Biochemical and Biophysical Research Communications **270,** 1–10 (2000)
doi:10.1006/bbrc.2000.2214, available online at http://www.idealibrary.com on IDEAL®

BREAKTHROUGHS AND VIEWS

Estrogen Receptors: How Do They Control Reproductive and Nonreproductive Functions?

Masami Muramatsu* and Satoshi Inoue†
**Future Program Research Division, Saitama Medical School, 38 Morohongo, Moroyama, Iruma-gun, Saitama 350-0495, Japan; and †Department of Geriatric Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan*

Received January 20, 2000

Three aspects of recent development in estrogen receptor research will be discussed in this review. First, since the discovery of the second estrogen receptor, ERβ, a new era has begun in this field. The presence of another receptor for estrogen having different tissue distribution and molecular specificity has posed a question as to the authenticity of the hitherto believed interpretation of the diverse actions of estrogen in different organs of both sexes. Ongoing studies, however, seem on the way of clarifying these new complex puzzles caused by the appearance of the new actor. Recent data with knockout mice for these genes are analyzed and discussed. Second, the mechanism of estrogen receptor action as a ligand-dependent transcription factor has been much more clarified these several years since the discovery of coactivators of steroid receptors which transmit the effect of ER to the transcription initiation complex. This may also open a way to understand the remodeling of chromatin to an active form which has long been sought. Third, the downstream genes of ER are now being isolated and characterized, which is mandatory for the global understanding of the estrogen action during the development and function of an individual animal. This approach, which has been most difficult, will now become more popular in future as newer technologies for this develop.

Abbreviations used: ER, estrogen receptor; GR, glucocorticoid receptor; AR, androgen receptor; PR, progesterone receptor; MR, mineralocorticoid receptor; TR, thyroid hormone receptor; VDR, vitamin D receptor; RAR, retinoic acid receptor; RXR, retinoid X receptor; PPAR, peroxisome proliferator activated receptor; ERE, estrogen response element; AF1, activation function 1; AF2, activation function 2; CNS, central nervous system; E_2, 17β-estradiol; LH, lutenizing hormone; LHR, lutenizing hormone receptor; −/−, homozygous deficient; ERR, estrogen receptor-related receptor; SFRE, SF-1 response element; ERAP, estrogen receptor associated protein; RIP, receptor interacting protein; SRC, steroid receptor coactivator; TIF, transcriptional intermediary factor; AIB1, amplified in breast cancer-1; ACTR, a novel coactivator; CBP, CREB-binding protein; CREB, cAMP response element binding protein; bHLH, basic helix loop helix; PAS, Period Arnt Sim; LBD, ligand binding domain; HAT, histone acetyltransferase; HDAC, histone deacetylase; REA, repressor of estrogen receptor activity; TBP, TATA binding protein; DRIP, VDR interacting protein; TRAP, TR associated protein; EGF, epidermal growth factor; IGF1, insulin-like growth factor-1; MAP kinase, mitogen-activated protein kinase; SRA, steroid receptor RNA activator; BRCA1, breast cancer-1; EFP, estrogen responsive finger protein; GBSC, genomic binding site cloning, RBCC, RING-finger B-box coiled coil; NR2D, *N*-methyl-D-aspartate receptor type 2D; Cox7 RP, cytochrome oxidase subunit 7 related protein; PML, promyelocytic leukemia.

ESTROGEN RECEPTOR HAS A BROTHER

Estrogen receptor is a member of the nuclear receptor superfamily which includes steroid receptors (GR, AR, PR, MR), thyroid receptor (TR), vitamin D receptor (VDR), retinoic acid receptor (RAR) and other receptors such as RXR and PPAR, all of which control gene transcription in the presence of each specific low molecular weight lipophilic natural or artificial bioactive substance as ligand (1, 2). A number of molecules with similar structures are found but without known ligands, hence being called orphan receptors. Some of these "receptors" may not have any physiological ligand, but increasing numbers of endogenous as well as exogenous ligands are being found for them, suggesting a vast variety of ligands are in cross-talk with others using these "orphans." They include, besides the typical ones mentioned above, metabolic products of kinases and other signaling pathways such as prostaglandins (3, 4) and bile acids (5–7).

Estrogen was the first to be established to work through a nuclear receptor ER (8) that binds to a unique DNA sequence named ERE (estrogen response element) and activate transcription ligand-dependently. Just how this type of transcription factors work on molecular terms had to await the molecular

0006-291X/00 $35.00

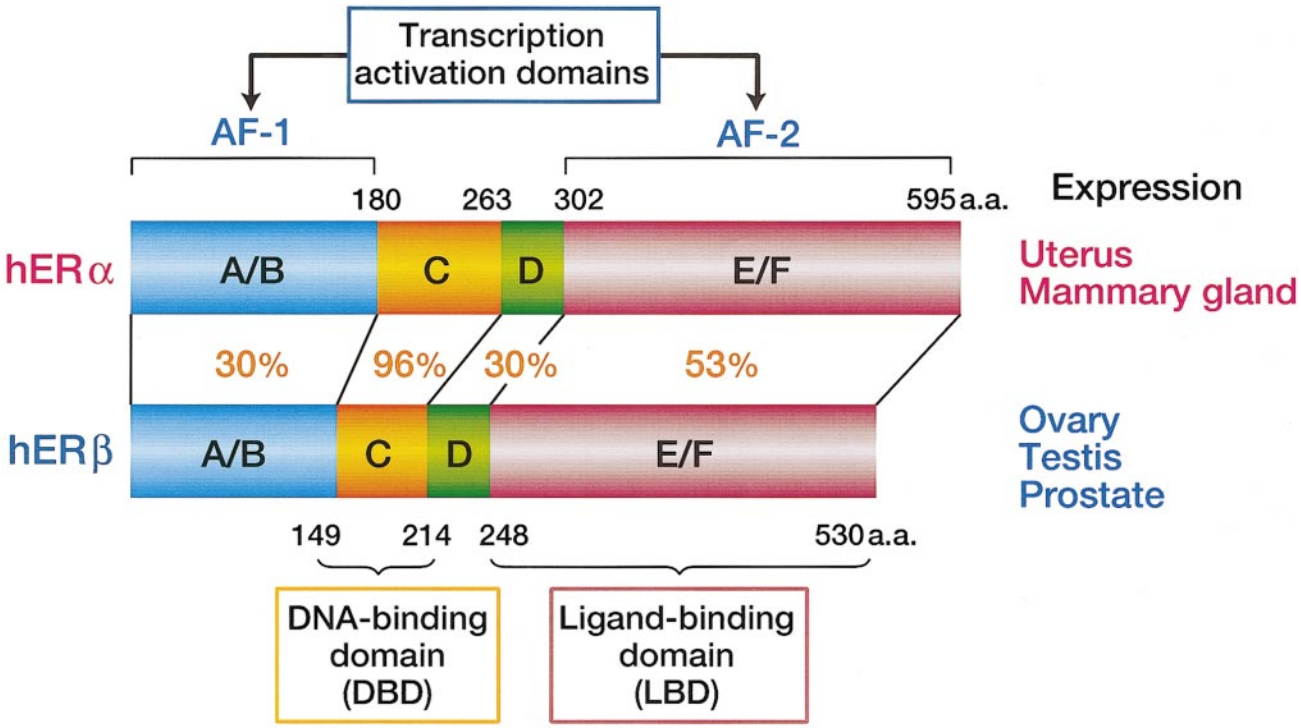

FIG. 1. Structural comparison of ERα and ERβ. Human ERs are compared. A to F regions represent classical nomenclature for the structurally similar amino acid sequences. Amino acid number from the N-terminus is written on each bar representing the ER molecule and the amino acid identity is expressed by percentages between the two ERs. A few major expression sites are presented on the right-hand column.

