CONTEMPORARY ENDOCRINOLOGY™

Gene Engineering in Endocrinology

Edited by

Margaret A. Shupnik

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PREFACE

During the past five to ten years, a variety of tools has been developed in the disciplines of both gene engineering, and molecular and structural biology. Some of these advances have permitted scientists not only to identify and characterize genes, but also to target these genes by disruption, thus eliminating their function in living animals, and to determine the biological responses to altered gene products. This has particular significance in endocrine systems, in which feedback mechanisms between the hypothalamus, pituitary, and end organs are critical in normal physiology. Interpretation of the physiological significance, or the site of action of specific molecules in this context, has been difficult prior to transgenic technology. Major advances have occurred specifically in the areas of growth and development, and of reproduction.

Coupled with analysis of naturally occurring mutations in humans, the use of transgenic animals and in vitro systems has recently allowed endocrinologists to understand the importance of specific thyroid hormone receptor isoforms in vivo, the molecular basis for generalized resistance to thyroid hormones via mutations in the nuclear receptor, and mechanisms for suppressing gene transcription. Previously designated "orphan receptors," such as steroidogenic factor-1, were demonstrated to have critical roles in development and reproduction. Other nuclear receptors—including those for thyroid hormone, estrogens, androgens, and progesterone—were shown to bind to coactivator and corepressor proteins that modified their transcriptional activity, and contributed to the cell-specific effects of the hormones. Previous dogma on the independence of steroid and peptide hormone mechanisms of action was shown to be simplistic. In fact, intracellular signaling pathways initiated by peptides modify steroid receptors directly and modulate their activity. These pathways also modify other transcription factors that, alone or in partnership with other proteins, regulate cell-specific patterns of gene expression. The application of transgenic and molecular techniques to the study of reproductive endocrinology illuminated the importance of estrogen in both males and females, the genetic basis for androgen insensitivity, gender-specific roles of the gonadotropins in normal reproduction, and the critical role played by activins, inhibins, and related growth factors.

In view of these tremendous advances, and the ability to draw clinical endocrine correlates from these findings, *Gene Engineering in Endocrinology* was assembled to include contributions from many leaders in these areas. The intent of our book is to place this new information in physiological perspective and to review the most recent work, as well as to indicate the areas of interest and questions that need still to be addressed in future research. The chapters describe studies performed with many types of molecular methods, and the use of animal and cellular model systems to explore the molecular basis of growth, development, and reproduction. Gene manipulation and disruption or "knockout" results are discussed in the context of the impact of specific genes on these physiological systems, and the developmental or physiological time period at which the mutation becomes critical. The molecular studies are compared, when possible, with naturally occurring human and animal gene mutations, in order to compare complete elimination of gene function with an altered gene product.

Gene Engineering in Endocrinology is aimed at a broad spectrum of readers, including those who are currently interested and actively working in molecular endocrinology, and clinical endocrinologists interested in relating molecular mechanisms to clinical endocrinology.

Margaret A. Shupnik, PhD

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1 Differential Cell Signaling and Gene Activation by the Human Growth Hormone Receptor

From Cell Surface to Cell Nucleus

Corinne M. Silva, PhD

CONTENTS

INTRODUCTION

Secretion of Growth Hormone

Growth hormone (GH) is a 22-kDa peptide that is released from the anterior pituitary in a pulsatile fashion. GH secretion is under the control of the hypothalamic hormones growth hormone releasing factor (GRF) and somatostatin (SS). GRF exerts positive effects on GH secretion and synthesis and SS inhibits GH secretion. For review of the regulation of GH secretion see Hartman et al. *(1)*. In addition to GRF and SS, a synthetic peptide derived from enkephalin was developed in the early 1980s and was shown to have a positive regulatory effect on GH secretion *(2)*. Because of its action, this peptide was named growth hormone–releasing peptide (GHRP). Recently, a specific receptor for GHRP has been cloned and found to be expressed in both the hypothalamus and pituitary, thus supporting early results suggesting that GHRP acts at both levels *(3)*. To date, the proposed natural ligand for this receptor has not been identified. Importantly, the pulsatile secretion of GH, including the frequency and amplitude of pulses, is influenced by a number of physiological factors. These factors include age, gender, body composition, nutrition, exercise, and sleep *(4)*. The mechanisms for these effects and the interplay among these factors is currently being investigated *(5)*.

