist activity of both ER subtypes (but not that of other receptors) by competitive reversal of SRC-1 enhancement. The cofactor was named REA for “repressor of estrogen receptor activity.” REA may, therefore, represent a bivalent cofactor with corepressive functions on both estrogen- and antiestrogen-bound ERs. Another candidate corepressor is the RSP5/RPF1 protein, which specifically represses the tamoxifen-mediated partial agonist activity of ERα (255).

H. Non-AF-2 Interacting Coactivators

Although the involvement of corepressors in antagonist signaling may in part explain their antiestrogenic activity in breast cancer treatment, it is not easy to envisage how corepressors alone account for the agonistic activities of antiestrogens. The existence of novel coactivators that bind to ERs only in the presence of antagonists has been postulated, and a few candidates have been identified. One such coactivator, called L7-SPA, was identified using RU487-bound progesterone receptor as bait in yeast two-hybrid screenings, and subsequently demonstrated also to bind and enhance the agonistic properties the tamoxifen-bound ERα (349). The hinge domain has been mapped as interaction site on PR, and it is likely that L7-SPA recognizes features that are conserved between antagonist-bound steroid receptors. In this respect, L7-SPA shares interaction features with corepressors, and this lends support to the idea that part of its function is competitive antagonism of corepressor function. As mentioned below, identification of synthetic peptides, which interact specifically with the antagonist-bound ER, suggest that there could be multiple sites on ER where coactivators in cells could interact only when the receptor is bound to an antagonist.

I. Differences Between ERα and ERβ With Regard to Cofactor Recruitment

One major unanswered question is whether or not ligand-bound ERα and ERβ contact different coactivators/cofactors. Because of the homology in their AF-2 domains, it was anticipated that the two ER subtypes would be similar in coactivator recruitment, but certain differences have been reported. For example, affinity of the ERα interaction with SRC-3 is much higher than that observed for the ERβ (344), and in contrast to ERα, ERβ apparently interacts in a ligand-independent manner with SRC-1 and SHP in vitro (78, 317, 321).
Furthermore, as mentioned above, the TRAP/DRIP complex apparently binds only weakly to ERα.

Such differences may be associated with distinct binding specificities of the NR-box interaction motifs (84, 216, 225). Clearly, preferential binding of certain coactivators to one of the ERs should have consequences for E2 signaling. In addition, because there are differences in the ligand-binding specificities of the two ERs, and because ligands can alter receptor conformations, ligand-binding specificity is likely to affect cofactor binding affinities and specificity. Indeed, such differences in receptor conformation have been found by an affinity-selection approach. This method was used to select peptides that specifically bind to ER subtypes in the presence of agonists or antagonists (255, 265). These experiments clearly showed that different agonists and antagonists induce different ER conformations, which in turn result in the recruitment of different ER-binding peptides.

J. Cofactors and Cancer

Although no cause and effect relationship between cofactors and carcinogenesis has been established, gene amplification and overexpression of the ER coactivators SRC-3, AIB3/ASC-2/RAP250, and TRAP220/PBP have been found in breast and ovarian cancer (8, 198, 408). Additionally, changes in the expression levels of conventional corepressors, which associate with antagonist-bound ERs, may contribute to the phenomenon of tamoxifen resistance in breast cancer treatment. There could, for example, be imbalances or changes in the ratio between corepressors and coactivators (194). Interestingly, ERα and SRC-1 are coexpressed in stromal cells but not in epithelial cells in the mammary gland (324). The response of the stroma to E2 differs from that of the epithelium, perhaps indicating that association between ERs and different coactivators may influence the responsiveness to E2. Lately, it has been suggested that transcriptional cross-talk between ERs and other major players in breast cancer such as BRCA1 or cyclin D1 occurs at the cofactor level via ligand-independent recruitment of acetyltransferases (100, 229). Although it is currently not known whether cofactor levels are related to carcinogenesis, it seems likely that changes in the levels or activity of cofactors could have profound effects on target gene expression. Such changes may shift the balance from differentiation to proliferation and contribute to the development of neoplastic diseases.

VIII. ESTROGEN RECEPTOR-ARYL HYDROCARBON RECEPTOR INTERACTIONS

More than two decades ago, it was first reported that ligands for the aryl hydrocarbon receptor (AhR) can counteract the effects of estrogens in intact animals (173). Like ER, AhR is a ligand-inducible transcription factor, but in contrast to ER, an endogenous ligand has yet to be identified. AhR is a member of the bHLH/PAS class of transcription factors, members of which are involved in development, oxygen homeostasis, and circadian rhythm (for review, see Ref. 80). AhR is the only ligand-activated bHLH/PAS protein identified, and because the majority of ligands are classified as environmental contaminants, AhR functions as a toxin sensor. Ligands for AhR include environmental polycyclic aromatic hydrocarbons (PAHs), 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) (275), related polychlorinated dibenzo-p-dioxins (PCDDs) and dibenzofurans (PCDF) (for review, see Ref. 276), and dietary indole carbinols present in cruciferous vegetables (35, 41, 65, 373). After ligand binding, AhR is found in the cell nucleus as a heterodimer with the AhR nuclear translocator Arnt (139). The AhR-Arnt heterodimer binds to specific genomic enhancer sequences termed dioxin- or xenobiotic-responsive elements (DREs or XREs), and this interaction leads to transcriptional activation. Those genes that are transcriptionally activated as a result of AhR interactions with 5′-upstream DREs have been described as members of the Ah gene battery and include phase I drug-metabolizing enzymes such as cytochromes P-450IA1 (CYP1A1), CYP1A2, and CYP1B1 and phase II enzymes including glutathione-S-transferase Ya subunit, UDP glutathione transferase, aldehyde dehydrogenase, and NAD(P)H quinone oxidoreductase (NQOR) (for review, see Ref. 126). In addition to the Ah battery, a number of E2-regulated genes (see below) are regulated by AhR at either the transcriptional or posttranscriptional level.