cloning of the receptor cDNA and the development of molecular biological technology for the analyses of transcription in general. A number of nuclear receptors are known to have subtypes coded for by similar but distinct genes and isoforms that are produced in major part by alternative splicing. As the former examples, TRα, β; RARα, β, γ; RXRα, β, γ; PPARα, β (δ), γ etc. may be raised. Interestingly, classical steroid receptors, (ER, GR, PR, AR and MR) were long thought not to have subtypes just because they were not easily identified.

In 1996, however, Kuiper *et al.* (9) found in rat prostate cDNA library a new type of ER and designated ERβ, the classical one thus being renamed ERα. Subsequently, human and mouse ERβs have been cloned by Mosselman *et al.* (10) and Tremblay *et al.* (11), respectively, but all their molecules lacked 53 amino acids at the N-terminus. The first complete ERβ cDNA was cloned from human by Ogawa *et al.* (12) and found to contain 530 amino acids (M_r 59.2 kDa). Figure 1 shows the structural comparison of the ERα and ERβ. ERβ has an amino acid identity of 96% to ERα in the DNA-binding domain (C), which suggests strongly that ERβ would recognize and bind with the ERE, the same site as ERα. Binding experiments have demonstrated this. The ligand binding domain (E) has much less homology between ERβ and ERα (53%). However, K_d values of ERβ and ERα to 17β estradiol (E_2) do not differ much (e.g., 0.6 nM vs 0.2 nM) as determined at different laboratories (9, 13). The N-terminal A/B domain and C-terminal E/F domain are known to have transactivation function 1 (AF1) and transactivation function 2 (AF2), respectively (see next section for detail). The fact that these domains are much less conserved; e.g., A/B domain is only 30% conserved, appears to indicate that proteins interacting with ERβ for its essential function are considerably different from those interacting with ERα.

The crucial issues here are what their functional differences and eventual roles are in different organs and at developmental stages. The difference of tissue distribution was found rather remarkable. In general, whereas ERα is expressed in major female organs such as ovary, uterus, vagina, mammary gland and certain areas of CNS especially in hypothalamus, ERβ is not always expressed significantly in those cells except for ovary but expressed rather abundantly in male organs and different areas of CNS including some part of hypothalamus and cerebral cortex. More specifically, in the rat, ERβ mRNA was found to be expressed abundantly in prostate and ovaries and less abundantly in uterus, lung, testis, brain and artery (14). Even in the prostate it is expressed strongly in epithelial cells but only weakly in stromal cells where ERα is prevalent

(9). It is to be noted that in humans ERβ mRNA is more abundantly expressed in testis rather than in prostate (10, 13) suggesting the presence of species difference in the distribution.

We utilized specific antibodies for the study of ERα and ERβ expression at the protein level (15, 16). In ovary, ERβ protein is expressed more strongly in granulosa cells than in theca cells (15). In contrast, ERα is expressed in both cell types and also in interstitial cells (14). We also revealed the ontogenetic changes in the expression of this family of receptor such that in pituitary ERβ is abundantly expressed from 12 days of gestation, while ERα appears only from 17 days. In adult, ERα is widely distributed in anterior lobe of pituitary, whereas ERβ is quite restricted to certain regions of anterior lobe (16).

The ultimate role of these receptors may best be exemplified in the knockout experiments of each receptor gene. The major phenotypes of these animals are summarized in Table 1.

The first ERα knockout mice were produced by Lubahn *et al*. (17) in 1993. They were found to be not lethal but infertile in both sexes. The basic female reproductive organs such as uterus, ovary and mammary gland were almost normally formed during the pre- and the neonatal stages, which may have suggested the presence of some other signaling pathways of E_2 including that by ERβ. But, the development of these organs after puberty was severely impaired indicating the insensitivity of these tissues to E_2 (17). These organs remained immature. Females did not show lordosis when mounted by a male, became rather aggressive and showed infanticide frequently (18). It was remarkable that male mice showed considerable abnormality in spermatogenesis (19) as well as in sexual behavior and/or function (Table 1), indicating that ERα was playing a significant role even in male animals (19, 20). When the first male patient of ERα null mutation was found, the major symptom was surprisingly the extraordinary long longitudinal bones with epiphyseal unclosure and a lowered bone mass (21). This was, however, not the case in the ERα knockout mice (14). Although they had similarly lowered bone mineral density, they had rather shorter longitudinal bones especially in female mice. This discrepancy may be due to the difference in bone physiology between humans and mice or to the special genetic background of the patient. Incidentally, no female patient with either ERα or ERβ deficiency has been reported up to the present time. In any event, some aberrant bone metabolism may be expected considering the osteoporosis frequently occurs after menopause. We have evidence that a transgenic rat expressing a dominant negative ERα that is truncated at a certain region of the C-terminus can maintain bone density but has a much lower capacity to recover from the bone loss when ovariectomized rats are treated with E_2 (22, unpublished observations). In fact, several alternatively spliced isoforms of ERβ have been reported including $ER\beta_2$ (23) and ERβcx (13), the latter appearing to work as a dominant negative regulator of ERα but not of ERβ. These splice isoforms must be taken into account in future studies since $ER\beta_2$ was reported to be much more resistant to the competition by genistein, a phytestrogen, than $ER\beta_1$ (original form) for E_2 binding although its Kd to E_2 is exceedingly high (23).

It should be noted that the secondary effects of many other factors especially in the hypothalamic–pituitary axis must be taken into account when these knockout phenotypes are analyzed. In fact, an extensive search for the cause of phenotypic changes in ERα knockout mice has shown that in adults much of them are derived from the high circulating LH that interacts with functional LHR of ovarian theca and granulosa cells, resulting in the failure of the normal maturational events in the ovary (14).