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Target Tissues and Effects of GH

After secretion from the pituitary, GH exerts its effects on many tissues throughout the body. In addition to its obvious effects on growth, as its name implies, GH has profound effects on lipid, carbohydrate, and protein metabolism. The integral role of GH in metabolism and overall growth of the organism is well established clinically. GH acts both directly and indirectly through the regulation of hepatic insulin-like growth factor-1 (IGF-1) to stimulate longitudinal bone growth at the epiphyseal growth plate. The regulation of growth by GH involves effects on tissue differentiation, cell proliferation, and protein synthesis. The well-known and studied target tissues of GH are adipose, muscle, and liver. Overall, GH is anabolic, lipolytic, and diabetogenic; i.e., it increases protein synthesis, lipolysis, and hepatic glucose production while it decreases lipogenesis and glucose uptake *(6)*. GH acts as a modulator, meaning that its action on a specific cell depends on the cellular milieu. Thus, in addition to the stimulation of growth, GH plays an integral role in the overall metabolism of an organism.

GH RECEPTOR

Cytokine Receptor Family

To exert its effects at the cellular level, GH must act through its receptor. GH receptors (GHRs) are found in most tissues throughout the body, with liver being the major site of GH receptors *(7)*. The GHR, a single membrane–spanning receptor, was cloned in 1987 and consists of an extracellular hormone binding domain (246 amino acids), a short membrane spanning region (24 amino acids), and a fairly large intracellular domain (350 amino acids) *(8)*. At that time, it became obvious that this single transmembrane–spanning protein had no apparent homology to other already defined receptor families with known signaling mechanisms. Clearly the intracellular portion of the GHR did not contain a catalytic tyrosine kinase domain as was known to be the case for a number of the growth factor receptors (epidermal growth factor [EGF], platelet-derived growth factor [PDGF], IGF-1). This lack of a tyrosine kinase domain was particularly interesting given that there were reports of GH-activated tyrosine phosphorylation of the GHR. It soon became clear that the GH receptor shared structural homology in its extracellular ligand binding domain with members of a newly defined family of receptors, the cytokine receptors *(9)*. The receptors in this family are all single transmembrane, share homology in their extracellular domain, stimulate tyrosine phosphorylation, but contain no intrinsic tyrosine kinase domain. This large family includes the receptors for GH, prolactin (PRL), thrombopoietin (TPO), erythropoietin (EPO), interleukins *(2–7,9,11–13,15)*, colony-stimulating factors (G-CSF and GM-CSF), leukemia inhibitory factor, oncostatin M, ciliary neurotrophic factor, and the more distantly related interferons (IFN-γ and IFN-α/β). This family of cytokine receptors has expanded greatly over the past few years. In general, the ligands of these receptors are involved in regulation of the immune system, in hematopoiesis, and, in some cases, development. All these receptors are single transmembrane and are categorized into four subtypes based on motifs in their extracellular domain. Some of the receptors have a single subunit (such as the GHR) and some have several, sharing a common signaling subunit. Nevertheless, all share a common pathway of signaling, as discussed subsequently. For review of the cytokine receptor superfamily see Wells and de Vos *(10)*.