A. Antiestrogenic Effects of AhR Ligands

In vivo, the most distinct antiestrogenic effects of TCDD in rodents are decreased uterine weight (113, 304), reduction in the incidence of mammary and uterine cancer (41, 172, 173), and inhibition of E2 induction of PR, EGF receptor, and c-fos protooncogene in immature or ovariectomized rodent uterus (18, 19, 297). In humans, accidental exposure to TCDD, as occurred in 1979 in Seveso, has resulted in a decrease in mammary and endometrial cancer (31, 32). In vitro, several genes have been identified as targets for cross-talk between ER and AhR. Among E2-regulated genes that are affected by TCDD treatment are cathepsin D (180), pS2 (402), prolactin receptor (211), c-fos (87, 88), hsp27 (278), TGF-α and TGF-β (111), as well as PR (129). E2-induced cell proliferation and postconfluency production of foci are also decreased by TCDD (34, 116, 390).

TCDD is not a typical ER antagonist, because it does not compete with E2 for binding to ER (130). Instead, ligands for AhR might mediate their antagonist effects on ER signaling by several separate pathways. These include the following.
I) The first pathway is by increasing the rate of metabolism of E2. TCDD increases the degradation of E2 by inducing CYP1A1 and CYP1B1, enzymes involved in the metabolic inactivation of estrogens (131, 304, 337, 338, 397a).

II) The second pathway is by decreasing levels of ERs. In vitro experiments have shown that TCDD treatment of MCF-7 human breast cancer cells and mouse hepatoma (Hepa 1c1c7) cells resulted in a dose-dependent decrease in levels of ERα. There was no decrease in ER in Hepa cells expressing a mutated AhR (130, 403). In vivo, TCDD suppressed ERα mRNA by 60% in an AhR-responsive mouse strain (C57BL/J6), but not in DBA/2J mice, a nonresponsive strain (353). Both the ERα (390) and ERβ (unpublished observations) genes contain DREs in their 5’-flanking regions. In addition to transcriptional regulation of ER, downregulation of ER by TCDD may be achieved by changes in receptor synthesis, recycling, and/or degradation, as is the case in mammary carcinoma cell lines where TCDD treatment decreases the level of ER protein by a proteasome-dependent pathway (304).

III) The third pathway is by suppressing transcription of E2-induced genes. As discussed above, ER can interact with DNA at ERE, Sp1, and AP-1 sites. A consensus DRE sequence has been identified that confers the antiestrogenic effect of liganded AhR complex on several genes. This inhibitory DRE (iDRE) has the core sequence GCGTG, which binds the AhR complex. In the cathepsin D gene (379), an iDRE lies between the ERE-half site and the Sp1 site in the ERE/Sp1 motif, disrupting the formation of the ERα/Sp1-DNA complex (347). In the fos gene, liganded ERα does not bind directly to DNA, but to DNA-bound Sp1. In this case an iDRE juxtaposes the Sp1 site, suggesting competition between Sp1 and AhR for DNA binding (87, 88). In the p52 gene an iDRE overlaps the AP-1 site in the ERE/AP-1 motif (54, 402), and in the hsp27 gene an iDRE is located 100 bp downstream of the ERE/Sp1 motif close to the transcription start site, probably blocking access of the basal transcription machinery to the promoter (278).

IV) The fourth pathway is by competing for shared cofactors. AhR interacts with various factors in the basal transcription complex (250, 301, 397a), including TATA-binding protein and transcription factor IIF (TFIIIF) and TFIIIB (347), whereas Arnt makes contact with TFIIIF and CBP (170). Coactivators and corepressors might be involved not only in regulation of AhR, but also in cross-talk mechanisms between AhR and ER. Thus RIP140 (185, 210a), ERAP140, SMRT (250), and Sp-1 protein (171) are involved in both AhR- and ER-mediated transcription and represent sites where the two receptors might compete.

IX. ESTROGEN RECEPTOR-RELATED RECEPTORS: INTERPLAY WITH ESTROGEN RECEPTORS

The estrogen receptor related receptors α and β (ERRα, ERRβ) were first identified based on their amino acid identity to ERα (118). The ERRs constitute a subgroup of orphan receptors that are evolutionarily and functionally most closely related to the steroid receptor subfamily of nuclear receptors, in particular the ERs (192, 193). These receptors display similarity to the ERs particularly in the DBD (>65% amino acid identity), and to a lesser extent in the LBD (~35% identity), but they do not bind estrogen or estrogen-like compounds. To date, three different members of this family have been identified, ERRα and ERRβ (initially named ERR-1 and -2, Ref. 118) and recently, an ERRγ of human origin, probably orthologous to an ERR-3 (63, 98, 141).