More recently, knockout mice of ERβ were obtained and their phenotype analyzed (24). These homozygous mice were found to be fertile and there was no abnormal sexual behavior in both sexes. Female mice, however, showed a reduced ovarian function and fewer pups per pregnancy than normal female. Young male mice showed no apparent abnormality but when they got old, hyperplasia of prostate and bladder were noted frequently. There are few reports as to the arteriosclerosis, osteoporosis or abnormal lipid metabolism until present. Rather, it is reported that female ERβ knockout mice tend to have longer and denser bones (25). Estrogen administration inhibits the vascular injury response both in ovariectomized female ERα and ERβ knockout mice to the same extent as wild-type mice, suggesting that at least one of the ERs or another unidentified ER mediates the vascular protective effects of estrogen (26, 27). The overall symptoms on reproductive system of ERβ knockout mice are much less severe than those of ERα, but some specific involvement of the former in this system may also be present. On the other hand the unique spaciotemporal distribution of ERβ, e.g., cerebral cortex, some hypothalamic nuclei and testis and the knockout effects thereof appear to indicate the more subtle but nevertheless important function of ERβ in other systems than female reproduction. Incidentally, it is interesting to note that the phenotype of aromatase knockout mice resembles that of ERα knockout closely (28). The double-knockout of both ERα and ERβ has just been achieved revealing interesting results (29). Both sexes of these animals show apparently normal development of the reproductive tracts until pre-and neonatal stages but are infertile. Striking thing is that ERα −/− · ERβ −/− females exhibit abnormal

TABLE 1

Phenotypes of ERα and ERβ Knockout Mice

	ERα (−/−)		ERβ (−/−)
	Female	Male	Female*
Viability and fertility	Viable but infertile	Viable but infertile	Viable and subfertile (reduced litter size)
Reproductive tract	Uterus: Normal pre- and neonatal development. Thin and hypoplastic uterus. Insensitive to E_2 in adult. Presence of non-ER-mediated pathway for 4-OH-E_2 and methoxychlor. Ovary: Normal pre- and neonatal development. Multiple hemorrhagic cysts and anovulatory during adult. No corpora lutea.	Normal pre- and neonatal development. In adult, atrophy of seminiferous epithelium and decreased sperm counts. Disrupted sperm function as evidenced by inability to fertilize.	Uterus: Normal pre- and neonatal development with apparent normal response to ovarian E_2 cycling in adult. Ovary: Normal pre- and neonatal development. In adult, frequency of spontaneous ovulation is decreased and follicle maturation appears impaired.
Mammary gland	Normal prenatal development but insensitive to E_2-induced development during puberty. Responsive to progesterone and PRL. Susceptible to proto-oncogene Wnt-1 signal induced ductal hyperplasia and tumorigenesis.		
Neuroendocrine system	Apparently normal histology of pituitary but with elevated transcripts for the gonadotropin submits (α-gonadotropin submit, LH-β, FSH-β). Serum E_2, testosterone and LH levels elevated. Progesterone and FSH levels normal. PRL level low. Medial preoptic region of hypothalamus exhibits increased PR transcripts which reduce with ovariectomy but return with E_2 treatment. Rapid actions of E_2 on hippocampal neurons remain.	Apparently normal anterior pituitary but with elevated LH-β transcripts. Elevated serum E2, testosterone and LH, but normal progesterone and FSH levels.	Normal E_2 level. No particular abnormality is reported.
Behavior	Lack of lordosis and other E_2- and progesterone-induced sexual behavior, aggressive, infanticide.	Normal mounting and attraction. Lack of intromission and ejaculation. Reduced aggressiveness.	No abnormal sexual behavior.
Cardiovascular	Reduced E_2-induced angiogenesis. Lower levels of vascular NO. Reduced response to E_2-induced serum Apo E increase. Increased expression of L-type Ca^{2+} channels. Normal response to carotid artery injury model.		
Bone and others	Growth arrest of longitudinal bones. Impaired glucose tolerance. Normal B lymphopoiesis.		

* No apparent abnormality is reported for male ERβ (−/−) mouse. (Adapted and modified from Ref. 14.)

differentiation of follicle to a structure resembling seminiferous tubules of the testis. In addition, Sertoli-like cells appear and Mullerian inhibiting substance, sulfated glycoprotein-2 and Sox9 are expressed. This apparent transdifferentiation indicates that a postnatal sex reversal occurs in the ovaries when both ERα and ERβ are lost (29).

GROWING COFACTORS, COACTIVATORS, AND COREPRESSORS

Molecular anatomy that had been made on the cloned ERα (and also ERβ later) revealed different domains which function relatively independently of each other. DNA transfection experiments with reporter plasmids having ERE as enhancer showed two distinct transactivating domains in the N- and C-terminal regions designated AF1 and AF2, respectively (30, 31, see Fig. 1). The AF2 requires ligand (E_2) binding for its transactivation function, whereas the AF1 appears not to require the ligand. However, recent reports indicate that both AF1 and AF2 act cooperatively and synergistically rather than completely independently. Advanced protein-protein interaction assays including yeast two-hybrid screen have revealed a number of proteins that specifically bind to AF2. Some

of those proteins were found to be important coactivators of ERα. Halachmi *et al.* (32) were the first to find a 160 kDa coactivator protein which was designated ERAP160. Another protein RIP140 was also identified (33, 34) together with RIP160 which was a different entity from ERAP160. Their binding to AF2 is E_2-dependent and is required for transactivation by AF2. ERAP160 was later found to be a splice variant of SRC-1 which had been identified as a coactivator of PR (35). These proteins including TIF2, AIB1/ACTR and others (36–38) are members of a family of a steroid receptor coactivator (39), which may be termed p160/SRC-1 type. Another more general coactivator CBP/p300 which had been identified as a cofactor of cAMP response element-binding protein (CREB) was found to bind with a wide variety of nuclear receptors including ER and activate transcription (40, 41). p160/SRC-1 type coactivators have bHLH and PAS domain and characterized by the presence of multiple LXXLL signature motifs also called LXD or NR box (42, 43), which directly interact with LBD of nuclear receptors including ERs. Recent structural studies by X-ray crystallography reveals that so-called helix 12, α-helical component of AF2, changes its orientation in LBD cleft when bound with E_2, which permits the binding of p160/SRC-1 coactivator via LXXLL motifs (44, 45). The molecular mechanism of some E_2 antagonists such as tamoxifen and raloxifene is now interpreted by the effect of these compounds on the LBD which changes the position of helix 12 to occupy the hydrophobic cleft and preclude the interaction of p160/SRC. This is direct evidence in support of the biological functions of coactivators (46, 47). In fact, SRC-1 is demonstrated to bind with the C-terminus of CBP/p300 and activates transcription synergistically (48). Also, the latter molecule interacts directly with nuclear receptors in a ligand-dependent manner through AF2 (40, 41).

Most interestingly, both p160/SRC-1 family and CBP/p300 have histone acetyltransferase (HAT) activity (38, 49). There is also evidence that p/CAF, the mammalian homologue of the yeast HAT GCN5 is part of a large TAF (TBP-associated factors) complex (50) and interacts with some p160, CBP and even with nuclear receptor itself to activate transcription (38, 51). Although these are not yet directly shown for ERα or ERβ, this leads one to speculate a crucial role of these coactivators in remodeling chromatin to an active form that allows more efficient interaction of various transcription factors around promoter and enhancer (Fig. 2). In contrast to this p160/SRC-1-CBP/p300 coactivator complex, a novel DRIP/TRAP coactivator complex was recently identified for VDR and TR systems (52, 53). They consist at least of 9 subunits including DRIP205/TRAP220 (52–54), but have no HAT activity and appear to function by recruiting RNA polymerase II holoenzyme (preformed large complex) to the promoter site, suggesting that different mechanisms are operating between different coactivator complexes (46, 54). Although there is no concrete evidence showing that this latter complex of coactivators plays a role in ER signaling pathways, similar cofactors may well be involved in the regulation of transactivation considering the molecular proximity of these systems to steroid receptors.