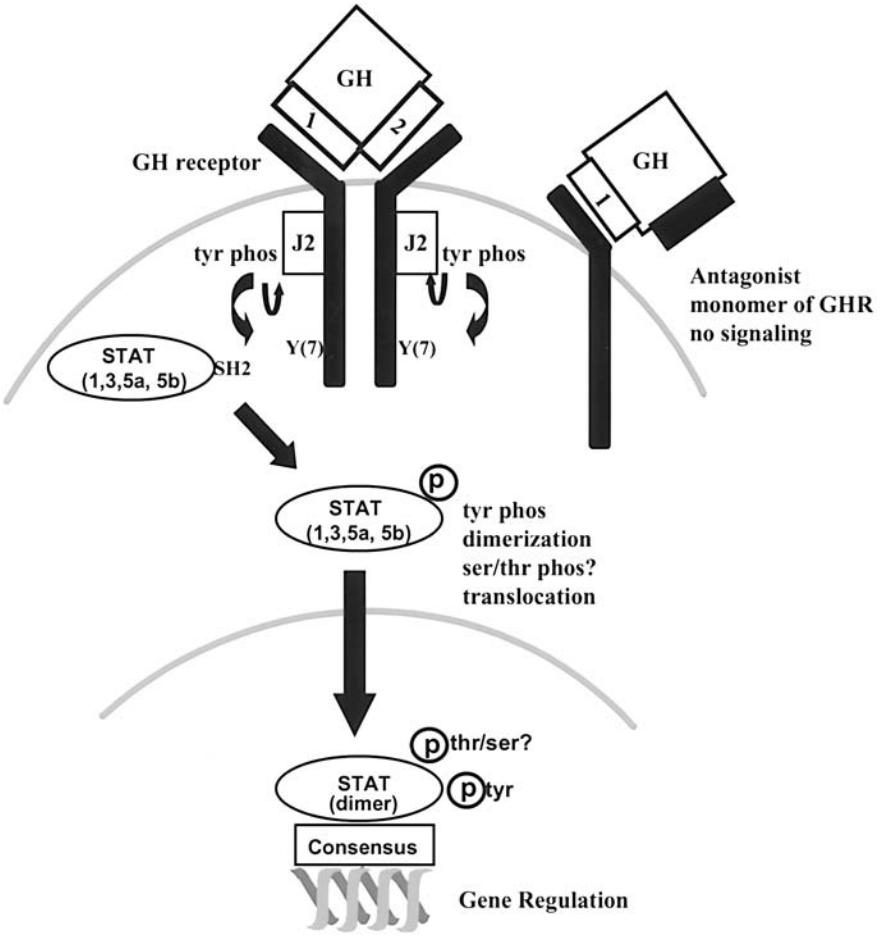

Fig. 1. Model of GHR signaling through the JAK/STAT pathway. GH has two binding sites (1) and (2) that bind two receptor molecules. The GH antagonist has an intact site 1 and a mutated site 2 and only binds a monomer of receptor (GHR): tyr phos, tyrosine phosphorylation; Y, tyrosine residue, the human GHR has seven (7). STAT, signal transducer and activator of transcription; J2, JAK2 tyrosine kinase; ser/thr, serine or threonine phosphorylation; consensus, site of STAT binding. (Adapted with permission from ref. *12a*.)

Receptor Dimerization

One major characteristic shared among cytokine receptors is the formation of hetero- or homodimers of receptor subunits *(10)*. The GHR has become the prototype for the process of homodimerization. Other receptors that form homodimers include the PRL, EPO, and TPO receptors. A series of biophysical studies by Cunningham and Wells *(11)* and crystal structure analysis by deVos et al. *(12)* resulted in the model of GH-induced receptor dimerization. Using the extracellular domain of the GHR, the GH-binding protein (GHbp), they showed that recombinant human GH has two sites of binding to the GHbp. Their data support a model of sequential binding whereby one GHbp binds to site 1 on the GH molecule, and then a second molecule of GHbp can bind to site 2 on GH, forming a GH:$(GHbp)_2$ complex (see the model of GHR in Figure 1). Elucidation of this model of GH binding has led to the development of GH

antagonists that have an intact site 1 and a mutated site 2 *(13)*. These antagonists disrupt dimer formation and bind only a monomer of receptor. More important, in vitro studies have shown that dimerization of the full-length GHR is necessary for the induction of intracellular signaling and the end points of GH action *(13,14)*. See model of the antagonist in Fig. 1.