Expression pattern analysis has revealed a structurally and temporally restricted expression of ERRβ during mouse embryogenesis. Essentially expression is confined to trophoblast progenitor cells in the developing chorion between day 6.5 and 7.5 days postcoitum (dpc). These structures eventually form the placenta (272), so it is not surprising that mice, homozygous for a targeted deletion of the ERRβ gene, die during early embryogenesis due to placental failure (213). In adult tissues, ERRβ expression levels appear to be low, and it is found only in a few organs (118, 272). ERRα, in contrast, displays a more ubiquitous expression in adult tissues (118, 323, 333). During mouse embryogenesis, expression of ERRα can be detected at day 8.5 in extra embryonic tissues, apparently sequential in time to expression of ERRβ, and also in the primitive heart. ERRα expression is further detected from 10.5 dpc in the heart of the embryo and later at 13.5 dpc in skeletal muscle. At later stages, expression of ERRα is detected in a number of tissues including adipose tissue and central nervous system (38, 39). Finally, although only a limited number of reports are available, ERRγ appears to be expressed in several adult and fetal tissues, in particular brain and kidney (63, 98, 141).

In addition to the structural similarities between ER and ERR, there are also certain functional similarities. ERRs activate transcription from ER-containing reporter constructs (141, 370, 371) and interact with heat shock protein 90 (Hsp90) (272). Hsp90 is part of a chaperone system, which is involved in signal transduction of a variety of hormone and growth factor receptors (reviewed in Ref. 281).

ERRα and ERRβ also bind to DNA sequences that are the recognition site for binding of the nuclear receptor, steroidogenic factor-1 (SF-1), which is known to bind to DNA as a monomer. The sequence (TCA AGG TCA) is known as an SFRE. Surprisingly, the ERRs bind prefer-
entially as homodimers to both the SFRE and to EREs (368). ERα, but not ERβ, can bind to SFREs and can activate transcription from SFRE reporter constructs, a feature that represents the first functionally discriminatory difference of DNA target recognition between the two ERs (371). A number of studies have demonstrated that ERRα can activate transcription of aromatase, lactoferrin, osteopontin, thyroid hormone receptor α, and human medium-chain acyl coenzyme A dehydrogenase genes (332, 368, 369, 375, 401). Furthermore, ERRα participates in regulation of the simian virus 40 (SV40) major late promoter (393, 410). No ligand has yet been identified for the ERRs, but transcriptional activity of ERRα is dependent on a serum factor that is present in culture media. This factor can be removed from serum by treatment with dextran-coated charcoal, and activity can be restored by addition of untreated serum. These data suggest that the serum factor may be a true ligand for ERRα (370). In contrast, ERR3 appears to be constitutively active in vitro (141). The ability of the ERRs to activate transcription of ERE containing genes must be of pharmacological concern, particularly during therapeutic use of anti-estrogens. Anti-estrogen compounds such as ICI-164,384 do not interfere with the action of ERRs (370) so that during antiestrogen therapy ERRs are free to activate ERE-regulated genes. The extent to which such transcriptional action can affect the outcome of antiestrogen therapy remains to be investigated.

X. TISSUE DISTRIBUTION OF ESTROGEN RECEPTOR TYPE β

In rodents, the tissues with the highest expression levels of ERβ are the prostate, ovary, and lungs, all tissues where, in the past, the mechanism of action of estrogen was difficult to understand because of the low content of ERα. Not surprisingly, therefore, these are the tissues that exhibit very pronounced dysfunction in ERβ knock-out mice (BERKO) (179). ERβ is also present in mammary glands, bone, uterus, central nervous system, and cardiovascular system, tissues which also express ERα. Because the two estrogen receptors may influence each other’s functions, estrogen action in tissues where they are co-expressed is very complex, and when one of the receptors is deleted, the resulting changes in physiological functions can be difficult to interpret. Recent reviews have covered estrogen actions in the central nervous system (184), cardiovascular system (101), and ovary (381), and these tissues are not discussed in this review.

XI. ESTROGEN ACTION AND ESTROGEN RECEPTOR TYPE β IN THE MALE

There is ample evidence for pronounced effects of exogenous E2 exposure in the developing and adult male urogenital tract. However, it has not been clear to what extent these effects are due to direct E2 actions mediated via ERs in reproductive tissues. Direct actions have to be distinguished from indirect effects, which are caused by actions of E2 on the hypothalamic-pituitary-gonadal axis and result in insufficient androgen production. Until 1995, because of the paucity of ERα expression in the male urogenital tract, it was thought that the effects of E2 were indirect. This view changed when ERβ was found to be widely expressed in developing and adult male urogenital tract of several animal species (42, 135, 136, 182, 205, 284, 299, 310, 326, 374). Phenotypes of mice lacking functional ERs, or aromatase, clearly indicate that lack of E2 action results in structural and functional alterations in the male reproductive system (86, 89, 295, 330), and it has become obvious that the role of direct E2 action in the urogenital tract needs to be critically reevaluated.