Nature frequently provides negative as well as positive regulation for one pathway. This is exactly the case for some nuclear receptors as shown by corepressors. At least two well-characterized corepressors termed N-CoR and SMRT exist for TR and RARα (55–57). They usually bind with these nuclear receptors at around the N-terminal end of E region involving helix 3, 4 and 5 in the absence of ligands and suppress the transactivation of transcription by the receptor (45). Both N-CoR and SMRT have a NR box (containing LXXLL)-like repeat, designated CoRNR ("corner") box, and interact with above-mentioned region of the nuclear receptors (58). Once the receptors bind with the cognate ligands, the corepressors are replaced by the recruited activator complexes and transactivation by the receptor is made possible. An intriguing observation is that these corepressors appear to form a complex with histone deacetylase (HDAC, 59, 60) which may well antagonize HAT active coactivators. This aspect of gene control will certainly be one of the central themes of investigation. Overexpression of RIP140 was reported to suppress ERα activity (34). RIP140 requires ligands for binding (33), which is therefore different from N-CoR/SMRT type complexes. Two liganded-ER binding proteins, TIF1α (61), a RING finger-B-box-coiled coil (RBCC) protein, and REA (62) isolated by two-hybrid screen using a dominant negative ER as bait are also suggested to have silencing activities. Recently, it is reported that acetylation of ACTR coactivator by p300/CBP induced by estrogen neutralizes the positive charges of two lysine residues adjacent to the core LXXLL motif and disrupts the association of HAT coactivator complexes with promoter-bound ER (63). This finding indicates a novel negative regulation of ER pathway. Future studies should clarify the relationship of these multiple repressing mechanisms for ER.

The most variable region of the nuclear receptor structure is the A/B domain that contains AF1. They are different in size and sequence among different receptor categories or even in subtypes. Therefore, this is thought to be one of the crucial regions which confer the tissue and target specificity to the receptor molecule. Kato *et al.* (64) was the first to show that the phosphorylation of Ser 118 in AF1 region of human ERα functionally activated AF1 and this was accomplished specifically by MAP kinase. This established for the first time a cross-talk between membrane-type growth factors (EGF/IGF1) and nuclear receptors. Tremblay *et al.* (65) also demonstrated that MAP ki-

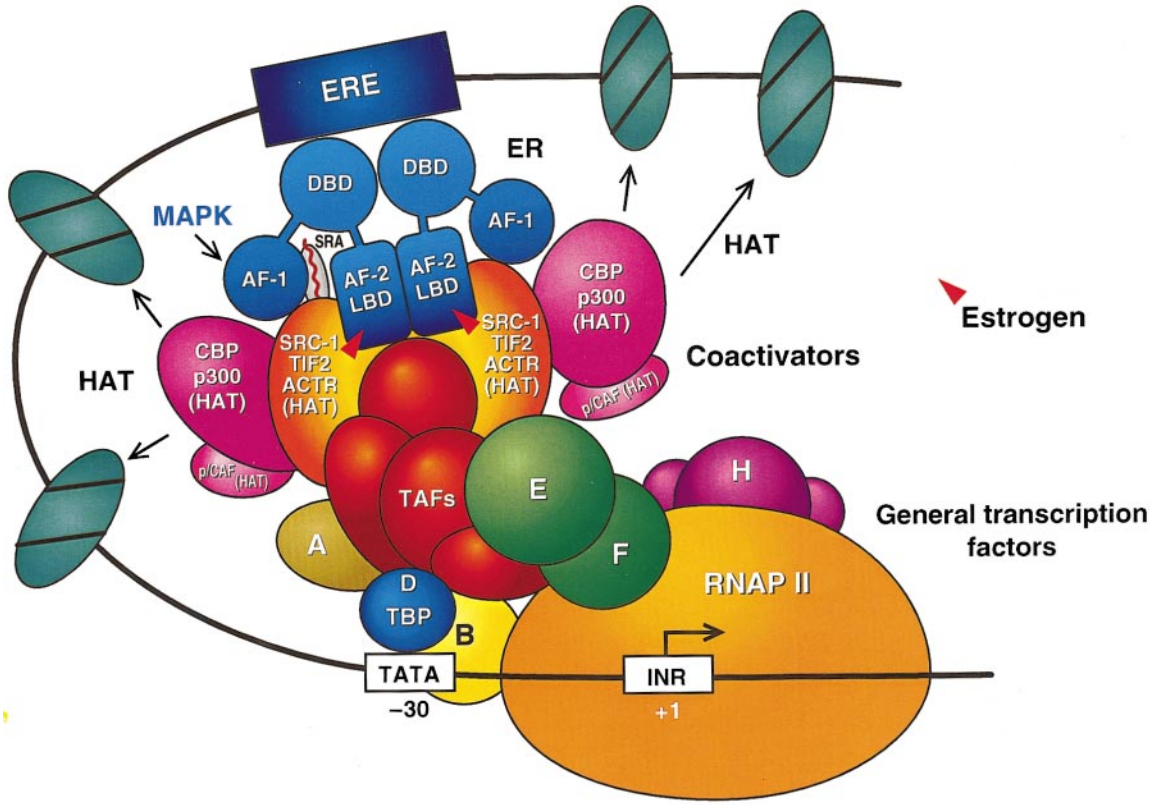

FIG. 2. A schematic model of the transcription initiation complex formed at the enhancer (ERE) and promoter site with the RNA polymerase II–general transcription factor complex and the ER-recruited coactivator complexes. Activation of nucleosome/chromatin structure by HAT activity and activation of transcription by ER dimer through a number of coactivators and MAP kinase, etc. are indicated. (see text for details).

nase induced phosphorylation of Ser106 and Ser124 in the AF1 domain of ERβ resulted in the stimulation of AF1 activity by recruiting SRC-1 to this region ligand-independently. N-terminal phosphorylation of PPARγ by MAP kinase is also known to down-regulate its transactivation (66, 67) by decreasing the ligand-binding affinity indicating the communication between AF1 and AF2. On the other hand, Endoh *et al.* has recently identified an RNA helicase p68 which specifically activates ERα by interacting with AF1 domain when it is phosphorylated by MAP kinase, thus introducing a new actor in this complex drama (68). How RNA helicase functions in transcription regulation provides interesting possibilities. Phosphorylation, as a major modification of proteins, must be working in various aspects of steroid receptor system and more work should be directed to the physiological roles of this regulation. Incidentally, the knockout mice of the gene SRC-1 showed only partial hormone insensitivity (69). This most probably means that this signaling pathway is redundantly regulated as experienced for many other important genes.