Signaling Pathways

Insight into the intracellular signaling mechanisms used by the cytokine family of nontyrosine kinase membrane–spanning receptors came originally from elucidation of the pathway involved in interferon signaling *(15,16)*. Major advances in that field came from genetic complementation studies showing that the Janus kinase family (JAK) of tyrosine kinases was required for interferon signaling *(17)*. This family of tyrosine kinases, which includes tyk2 and the JAKs 1, 2, and 3, are cytosolic tyrosine kinases of ~120 kDa and have a kinase domain as well as a pseudokinase domain *(18,19)*. The subsequent development of kinase-specific antibodies led to elucidation of the role of the JAK kinases in signaling by all cytokine receptors. Furthermore, a family of src homology-2 (SH2) domain–containing transcription factors, known as signal transducers and activators of transcription (STATs), were identified. There are seven defined members of this family: STAT1, -2, -3, -4, -5a, -5b, and -6. They all have a molecular mass of between 85 and 105 kDa and share homologous domains. All contain a conserved SH2 domain that binds to phosphorylated tyrosine residues at the cytokine receptor/JAK kinase complex, and all require phosphorylation on a single tyrosine residue at the C-terminus of the molecule for activation. STATs were found first to be involved in signaling by the interferons but have since been shown to be activated in response to greater than 35 other cytokines as well as some growth factors (e.g., EGF and PDGF). For a review of STAT proteins, see refs. *20* and *21*.

GHR SIGNALING

JAK/STAT Pathway

The first evidence that growth hormone signaling occurred through a pathway similar to that seen with other cytokines came from evidence that GH activated the JAK family tyrosine kinase, JAK2. A number of laboratories demonstrated that on binding of GH, and dimerization of the GHR, the JAK2 tyrosine kinase became associated with the receptor, leading to its autophosphorylation and activation *(22–24)*. As with other Type I cytokine receptors, the JAK2 tyrosine kinase associates with a proline-rich juxtamembrane region of the GHR, and this region has been shown to be absolutely required for binding of JAK2 and activation of GH signaling *(23,25)*. Association of the JAK2 kinase in the GHR dimer complex results in autophosphorylation of the JAK2 kinase on tyrosine as well as tyrosine phosphorylation of the intracellular, cytoplasmic domain of the GHR. The human GHR contains seven tyrosines in its intracellular domain *(8)*. However, the number of tyrosines varies depending on species: the rat GHR has 10, the rabbit has 9, and the pig has 8. A number of mutational studies have begun to define the role of these tyrosines in the signaling pathways activated by the GHR. The requirement for these tyrosines depends on the end point analyzed. For example, in one study, all seven tyrosines of the human GHR were either deleted or

mutated. Nevertheless, this mutated receptor was still able to activate JAK2 and stimulate proliferation in a mouse promyeloid cell line *(26)*.