A. Expression of ERs and Estrogen Action in Testis and Epididymis

In the testis, both ERα and ERβ are expressed, but the expression profiles of these two receptors are different. In rodent testes, ERα is localized in the nuclei of the Leydig cells (104), while ERβ is found in germ cells, Sertoli cells, and fetal Leydig cells (299, 310, 374). In the excurrent ducts of adult male rats, both ERα and ERβ are expressed (105, 136). Very little is known about the expression of ER proteins in the human testis. At early stages of gonadal development (during the first trimester), no ER protein was detected in spermatogonia or their supporting cells (290). However, midgestational human fetal testis expresses high levels of ERβ mRNA and small quantities of ERα mRNA (42). Human spermatozoa and adult testis express ERβ mRNA (132, 212). In fact, multiple ERβ transcripts have been detected in human testis (240, 256). In situ hybridization studies show that ERβ mRNA is localized in developing spermatids, while germ cells at earlier stages of differentiation, Sertoli cells, and Leydig cells are negative (93). A similar expression pattern for ERβ was observed in immunohistochemical studies with biopsy samples from young infertile men with obstructive azoospermia (217). Strong immunoreactivity for ERβ was detected in nuclei of the spermatogonia, spermatocytes, and early developing spermatids. No reaction was seen in elongating spermatids or mature spermatozoa, Sertoli cells, or Leydig cells. Furthermore, no ERα was observed in any of the samples. Because of the difficulty in obtaining age-matched biopsies from normal males, it is not yet known whether this distribution of ERs in the testis is normal or is related to infertility.

In situ synthesized E2 appears to play a crucial role in normal testicular and epididymal function. Multiple cell types in testis, as well as in the excurrent ducts, including
epididymal sperm, express aromatase (50, 134–136). This probably explains the high concentration of E2 in the testis (135). Both aromatase knock-out (AR KO), and ERα knock-out (ERKO) mice show age-related impairment in testicular function. ARKO mice are initially fertile, but with advancing age develop dysmorphic seminiferous tubules, spermatogenic arrest and Leydig cell hyperplasia, and consequently reduced fertility (295). Because there are no decreases in circulating gonadotropin or androgen concentrations in ARKO mice, it appears that local E2 production is critical for the maintenance of normal testicular function. In contrast, in ERKO mice, testicular dysfunction appears to develop in an indirect manner, due to disturbances in the reabsorption of luminal fluid in the epididymis. This leads to accumulation of fluid in the recurrent ducts, dilatation of rete testis and seminiferous tubules (89, 135), and pressure-related degeneration of seminiferous tubules. The overall result is disruption of spermatogenesis with age. The obvious differences between the testicular phenotypes of ARKO and ERKO mice indicate that ERα is not solely responsible for mediating E2 effects in testis, although it plays a critical role in regulation of efferent duct function. Aromatase and ERβ are colocalized in the same cells in the testis, suggesting that ERβ, rather than ERα, could mediate the actions of locally produced E2. However, Couse and Korach (77) report that male mice lacking functional ERβ preserve normal fertility and do not develop the structural abnormalities typically seen in ERKO mouse testis. Further studies with BERKO mice are warranted to investigate whether there are more subtle changes in testicular structure and function. In addition, studies with ERα/ERβ double knock-out mice (DERKO) (76) will aid in understanding the specific roles of the two ER subtypes in regulation of testicular development and function.

The potential role of estrogens in regulation of Leydig cell development and function has recently been reviewed (1). In fetal rats, Leydig cells express ERα and ERβ, whereas in adult rodents and men, only ERβ has been detected in Leydig cells (299, 310, 374). Aromatase is also expressed in Leydig cells in several animal species including humans (for review, see Ref. 50). During development, E2 inhibits development of Leydig cells from precursor cells, whereas in adults, E2 can block androgen production and Leydig cell regeneration (1). The Leydig cell hyperplasia seen in ARKO mice is consistent with these findings. It is not yet known whether there are any Leydig cell-related abnormalities in ERKO, BERKO, or DERKO mice, and the specific roles of ER subtypes in Leydig cells remain to be investigated.

Exposure to E2 during development causes multiple permanent disorders in the structure and function of the testis and epididymis. In rodents, these include defective epididymal fluid absorption; decreased expression of a water channel protein, aquaporin-1, in efferent ducts; dilatation of rete testis and seminiferous tubules; impaired spermatogenesis; reduction in Leydig cell number and size; reduction in testicular androgen production; and dose-dependent alterations in Sertoli cell number, germ cell volume, and germ cell apoptosis (2, 20, 105, 319). In some studies, in utero exposure of human males to DES was reported to cause developmental abnormalities similar to those described in laboratory animals (33, 339). However, the evidence is not conclusive, and in other studies, no reproductive abnormalities were found in sons of DES-treated women (195, 235, 392). The role of gonadal ERs in mediating the effects of E2 during development is not yet clear. It is not known to what extent these effects are due to reprogramming of the central nervous system and/or altered gonadotropin secretion. Studies with ER knockout animals will be helpful in understanding the mechanisms responsible for the immediate as well as the long-term effects of exposure to E2 during development.