Recently, an unusual newcomer has joined in the steroid receptor coactivator families. Lanz *et al.* (70) isolated using yeast two-hybrid screen a novel transcriptional coactivator termed SRA (steroid receptor RNA activator) which stimulated transactivation by PR via AF1. SRA exists as ribonucleoprotein complexes, at least some of which include SRC-1 but works ligand-independently. Most strikingly, SRA was found to act as an RNA transcript because 1) no translates appeared to be present in the cell, 2) SRA mutants with multiple stop codons could not abrogate the activator function on PR-dependent gene expression and 3) blockage of protein synthesis by cycloheximide did not affect the activation by cDNA transfection. This activation function was demonstrated using all tested nuclear receptors including GR, AR, ER, TR, RAR/RXR and PPAR but not with other transcription activators such as GAL4, SP1, E2F and E47 (70), indicating that SRA works selectively on steroid or nuclear receptor-mediated transactivation. Although a number of questions may be raised as to the mechanisms and the roles of SRA in steroid receptor specific transactivation, more detailed study on the interaction of SRA with other cofactors, known and unknown, will solve the enigma of this molecule.

In this regard, cyclin D1 was reported to activate ER ligand (E_2)-independently (71, 72). This was supposed

TABLE 2

Some Examples of ER-Target Genes Obtained by GBSC

Gene	Expression	Structure	Function
EFP	Uterus, breast	RING finger	Growth promotion
EBAG9	Breast cancer	Transmembrane	Growth inhibition
NR2D	Brain	Neural receptor	CNS activation
COX7RP	Ubiquitous	Enzyme subunit	Energy production

to bridge the SRC-1 to ER. Ligand-independent transactivation by steroid receptors would most probably be exerted through AF1 but its mechanism should be examined now in the context of the action of whole receptor molecule.

ANALYZING ESTROGEN RECEPTOR DOWNSTREAM GENES, CAUSE OF DIVERSITY

Estrogen has a diverse physiological effect that expands from female organogenesis and function to general maintenance of the healthy bone, blood vessels and even some part of the male functions as exemplified by the ERα-knockout mice (Table 1). How are all these phenomena effected? Because ERs are ligand-dependent transcription factors, the sum of the all the ER responsive genes must play the major role in this drama, although some non-genomic functions of estrogen may play some physiological roles indeed. This aspect, however, will not be dealt with in this short review because of the space limitations and those who are interested are referred to some of the recent papers (73, 74). Compared with the variety of the estrogen function, however, we have yet to admit that relatively few genes are known which are proven to be under the control of ERs. They include vitellogenin, PR, prolactin, pS2, c-fos, oxytocin and cathepsin D, whose genes have ERE in their control region and E_2 can enhance their transcription. There are more genes that are activated eventually by estrogen but without apparent ERE. They include EGF, EGFR, cyclin D1, BRCA1 and others, which may be called the secondary E_2-responsive genes in contrast to the first-mentioned group that may be termed the primary-responsive genes.

To obtain more primary-responsive genes for estrogen, the authors have developed a method, named "Genomic binding-site cloning (GBSC)," in which genomic DNA fragments are screened for the presence of functional ERE by binding with recombinant protein of DNA-binding domain of human ERα (75–77). Some examples that were obtained by this procedure are

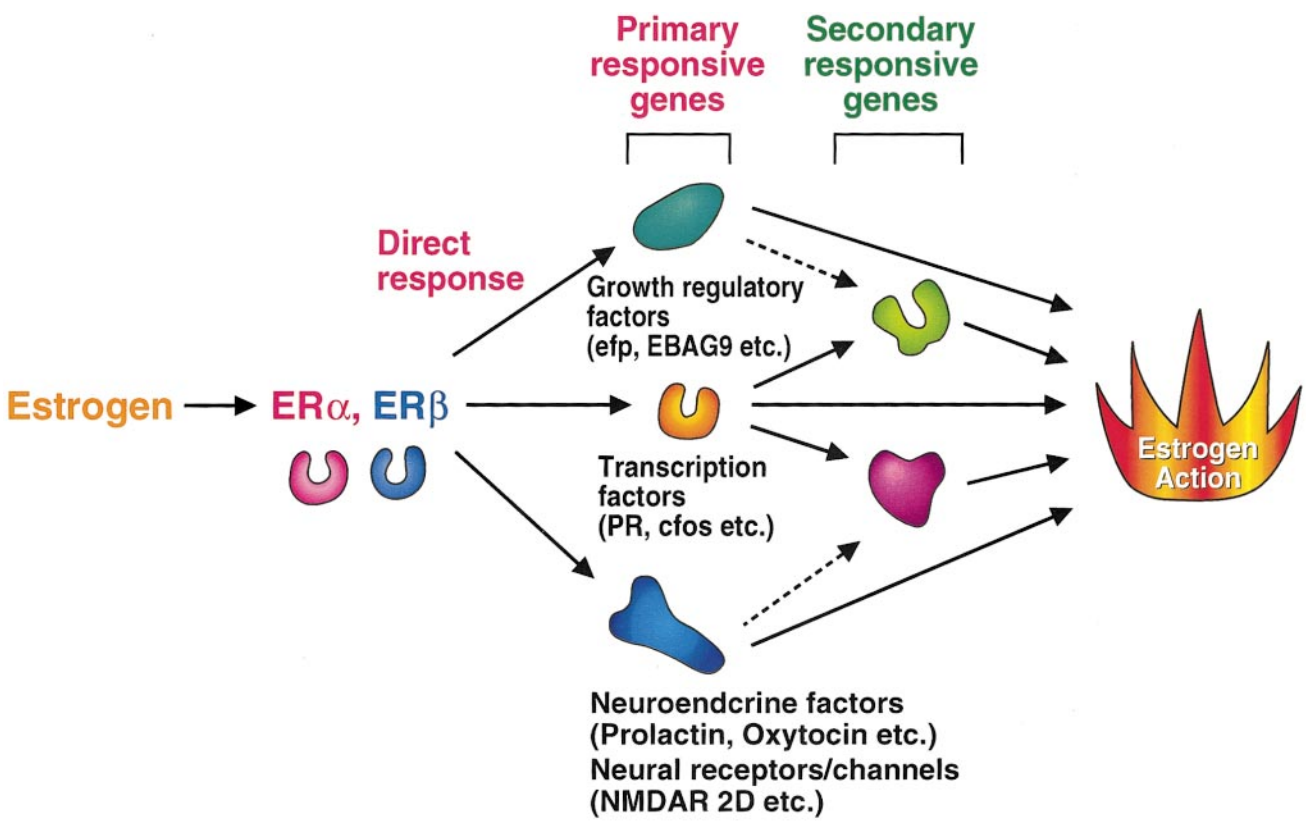

FIG. 3. A hierarchical cascade of estrogen action. (See text for details.)