Activation of the JAK2 tyrosine kinase is required for GH-stimulated activation of the STAT proteins. Depending on the cell type or model system used, GH activates STATs1, -3, -5a and -5b. Not all of these STATs are activated in every cell type. For example, in the mouse 3T3-F442A preadipocyte cell line, which has been extensively studied, GH has been shown to activate all of these STATs *(27)*. In the human IM-9 lymphocyte cell line, GH activates only STAT5a and STAT5b *(24,28)*, whereas in a human fibrosarcoma cell line, it appears that GH activates STAT1 and STAT3 but not STAT5 *(29)*. In some cases it has been demonstrated that particular phosphorylated tyrosine residues on the GHR are required for binding of the STAT protein (through its SH2 domain), allowing it to be tyrosine phosphorylated by the JAK2 kinase. In general, mutational studies have indicated that there are tyrosine residues in the distal intracellular domain of the GHR that are important in the activation of STAT5b *(30,31)*. For example, in the porcine GHR the phosphorylation of one of four tyrosines at the C-terminus of this receptor seems to be sufficient for STAT5 activation *(25,32,33)*. On the other hand, activation of STAT1 and STAT3 does not appear to be dependent on phosphorylation of these C-terminal tyrosines. Rather, evidence suggests that STAT1 and STAT3 may interact with tyrosine-phosphorylated JAK2, or an adapter molecule that requires JAK2 for binding *(25,34)*. In fact, a recent study has identified an adapter protein, SH2-Bβ, which associates with JAK2 and becomes tyrosine phosphorylated in response to GH treatment of 3T3-F442A cells *(35)*. Since this protein contains an SH2 domain, nine tyrosines, a proline-rich region, and serine/threonine phosphorylation sites, it could potentially play an important role in linking the JAK2/GHR complex to other signaling molecules and pathways.

After binding to the GHR/JAK2 complex, the STATs become tyrosine phosphorylated on a single tyrosine residue. This tyrosine phosphorylation allows dimers to form between the SH2 domain of one STAT and the phosphorylated tyrosine residue on a second STAT. Through unknown mechanisms, this dimerization leads to translocation of the dimer to the nucleus *(20)*. In some cases, the STATs also become serine phosphorylated. In the case of STAT1 and STAT3, a mitogen-activated protein kinase (MAPK) consensus site has been defined *(36,37)*. STAT5b is also serine phosphorylated, although the site and kinase involved has not been defined *(38)*. Nevertheless, this serine phosphorylation occurs in the C-terminal transactivation domain and affects transcriptional activation. In the nucleus, STAT dimers bind to consensus elements upstream of target genes. The role of these consensus sites in GH-stimulated gene transcription is discussed further under "Genes Activated by GH."

MAP Kinase Pathway

Other signaling pathways are also stimulated in response to GH activation of the JAK2 tyrosine kinase. One pathway that has been extensively studied is the MAPK pathway. Studies in the 3T3-F442A mouse preadipocyte cell line have demonstrated that GH stimulates activation of MAPK in a time-dependent manner *(39–41)*. Several studies now indicate that this activation occurs through binding of the adapter protein Shc to the GHR/JAK2 kinase complex. Tyrosine phosphorylation of Shc and the subsequent association of growth factor binding protein-2 (Grb2) leads to activation