B. Expression of ERs and Estrogen Action in Male Accessory Sex Glands

In adult male rodents, E2 induces structural and functional changes in both epithelial and stromal compartments in the accessory sex glands (29, 196, 222, 269, 309). In the prostate and seminal vesicles, some of the changes (e.g., the castration-like involution) are indirect and mediated via the central nervous system-gonadal axis, but it is apparent from experiments with castrated animals and with organ cultures that E2 may also act directly on the prostate (24, 103, 220, 222, 246, 309). ERα is present only in few cells in the prostate, mainly in stromal cells of the prostatic lobes and the posterior periurethral region. Epithelial cells are devoid of or contain very low levels of ERα (73, 282, 287). In adult animals, the effects of E2 on the central nervous system-gonadal axis are pronounced. It causes reduced androgen production and rapid involution of accessory sex glands. The direct actions of E2 in accessory sex glands, particularly the prostate, are stimulatory and/or growth promoting, but these effects are difficult to observe, in vivo, as they are usually masked by the castration-like effect of E2. In castrated rodents, the most notable changes caused by administration of E2 are growth of fibromuscular stroma, development of squamous epithelial metaplasia in ejaculatory and prostatic collecting ducts, infiltration of inflammatory cells, induction of the expression of c-fos proto-oncogene, and increased production of a major prostatic secretory protein, carbonic anhydrase II (128c, 135, 248, 263, 307). Long-term treatment of rodents with estrogens and androgens (or aromatizable androgens) leads to the development of adenocarcinomas in distinct prostatic sites (253, 196). In organ cultures of rat prostate, E2 treatment increases the relative amount of epithelium,
induces changes in epithelial cell shape and organization, and enhances expression of prostate-specific genes (103, 220, 248). These changes occur in the absence of androgens but may be modulated by simultaneous exposure to androgens.

C. Estrogens and Prostatic Disease

Developmental exposure to high doses of exogenous E2 induces multiple persistent structural and functional abnormalities in the accessory sex glands. These include reduction in overall gland size; focal epithelial hyperplasia, metaplasia, and dysplasia; altered hormonal sensitivity; altered expression of ERs and AR; alterations in stromal cell growth and function; disturbance of TGF-β signaling system; induction of protooncogenes; and inflammatory changes (9, 58, 59, 196, 282, 283, 288, 309, 331). In contrast, exposure to low doses of E2 has been reported to increase prostate size in adulthood (378). The effect of subtle changes in intrauterine hormone milieu is indicated by the stimulation of prostate growth in male fetuses by intrauterine proximity to female fetuses (354). Animal experiments suggest that E2 plays a role in the development of prostatic neoplasia, i.e., benign prostatic hyperplasia (BPH) and prostate cancer. Excessive E2 exposure leads to enhanced growth of both stromal and epithelial components and to abnormal cell function (9, 58, 59, 196, 282, 283, 307, 309, 331). In one study, ERα but not ERβ was found (37) in human prostate cancer and premalignant prostate lesions (high-grade prostatic intraepithelial neoplasia). Recent results (unpublished results, Fig. 6), however, indicate that there is widespread expression of ERβ in the nuclei of epithelial cells in human prostate cancer. There are always difficulties in interpretation of immunohistochemical data, and caution must be used, but the prostate poses a special problem in this regard. Because of the complexity of the gland, sections from the same prostate gland can display completely normal morphology, hyperplasia, and cancer, depending on the region sectioned. It is almost impossible to identify which part of the organ is represented in an immunohistochemical section. Regional differences in the sections used in various laboratories might account for some of the differences reported.

The specific roles of the two ER subtypes in the development and growth of normal and neoplastic prostate are not known. However, ER knock-out mice have revealed that lack of functional ERs leads to receptor-specific structural and/or functional alterations in the prostate. Recently, Couse and Korach (77) reported that the size of accessory sex glands, prostate, and seminal vesicles is increased in ERKO mice, and this phenotype becomes more pronounced when the animals age. We have also observed that the overall size of accessory sex glands is increased in ERKO mice. In addition, we have found morphological changes in the ventral prostate where the lumina are enlarged and the glandular epithelium flattened (Fig. 7). The exact cause of these changes has not been defined, but Couse and Korach (77) suggest that increased size may be due to the slightly elevated androgen concentrations in ERKO mice.

In contrast, the prostates of BERKO mice frequently show foci of epithelial hyperplasia and disorganization (406a) (Fig. 7). DERKO mice share some of the phenotypic characteristics of both ERKO and BERKO mice. The accessory sex glands are large, but the ventral prostate

![FIG. 6. Location of ERβ protein in the human prostate. In normal prostate (A), the majority of epithelial cell nuclei express ERβ, whereas the stromal nuclei are negative. In human prostate carcinoma (B), both ERβ-positive and -negative cancer cells are seen. Positive immunoreaction is indicated by brown color (arrows). Negative nuclei show only the counterstain, indicated by the blue color. Immunostaining was performed with a chicken antibody raised against the full-length human ERβ protein, which was modified by insertion into the ligand-binding domain of the 18-amino acid insert found in rat ERβ 503. Original magnifications: 100× (top panels) and 400× (bottom panels).]
shows foci of epithelial disorganization, whereas some acini are large and lined with flattened epithelium (Fig. 7). Although the role of ERβ in the prostate is not known, development of prostate hyperplasia in BERKO mice suggests a role in regulation of prostate growth. ERβ and the androgen receptor (AR) are widely expressed in prostate epithelium (181) and are colocalized in the same cells. Because castration causes downregulation ERβ expression (284), androgens may have a role in regulation of ERβ and, perhaps, ERβ in turn may modulate the growth stimulating effects of AR.