shown in Table 2. One of the first identified group is named EFP (estrogen-responsive finger protein) because it belongs to an RBCC protein family which consists possibly of several dozens of members including PML (acute promyelocytic leukemia) gene that is known to cause the above disease by reciprocal translocation with RARα gene (78–80). EFP gene product is distributed among female organs such as ovary, uterus, mammary gland and certain region of brain where ERα is expressed and positively regulated by estrogen (76, 81). EFP is overexpressed in some mammary tumors (unpublished observations). EFP-knockout mice (82) have hypoplastic uteri (about 60% of control weight) but fertile. The sensitivity of endometrium to E_2 measured by the growth recovery after ovariectomy is significantly reduced in EFP$-/-$ mice. Thus, EFP appears to augment the growth capacity of ER responsive organs in female. On the other hand, another gene cloned by GBSC is that of a membrane protein designated EBAG9 (83) and has a growth-suppressive activity and may cause growth inhibition or even apoptosis to the adjacent cells that have a receptor (84, unpublished observation). Together with the finding that EBAG9 is expressed frequently in breast, uterus and mammary carcinoma cells, this gene might be related to E_2 induced invasiveness or metastasis of hormone dependent tumors (83–85, unpublished observations).

Another facet of ER-related gene regulation became apparent from the finding of multiple half-site-type ERE in the gene of an excitatory glutamate receptor, *N*-methyl-D-aspartate receptor type 2D (NR2D) (86). This gene was also demonstrated to be activated in hypothalamus by E_2. Since the hypothalamic nuclei such as having this receptor are highly related to sexual behavior, the presence of ERE and reactivity to E_2 strongly suggest that NR2D is a key molecule in this phenomenon and its amount is regulated by ER.

The complex cascade and network of signaling originated from estrogen is summarized in Fig. 3. There is apparently direct action of E_2 by the primary response gene such as a number of enzymes like Cox7RP (Table 2) or other growth factors that are not yet critically identified. In addition, there must be a number of transcription factors and cofactors that are induced by ER, which include PR, c-fos and possibly EFP, if it is a transcription cofactor as inferred from TIF1, etc. They will greatly increase the diversity of control of different genes culminating in the phenotype defined by estrogen. On the top of it, there appears another control by neurons in CNS where both ER and NR2D are expressed (see above). The well known effect of estrogen on sexual behavior may well be controlled by the amount of NR2D in certain neuronal cells which govern various sensory, motor and other functions in a concerted manner to activate sexual behavior (86). Needless to say, a number of neuroendocrine peptides many of which are yet to be identified must be in this circuit.

CONCLUSIONS

We have here briefly reviewed recent developments in the molecular biology of steroid hormone, particularly estrogen. There are a number of issues to be resolved in the near future.

First, the biological significance of the presence of ERβ in addition to ERα must be and will be solved relatively shortly. Second, the molecular functions of ER's will be clarified in relation to various coactivators and corepressors, and in the context of nuclear receptors in general. Third, finer mechanisms of transcription activation at the level of chromatin/nucleosome and cognate associated protein complexes must also be revealed. Fourth, for this purpose, the development of technologies *in vivo* (at cellular and animal levels) and *in vitro* (cell free and reconstituted systems constructed by recombinant molecules) are mandatory. Fifth, for each nuclear receptors, search for ligands and target genes must be of prime importance. We have just described a relatively naive method, GBSC, which actually yielded a number of interesting genes. Other newer methods such as differential DNA array screen etc. may become feasible soon as the human and mouse genome projects proceed. The global understanding of gene regulation by nuclear receptors including ER will provide useful means against human common diseases such as gynecological cancers, osteoporosis, arteriosclerosis and senile dementia in not so far future.

ACKNOWLEDGMENT

The authors thank Dr. Shigeaki Kato for his critical reading of the manuscript.

REFERENCES

1. Evans, R. M. (1988) *Science* **240,** 889–895.
2. Mangelsdorf, D. J., Tummel, C., Beato, M., Herrlich, P., Schutz, G., Umesono, K., Blumberg, B., Kastner, P., Mark, M., Chambon, P., and Evans, R. M. (1995) *Cell* **83,** 835–839.
3. Forman, B. M., Tontonz, P., Chen, J., Brun, R. P., Spiegelman, B. M., and Evans, R. M. (1995) *Cell* **83,** 803–812.
4. Kliewer, S. A., Lenhard, J. M., Wilson, T. M., Patel, I., Morvis, D. C., and Lehaman, J. M. (1995) *Cell* **83,** 813–819.
5. Wang, H., Chen, J., Hollister, K., Sowers, L. C., and Forman, B. M. (1999) *Mol. Cell* **3,** 543–553.
6. Parks, D. J., Blanchard, S. G., Bledsoe, R. K., Chandra, Conslev, T. G., Kliewer, S. A., Stimmel, J. B. Wilson, T. M., Zavacki, A. M., Moore, D. D., and Lehmann, J. M. (1999) *Science* **284,** 1365–1368.
7. Makishima, M., Okamoto, A. Y., Repa, J. J., Tu, H., Learned, R. M., Luk, A., Hull, M. V., Lusting, K. D., Mangelsdorf, D. J., and Shan, B. (1999) *Science* **284,** 1362–1365.
8. Jensen, E. V., and DeSombre, E. R. (1973) *Science* **182,** 126–134.
9. Kuiper, G. G., Enmark, E., Pelto-Huikko, M., Nilsson, S., and

Gustafsson, J. Å. (1996) *Proc. Natl. Acad. Sci. USA* **93** 5925–5930.