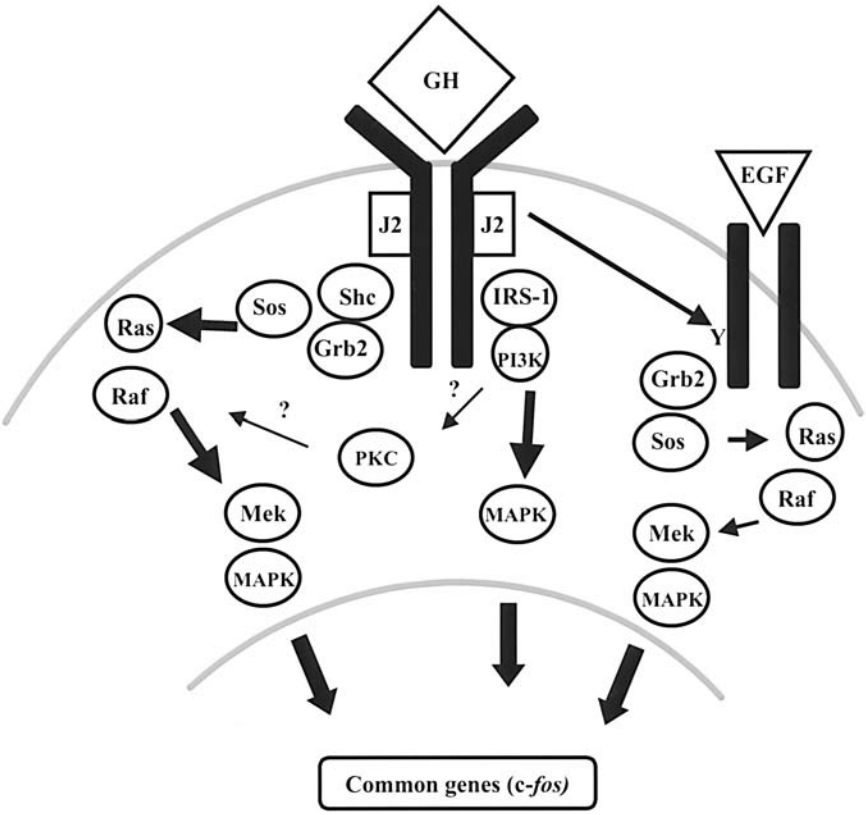

Fig. 2. Model of GHR signaling pathways leading to MAP kinase activation. Known and potential pathways of signaling from the GHR that lead to activation of the MAP kinase pathway. J2, JAK kinase; EGF, epidermal growth factor; PKC, protein kinase C; PI3K, phosphatidylinositol 3-kinase; IRS-1, insulin receptor substrate-1. See "MAP Kinase Pathway" for a discussion of MAPK pathway signaling molecules.

of the guanosine 5′-triphosphate exchange protein (Sos), and activation of the serine/threonine kinase (Raf), which activates the MAPK kinase (Mek), which directly activates MAPK *(42)*. Activation of all signaling molecules in the Shc/Grb2/Sos/Raf/Mek pathway has been demonstrated to occur in response to GH treatment *(43,44)*. (*see* Fig. 2).

However, recent studies have indicated that the Shc/Grb2/Sos/Ras/Raf pathway is not the only pathway that leads to activation of MAPK in response to GH. Like other cytokines, such as the interferons (α and γ) and the interleukins (2, 4, 7, and 15), GH has been shown to stimulate the tyrosine phosphorylation of the insulin receptor substrate-1 (IRS-1) *(45–48)*. Tyrosine phosphorylation of IRS-1 leads to the association of the phosphatidylinositol 3-kinase (PI3K), and GH has been shown to increase PI3K activity in 3T3-F442A cells *(49)*. Furthermore, inhibition of PI3K with wortmannin attenuates GH stimulation of MAP kinase in 3T3-F442A cells, indicating a role for this pathway. Additional studies using antisense oligodeoxynucleotides have indicated that a specific isoform of protein kinase C (PKC), PKCδ, is involved in the activation of the MAPK pathway by GH in the 3T3-F442A preadipocyte *(50)*. The site of PKC involvement is

not known; however, inhibition of either PKC or PI3K only partially inhibits GH stimulation of MAPK.

There is now evidence that the EGF receptor is involved in GH-stimulated activation of MAP kinase *(51)*. In these studies, it was shown that GH treatment results in JAK2-stimulated tyrosine phosphorylation of the EGF receptor on a single site, which results in binding of Grb2 and activation of the MAP kinase pathway. More important, an EGF receptor mutated at this tyrosine significantly decreases GH stimulation of MAP kinase in 3T3-F442A cells. Thus, activation of MAP kinase by GH does not occur through one pathway but is a dynamic integration between adapter proteins, signaling molecules, and even other receptors (*see* Fig. 2).

Genes Activated by GH

GH is known to specifically regulate a number of genes, including c-*fos*, c-*myc*, c-*jun*, IGF-1, spi 2.1, lipoprotein lipase, somatostatin, insulin, and cytochrome P-450s *(52,53)*. The mechanisms by which GH regulates these genes are beginning to be elucidated. For example, STAT5b has been shown to be involved in the regulation of the serine protease inhibitor (Spi 2.1) gene as well as the insulin-1 gene *(25,54)*. This regulation occurs through binding of GH-activated STAT5b to consensus elements found upstream of these genes *(24,55,56)*.