D. Expression of ERs and Estrogen Action in Lower Urinary Tract

Urinary bladder function in rodents shows marked gender-related differences (68, 207) and changes dramatically with sexual maturity (68). Furthermore, neonatal estrogen exposure induces permanent functional abnormalities in the lower urinary tract of male and female mice (207). In female rats and rabbits, the function of the urinary bladder and urethra in vitro is affected by E2 (26, 40, 137, 207, 320). However, the mechanisms of E2 action, and the role of ERs, are not yet understood. Many of the rapid E2-induced changes in the function of lower urinary tract have been postulated to be due to non-receptor-mediated actions. However, ER, in particular ERβ, is present in the lower urinary tract, indicating that ER-mediated actions are possible as well. Several studies confirm the presence of ERs in distinct sites of the male lower urinary tract. In the canine and rabbit prostatic urethra, both epithelium and stroma display specific ERα staining (137, 314). In the mouse, there are ERα-positive cells in the stroma of the bladder base, bladder neck, and posterior periurethral region (207). No ER-positive cells were observed in human bladder biopsy material (36a, 37).

Twenty-seven years ago it was found that the male baboon bladder could accumulate radiolabeled E2 (382), but no ERs could be detected. More recently, it has been clearly shown that male rat urinary bladder and rat and monkey prostatic urethra express ERβ (184, 269, 310). ERβ-positive cells have been detected in urothelium, detrusor muscle, and rhabdosphincter, while fibroblasts are mainly ERβ negative but may express ERα. Enlarged bladder, thickened bladder wall, and occasionally bladder stones have been detected in neonatally estrogenized mice, suggesting the presence of E2-related infravesical obstruction. In accordance with this, neonatally estrogenized mice had lower voided urine volumes, higher voiding frequencies, and decreased ratio of the urinary flow rate to the bladder pressure (204). In male rats, neonatal E2 treatment causes symptoms typical of rhabdosphincter dyssynergia, i.e., inappropriate contraction or failure of relaxation of the urethral smooth and/or striated muscle during detrusor contraction (339). This is indicated by altered electromyographic activity and impaired urinary flow rates. Neonatally estrogenized animals also developed changes in the bladder structure typical of obstruction.

XII. ESTROGEN AND THE MAMMARY GLAND

The female mammary gland undergoes a surge of cell division during puberty, and throughout adult life there is cyclical proliferation and involution during estrous cycles.
E2 is obligatory for normal development as well as for induction and progression of mammary carcinoma. The role of ER in this process remains unclear (143). During pubertal growth and during the estrous cycle the majority of proliferating cells both in terminal end buds and ducts are ERa negative (69, 70, 404). Induction of the progesterone receptor (PR) by E2 does occur in ERa-containing cells, and this induction occurs at much lower plasma levels of E2 than are required for epithelial cell proliferation (70). These observations have led to the concept (391) of two distinct types of responses to E2 in the breast: 1) an indirect action in the mammary epithelium which occurs via ER-containing stromal cells and 2) a direct effect on ERa-containing cells that occurs at low E2 concentrations and results in induction of PR and differentiation of the epithelium. The stroma, upon E2 stimulation, produces growth factors that cause replication of epithelial cells. From studies involving ERKO mice it is clear that ERb, in the absence of ERa, cannot mediate E2-dependent growth and development of the mammary gland (75, 77). In addition, with the use of reconstitution experiments with ERKO mouse breasts (81) it has been shown that the presence of ERa in breast stroma, but not in the epithelium, is sufficient for E2-dependent ductal growth.

Surprisingly, when ERb was localized in the mammary gland, it was discovered that very few of the proliferating cells express ERb. Approximately 60% of proliferating cells contained neither ERa nor ERb (306). This observation raises the question, Why do ERa-containing cells not divide? The answer to this question is not known, but if growth of epithelial cells is due to stimulation by growth factors, it appears that ERa-containing cells do not have the capacity to respond to growth factor stimulation. This then raises another question, Why do ERa-containing breast cancer cells divide in response to E2? There is no clear answer to this question either, but there is evidence that one of the changes occurring in breast cancer is induction of growth factor receptors (97, 210). It is possible that ERa suppresses expression of certain growth factor receptors in normal mammary epithelium and that upon E2 withdrawal, as occurs during menopause, there is expression of growth factor receptors on ER-positive cells. Once this has occurred, ER can be activated by growth factor-stimulated tyrosine kinases (90, 114, 219, 273) and the normal regulation of cell growth is lost. Why then do antiestrogens offer temporary help in breast cancer? It could be speculated that blocking of ER with tamoxifen inhibits growth factor production in stromal cells, and this temporarily limits the growth of epithelial cells. However, blocking of ERs in this system has the unwanted effect of increasing growth factor receptors and eventually the epithelial cells become more sensitive to growth factors.