10. Mosselman, S., Polman, J., and Dijkema, R. (1996) *FEBS Lett.* **392,** 49–53.
11. Tremblay, G. B., Tremblay, A., Copeland, N. G., Giller, D. J., Jenkino, N. A., Labrie, F., and Giguere, V. (1997) *Mol. Endocrinol.* **11,** 353–365.
12. Ogawa, S., Inoue, S., Watanabe, T., Hiroi, H., Orimo, A., Hosoi, T., Ouchi, Y., and Muramatsu, M. (1998) *Biochem. Biophys. Res. Commun.* **243,** 122–126.
13. Ogawa, S., Inoue, S., Watanabe, T., Orimo, A., Hosoi, T., Ouchi, Y., and Muramatsu, M. (1998) *Nucleic Acids Res.* **26,** 3505–3512.
14. Couse, J. F., and Korack, K. S. (1999) *Endocrine Rev.* **20,** 358–417.
15. Hiroi, H., Inoue, S., Watanabe, T., Goto, W., Orimo, A., Momoeda, M., Tsutsumi, O., Taketani, Y., and Muramatu, M. (1999) *J. Mol. Endocrinol.* **22,** 37–44.
16. Nishihara, E., Nagayama, Y., Inoue, S., Hiroi, H., Muramatsu, M., Yamashita, S., and Koji, T. (2000) *Endocrinology,* in press.
17. Lubahn, D. B., Moyer, J. S., Golding, T. S., Couse, J. F., Korach, K. S., and Smithies, O. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 11162–11166.
18. Ogawa, S., Eng. V., Taylor, J., Lubahn, D. B., Korach, K. S., and Pfaff, D. W. (1998) *Endocrinology* **139,** 5070–5081.
19. Eddy, E. M., Washburn, T. F., Bunsh, D. O., Goulding, E. H., Gladen, B. C., Lubahn, D. B., and Korach, K. S. (1996), *Endocrinology* **137,** 4796–4805.
20. Ogawa, S., Washburn, T. F., Taylor, J., Lubohn, D. B., Korach, K. S., and Pfaff, D. W. (1998) *Endocrinology* **139,** 5058–5069.
21. Smith, E. P., Boyd, J., Frank, G. R., Takahashi, Ito., Cohen, R. M., Specher, B., Williams, T. C., Lubahn, D. B., and Korack, K. S. (1994) *N. Engl. J. Med.* **331,** 1056–1061.
22. Ogawa, S., Inoue, S., Orimo, A., Hiroi, T., Ouchi, Y., and Muramatsu, M. (1998) *FEBS Lett.* **423,** 129–132.
23. Petersen, D. N., Tkalceric, G. T., Koza, Taylor, P. H., Turi, T. G., and Brown, T. A. (1998) *Endocrinology* **139,** 1082–1092.
24. Krege, J. H., Hodgin, J. B., Couse, J. F., Emmark, E., Warner, M., Mahler, J. F., Sar, M., Korach, K. S., Gustafsson, J. Å., and Smithies, O. (1998) *Proc. Natl. Acad. Sci. USA* **95,** 15677–15682.
25. Windahl, S. H., Vidal, O., Andersson, G., Gustafsson, J. Å., and Ohlsson, C. (1999) *J. Clin. Invest.* **104,** 895–901.
26. Iafrati, M. D., Karas, R. H., Aronovitz, M., Kim, S., Sullivan, T. R., Jr., Lubahn, D. B., O'Donnell, T. F., Jr., Korach, K. S., and Mendelsohn, M. E. (1997) *Nat. Med.* **3,** 545–548.
27. Karas, R. H., Hodgin, J. B., Kwoun, M., Krege, J. H., Aronovitz, M., Mackey, W., Gustafsson, J. Å., Korach, K. S., Smithies, O., and Mendelsohn, M. E. (1999) *Proc. Natl. Acad. Sci. USA* **96,** 15133–15136.
28. Fisher, C. R., Graves, K. H., Partow, A. F., and Simpson, E. R. (1998) *Proc. Natl. Acad. Sci. USA* **95,** 6965–6970.
29. Couse, J. F., Hewitt, S. C., Bunch, D. O., Sar, M., Walker V. R., Davis, B. I., and Korach, K. S. (1999) *Science* **286,** 2328–2331.
30. Kumar, V., Geen, S., Stack, G., Berry, M., Jin, J. R., and Chambon, P. (1987) *Cell* **51,** 941–951.
31. Lees, J. A., Fawell, S. E., and Parker, M. G. (1989) *Nucleic Acids Res.* **17,** 5477–5488.
32. Halachmi, S., Marden, E., Martin, G., Mackay, H., Abbondanza, C., and Brown, M. (1994) *Science* **264,** 1455–1458.
33. Cavailles, V., Dauvois, S., Danielian, P. S., and Parker, M. G. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 10009–10013.
34. Cavailles, V., Dauvois, S., L'Horset, F., Lopez, G., Hoare, S., Kushner, P. J., and Parker, M. G. (1995) *EMBO J.* **14,** 3741–3751.
35. Onate, S. A., Tsai, S. Y., Tsai, M. J., and O'Malley, B. W. (1995) *Science* **270,** 1354–1357.
36. Voegel, J. J., Heine, M. J. S., Zechel, C., Chambon, P., and Gronenmeyer, H. (1996) *EMBO J.* **15,** 3667–3675.
37. Anzick, S. L., Kononer, J., Walker, L. R., Azorsa, O. D., Tanner, M. M., Guan, Y. X., Sauter, G., Kallioniemi, P. O., Trent, M. J., and Meltzer, S. P. (1997) *Science* **277,** 965–968.
38. Chen, H. W., Lin, R. J., Schlitz, R. L., Chakrawarti, D., Nash, A., Nagy, L., Privalski, M. L., Nakatani, Y., and Evans, R. M. (1997) *Cell* **90,** 569–580.
39. Horwitz, K. B., Jackson, T. A., Bain, D. L., Richer, J. K., Takinoto, G. S., and Tung, L. (1996) *Mol. Endocrinol.* **10,** 1167–1177.
40. Kamei, Y., Xu, L., Heinzel, T., Torchia, J., Kurokawa, R., Gloss, B., Lin, S. C., Heyman, R. A., Rose, D. W., Glass, C. K., and Rosenfeld, M. G. (1996) *Cell* **85,** 403–414.
41. Chakravarti, D., Lamorte, V. J., Nelson, M. C., Nakajima, T., Schulman, I. G., Juguilon, H., Montiminy, M., and Evans, R. M. (1996) *Nature* **383,** 99–103.
42. Heery, D. M., Kalkhoven, E., Hoare, S., and Parker, M. G. (1997) *Nature* **387,** 733–736.
43. Wurtz, J. M., Bourguet, W., Renaud, J. P., Vivat, V., Chambon, P., Moras, D., and Gronenmeyer, H. (1996) *Nature Struct. Biol.* **3,** 87–94.
44. Brozozowski, A. M., Pike, A. C., Dauter, Z., Hubbard, R. E., Bonn, T., Enastrom, O., Ohman, L., Greene, G. L., Gustafsson, J. Å., and Carlquist, M. (1997) *Nature* **389,** 753–758.
45. Shiau, A. K., Barstad, D., Loria, P. M., Chen, L., Kushner, P. J., Agar, D. A., and Greene G. L. (1998) *Cell* **95,** 927–937.
46. Xu, L., Glass, C. K., and Rosenfeld, M. G. (1999) *Curr. Opin. Genet. Dev.* **9,** 140–147.
47. Freedman, L. P. (1999) *Cell* **97,** 5–8.
48. Yao, T. P., Ku, G., Zhou, N., Scully, R., and Livingston, D. M. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 10626–10631.
49. Spencer, T. E., Jenster, G., Burcin, M. M., Allis, D. C., Zhou, J., Mizzen, C. A., Mckenna, N. J., Onate, S. A., Tsai, S. Y., Tsai, M. J., and O'Malley, B. W. (1997) *Nature* **389,** 194–198.
50. Ogryzko, V. V., Kotani, T., Zhang, X. L., Rchlitz, R. L., Howard, T., Yong, X. I., Howard, B. H., Qin, J., and Nakatani, Y. (1999) *Cell* **94,** 35–44.
51. Blanco, J. C. G., Minucci, S., Lu, J. M., Yang, X. J., Walker, K. K., Chen, H. W., Evans, R. M., Nakatani, Y., and Ozato, K. (1998) *Genes Dev.* **12,** 1638–1651.
52. Rachez, C., Suldan, Z., Ward, J., Chang, C. P., Burakov, D., Erdjument-Bromage, H., Tempst, P., and Freedman, L. P. (1999) *Proc. Natl. Acad. Sci. USA* **93,** 8329–8333.
53. Fondell, J. D., Ge, H., and Roeder, R. G. (1998) *Proc. Natl. Acad. Sci. USA* **93,** 8329–8333.
54. Gu, W., Malik, S., Ito, M., Yuan, C.-X., Fondell, D. J., Zhang, X., Martinez, E., Qin, J., and Roeder, R. G. (1999) *Mol. Cell.* **3,** 97–108.
55. Horlein, A. J., Naar, A. M., Heinzel, T., Torchia, J., Glass, B., Kurokawa, R., Ryan, A., Kamei, Y., Sonderstrom, M., Glass, C. K., and Rosenfeld, M. G. (1995) *Nature* **377,** 397–403.
56. Kurokawa, R., Sonderstrom, M., Horlein, A., Halachmi, S., Brown, M., Rosenfeld, M. G., and Glass, C. K. (1995) *Nature* **377,** 451–454.
57. Chen, J. D., and Evans, R. M. (1995) *Nature* **377,** 454–457.
58. Hu, X., and Lazar, M. A. (1999) *Nature* **402,** 93–96.
59. Heinzel, T., Lavinsky, M. R., Mullen, T.-M., Soderstrom, M., Laherty, D. C., Torchida, J., Yang, W.-M., Brand, G., Ngo, D. S., Davie, R. J., Seto, E., Eisenman, N. R., Rose, W. D., Glass, K. C., and Rosenfeld, G. M. (1997) *Nature* **387,** 43–48.
60. Nagy, L., Kao, H.-Y., Chakravarti, D., Lin, J. R., Hassig, A. C., Ayers, E. D., Schreiber, L. S., and Evans, M. R. (1997) *Cell* **89,** 373–380.