In the rat mammary gland, both ERα and ERβ are expressed, but the presence and cellular distribution of the two receptors are distinct. ERβ is present in ~70% of epithelial cell nuclei. The percentage of ERα-positive nuclei varies according to the developmental state of the mammary gland. It is 30% at puberty and decreases throughout pregnancy to a low of 5% at day 14 of pregnancy. During lactation there is induction of ERα with up to 70% of the nuclei positive at day 21. Cells coexpressing ERα and ERβ are rare during pregnancy, a proliferative phase, but represent up to 60% of the epithelial cells during lactation, a nonproliferative, E2-insensitive phase (327, 328). That colocalization of ERα and ERβ is associated with insensitivity to E2 is perhaps not surprising in view of the existence of ERβ isoforms that can inhibit ERα activity (257, 306a). One important question that has arisen since the cloning of ERβ is whether it would be of clinical value to measure ERβ, along with ERα, in breast cancer. It seems that it will not be sufficient to simply measure whether or not ERβ is expressed. It may be necessary to define which isoforms of ERβ are present in cells and whether or not they are colocalized with ERα. Depending on the ERβ isoform, high levels of ERβ might mean insensitivity to E2 or inhibition of ERα activity if the receptors are colocalized, or it might simply mean that an estrogen receptor is present in so-called “estrogen receptor negative cells.”

XIII. ROLE OF ESTROGEN RECEPTORS IN BONE

Estrogens and androgens play important roles in bone metabolism and homeostasis. During adolescence, they are involved in modeling of bone. They initiate pubertal growth and later limit longitudinal bone growth by inducing closure of the epiphyseal growth plate. In adults, sex steroids appear to influence the remodeling of bone. E2, in particular, is crucial for maintenance of bone mass in females (234) as is evident from the rapid loss of trabecular bone and development of osteoporosis that occurs after ovariectomy or at menopause (364, 366).

Several factors, known to be important in regulating differentiation and function of osteoblasts and osteoclasts, are regulated by E2. In osteoblasts, E2 stimulates synthesis and secretion of the anabolic growth factor IGF-I and inhibits that of the cytokines, IL-1, tumor necrosis factor (TNF), and IL-6 (298) that are involved in bone resorption. (96). E2 also stimulates synthesis and secretion of osteoprotegin (OPG), a protein with a critical role in inhibition of the function of osteoclasts (138, 154). OPG, together with osteoclast differentiation factor (ODF) and receptor activator of NFκB (RANK), all members of the TNF-α or TNF-β receptor superfamilies, form
a network that regulates osteoclastic differentiation and function (5, 154, 157, 329). RANK, a membrane-bound TNF receptor, is expressed on osteoclast precursors. It interacts with ODF present on the surface of osteoblast/stroma cells, and this interaction permits differentiation and subsequent activation of osteoclasts. OPG, secreted from osteoblasts, is an endogenous, soluble decoy receptor for ODF. It competes with RANK for the binding of ODF and thus inhibits osteoclast formation. The physiological importance of these factors has been demonstrated in transgenic mice. Mice overexpressing OPG lack osteoclasts and develop osteopetrosis. In contrast, mice in which the OPG gene is inactivated have severe osteoporosis (45, 329).

Suppression of osteoclastic bone resorption and stimulation of osteoblastic bone formation form the basis for the bone-preserving effects of E2. These diverse actions of E2 on bone raise the question whether the two ERs have distinct functions and whether cellular localization of the two receptors differs in bone. In humans and rodents, both ERα and ERβ have been detected in osteoblast-like cell lines (6, 17, 35, 94, 174, 261), in osteoblasts and osteocytes in bone tissue (43, 144, 147, 186, 261, 262, 394), and in chondrocytes in the epiphysial growth plates (167, 186, 252, 376). The presence of ERs in mature osteoclasts is more controversial (147, 252, 262, 270).

At the mRNA level, ERα is ~10-fold more abundant than ERβ in trabecular bone, but neither ER mRNA has been detected in cortical bone (209). In long bones of immature male and female rats ERα levels are higher than those of ERβ, and ERβ mRNA is expressed in trabecular osteoblasts (348). Current information suggests that both ERα and ERβ have overlapping distribution in cells of the osteoblastic and chondrocytic lineages. Effects of E2 on osteoclasts appear to be indirect, through elaboration of regulatory factors such as OPG by osteoblasts or by bone marrow stromal cells.

In immature rodents, gonadectomy leads to attenuation of the sexual dimorphism normally seen in bone (364, 365). Limb length in males is decreased, while in females it is increased. In adult rodents, gonadectomy leads to increased bone turnover as well as decreased trabecular bone volume and cortical thickness (348, 163, 372), all of which can be reversed by either estrogen or androgen replacement (163, 348, 366, 372). Neither ERKO nor BERKO mice show any significant alterations in bone phenotype before the onset of puberty (377, 394). However, in adult mice, both receptors appear to be necessary for normal bone development. Loss of ERα is associated with decreased longitudinal and radial limb growth and cortical osteopenia as a result of decreased radial growth in both sexes (312, 377). Female ERKOs have elevated levels of serum E2, and ovariectomy of both ERKO and wild-type female mice results in loss of trabecular bone (312). There is a decrease in serum levels of IGF-I and osteocalcin in male ERKOs, suggesting involvement of the growth hormone-IGF-I axis in the bone phenotype (377).

In contrast to the ERKO phenotype, adult (3-mo-old) female BERKOs have an increased limb length and increased (30%) cortical bone mineral content. Trabecular bone is normal (377, 395). No abnormalities have been found in the bones of adult male BERKO mice, and the bone phenotype in DERKO mice is similar to that ERKO mice (unpublished observations).

These findings suggest that ERα mediates the growth-promoting effects of E2 but is not involved in maintenance of trabecular bone. The major role of ERβ during pubertal growth is to terminate the growth spurt in females, limiting longitudinal and radial bone growth. A normally functioning ERβ may, therefore, be responsible for the shorter bones and lower peak bone mass normally seen in female rodents. This is also possibly true in women.

In the human population, one subject (a male) has been reported lacking functional ERs protein (336) and two have been described with total lack of E2 due to mutations in the aromatase gene (49, 241). In contrast to the male mice, these men all showed continued linear growth through adulthood due to unclosed epiphyses. They had low bone age and severe osteoporosis associated with decreased trabecular bone density. Aromatase-deficient males responded to E2 treatment by growth plate closure and increased bone mineral density. Further studies are clearly needed to clarify the cellular and molecular mechanisms behind the phenotypic alterations observed in the ER knock-out animals and, most interestingly, why the bone phenotypes in these mouse models differ from those of men with similar defects.

XIV. EVOLUTION OF NUCLEAR RECEPTORS

Nuclear receptors constitute an ancient protein family, found in most or all metazoan animals. Members of the ERR orphan receptor family probably represent the earliest precursors of the ERs, since they are found in very primitive animals such as corals.

A strikingly similar set of steroid receptors is present in most vertebrates, ranging from fish to mammals. What complicates the picture is that some species, particularly fish and some amphibians, for example, Xenopus, have undergone tetrtraploidization at some relatively recent time point during evolution.

A. ERs in Fish

All fish studied so far have an ERβ homolog (60, 352, 355). In addition to ERβ, they all have another ER, but it is not clear if this receptor is a true ERα homolog or repre-
sents an additional ER subtype. These “non-ERβ”-ER sequences are much less similar to mammalian ERα than the fish ERβs are to mammalian ERβ and are as different from ERα as from ERβ (Fig. 8). This could be regarded as supportive evidence for a third ER subtype, an “ERγ.” The ERRγ subtype, for example, is much more similar to ERRβ than to ERα (141). Support for the notion of a gene that diverged from a ERα/ERβ precursor gene (Fig. 9) comes from analysis of the genomic structure of fish ER. The exon/intron organization of the fish “non-ERβ”-ER differs from that of both ERα and ERβ, in that the D domain is divided into two exons (206, 350).

At present, we can only make guesses about the total number of nuclear receptor genes in various species. One model that has been presented is the “one-to-four rule.” According to this model, every invertebrate gene should have four vertebrate paralogues (258). Exceptions to this rule would stem from subsequent deletion of parts of the genome. Attempts have been made to fit the known nuclear receptor genes into such a scheme (258), and although the available data are probably incomplete, most nuclear receptor genes seem to appear in groups of three rather than of four homologous genes. The exceptions to this are in fact the steroid receptors and their relatives. Thus one unknown and probably extinct precursor has evolved into four related steroid receptors: the glucocorticoid, mineralocorticoid, progesterone, and androgen receptors. Furthermore, there are four known members of the ERR family, three expressed genes (117) and one pseudo gene (333), but only one identified Drosophila ERR. To date, there are two known mammalian subtypes of ER. According to the one-to-four rule, the possibility thus exists that more ERs remain to be discovered. Whether or not a functional ERγ exists in mammals is an issue that must be resolved soon, since the biological functions of E2 cannot be understood unless all the participants in the system are known. Analysis of E2 responsiveness in DERKO mice, which have neither functional ERα nor ERβ, will be invaluable in resolving this issue.

**XV. CONCLUDING REMARKS**

Understanding of E2 physiology and mechanisms of action has undergone a paradigm shift during recent years following the discovery of the second estrogen receptor ERβ. Even though much remains to be done for a full understanding of the role of this new receptor, it is safe to conclude that ERβ is of importance for E2 signaling. For instance, the function of the ovaries is severely impaired in the absence of ERβ, and other aspects of reproductive physiology appear to be affected. It can be anticipated that continued analysis of BERKO mice will reveal many other interesting deficiencies and malfunctions. One important characteristic of ERβ seems to be its capacity to modulate the biological activity of ERα (388) in the sense that ERα is counteracted by ERβ as if ERα/ERβ would represent an example of the yin-yang principle. Of particular interest is that this quenching effect of ERβ on ERα function seems to be exerted particularly efficiently by some of the many isoforms of ERβ that exist, rather than by the originally discovered receptor, now designated ERβ1. There is accumulating evidence that, in ERβ knock-out mice, those phenotypic features that are related to unrestrained ERα activity (388) are exaggerated in mice whose diet contains high levels of phytoestrogens. This could be one of the reasons for the differences in the severity of BERKO phenotype observed in different laboratories. Furthermore, the realization that there are two ERs with somewhat different tissue distribution has strongly boosted the interest of the pharmaceutical industry to try to develop tissue-specific E2 agonists and antagonists, to be used in a multitude of clinical states, e.g., menopausal symptoms such as osteoporosis, cardiovascular disease, urinary incontinence and recurrent urinary infections, hot flashes and mood swings (substitution therapy), as well as breast cancer (estrogen antagonists) and age-related central nervous system degenerative changes.

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**FIG. 8.** Homologies between the different domains of the mouse ERβ, human ERα, and fish ER. Comparison of exon/intron boundaries in these ERs are shown.
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FIG. 9. Phylogenetic tree showing all cloned estrogen receptors.


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