61. Nielsen A. L., Ortiz, J. A., You, J., Oulad-Abdelghani, M., Khechumian, R., Gansmuller, A., Chambon, P., and Losson, R. (1999) *EMBO J.* **18,** 6385–6395.
62. Montano, M. M., Ekena, K., Delage-Mourroux, R., Chang, W., Martini, P., and Katzenellenbogen, B. S. (1999) *Proc. Natl. Acad. Sci. USA* **96,** 6947–6952.
63. Chen, H., Lin, R. J., Xie, W., Wilpitz, D., and Evanx, R. M. (1999) *Cell* **95,** 675–686.
64. Kato, S., Endoh, H., Masuhiro, Y., Kitamoto, T., Uchiyama, S., Sasaki, H., Masushige, S., Goto, Y., Nishida, E., Kawashima, H., Metzger, D., and Chambon, P. (1995) *Science* **270,** 1491–1494.
65. Tremblay, A., Tremblay, G. B., Labrie, F., and Giguese, V. (1999) *Mol. Cell.* **3,** 513–519.
66. Hu, E., Kim, J. B., Sarraf, P., and Spiegelman, B. M. (1996) *Science* **274,** 2100–2103.
67. Shao, D., Rangwala, S. M., Bayley, S. T., Krakow, S. L., Reginato, M. J., and Lazar, M. A. (1998) *Nature* **396,** 377–380.
68. Endoh, H., Maruyama, K., Masuhiro, Y., Kobayashi, Y., Goto, M., Tai, H., Yanagisawa, J., Metzger, D., Hashimoto, S., and Kato, S. (1999) *Mol. Cell. Biol.* **19,** 5363–5372.
69. Xu, J., Qiu, Y., De Mayo, F. J., Tsai, S. Y., Tsai, M. J., and O'Malley, B. W. (1998) *Science* **279,** 1922–1925.
70. Lanz, R. B., Mckenna, N. J., Onate, S. A., Albrecht, U., Wong, J., Tsai, S. Y., Tsai, M. J., and O'Malley, B. W. (1999) *Cell* **97,** 17–27.
71. Zwijsen, R. M. L., Wientjens, E., Klompmaker, R., Van der Smani, J., Bernards, R., and Michalides, R. J. A. M. (1997) *Cell* **88,** 405–415.
72. Zwijsen, R. M., Buckle, R. S., Hijmans, E. M., Loomans, C. J. M., and Bemands, R. (1998) *Genes Dev.* **12,** 3488–3498.
73. Grazzini, E., Guillon, G., Mouillac B., and Zingg, H. H. (1998) *Nature* **392,** 509–512.
74. Razandi, M., Pedram, A., Greene G. L., and Levin, E. R. (1999) *Mol. Endocrinol.* **13,** 307–319.
75. Inoue, S., Kondo, S., Hashimoto, M., Kondo, T., and Muramatsu, M. (1991) *Nucleic Acids Res.* **19,** 4091–4096.
76. Inoue, S., Orimo, A., Hosoi, T., Kondo, S., Toyoshima, H., Kondo, T., Ikegami, A., Ouchi, Y., Orimo, H., and Muramatsu, M. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 11117–11121.
77. Inoue, S., Kondo, S., and Muramatsu, M. (1999) *in* Transcription Factors. A Practical Approach (D. S. Latchman, Ed.), 2nd ed., pp. 165–179. Oxford.
78. Kakizuka, A., Miller, W. H., Umesono, K., Warrel, R. P., Frankel, S. R., Murty, V. V., Dmitrovsky, E., and Evans, R. M. (1991) *Cell* **66,** 663–674.
79. de The., Lavau, C., Marchio, A., Chomienne, C., Degos, L., and Dejean, A. (1991) *Cell* **66,** 675–684.
80. Goddard, A. D., Borrow, J., Freemont, P. S., and Solomon, E. (1991) *Science* **254,** 1371–1374.
81. Orimo, A., Inoue, S., Ikeda, K., Noji, S., and Muramatsu, M. (1995) *J. Biol. Chem.* **270,** 24406–24413.
82. Orimo, A., Inoue, S., Minowa, O., Tominaga, N., Tomioka, Y., Sato, M., Kuno, J., Hiroi, H., Shimizu, Y., Suzuki, M., Noda, T., and Muramatsu, M. (1999) *Proc. Natl. Acad. Sci. USA* **96,** 12027–12032.
83. Watanabe, T., Inoue, S., Hiroi, H., Orimo, A., Kawashima, H., and Muramatsu, M. (1998) *Mol. Cell. Biol.* **18,** 442–449.
84. Nakashima, M., Sonoda, K., and Watanabe, T. (1999) *Nature Med.* **8,** 938–942.
85. Sonoda, K., Nakashima, M., Kaku, T., Kamura, T., Nakano, H., and Watanabe, T. (1996) *Cancer* **77,** 1501–1509.
86. Watanabe, T., Inoue, S., Hiroi, H., Orimo, A., and Muramatsu, M. (1999) *Mol. Brain Res.* **63,** 375–379.