Regulation of c-*fos* by GH occurs through two upstream elements, the *sis*-inducible element (SIE) and the serum response element (SRE). Several studies have demonstrated that GH induces binding of STAT1 and STAT3 to the SIE *(57–59)*. DNA-binding analysis (electrophoretic mobility shift assay, EMSA) demonstrates that these two proteins form three complexes containing hetero- and homodimers. By contrast, STAT5a and STAT5b do not bind to the SIE (28). GH also regulates the transcription of c-*fos* through the SRE *(59)*. Recent studies have demonstrated that both the serum response factor and ternary complex factor known as Elk-1 are involved in GH regulation through the SRE *(60)*. One mechanism involved in this regulation is the GH stimulation of the serine phosphorylation of Elk-1. Phosphorylation of Elk-1 on serine leads to increased transcription of the c-*fos* gene. Interestingly, the site on Elk-1 that is phosphorylated in response to GH is the same as that induced by MAPK; however, a direct role for GH activation of MAPK in Elk-1 phosphorylation has not yet been demonstrated.

A number of liver gene products including two cytochrome P-450 genes that are involved in steroid metabolism (*CYP2C11*, male specific; *CYP2C12*, female specific) are regulated by GH in a sex-dependent manner *(61)*. Differences in GH secretion are most apparent in rodent models in which GH is secreted in a well-defined pulsatile manner in males and in a more continuous pattern of secretion in females *(62)*. Recent studies have characterized the role of the transcription factor STAT5b in the transduction of the male-specific GH pulses to the regulation of male-specific P-450 genes. Subramanian et al. *(63)* first described a role for the STAT5 transcription factor in the sexually dimorphic regulation of the P-450 gene (*CYP3A10*/6β-hydroxylase) by GH in male hamsters. Using an in vivo rat model, Waxman et al. *(64)* conducted a series of studies that demonstrate that intermittent but not continuous exposure to GH triggers tyrosine phosphorylation and nuclear translocation of a STAT5–related DNA-binding protein *(64)*. By contrast, STAT1 and STAT3, which are also activated in rat liver, are independent of the temporal pattern of GH *(65)*. More recent studies using an immortalized

rat hepatocyte cell line have shown that STAT5b is responsive to pulsatile GH and that this protein undergoes both tyrosine and serine/threonine phosphorylation in response to GH *(38)*. Thus, STAT5b, but not STAT1 or STAT3, is involved in transducing the signal of pulsatile GH to regulation of male-specific gene transcription. Interestingly, the off time of GH secretion is important for the ability of STAT5b to be reactivated *(38)*. Recent evidence suggests that the SH2 domain tyrosine phosphatase SHP-1 is involved in the dephosphorylation and thus recycling of STAT5b for stimulation by the next GH pulse *(66)*.

Turning Off the Signal

As mentioned previously, just as important as turning on the signaling mechanism through the GHR-activated JAK/STAT pathway is turning off the signal. Some of the mechanisms involved in turning off GHR signaling are now being elucidated. The SH2-containing tyrosine phosphatase (SHP-1) was first described to be involved in turning off the signal from the EPO receptor *(67)*. Through its SH2 domain, the phosphatase binds to C-terminal tyrosine-phosphorylated residues on the EPO receptor, becomes activated, and dephosphorylates the JAK2 kinase (and potentially other tyrosine-phosphorylated substrates). In addition to the association of SHP-1 with STAT5b (mentioned previously), there is also evidence that SHP-1 associates with specific residues at the C-terminus of the GHR, leading to inactivation of JAK2 *(33)*. These studies support those of Sotiropoulos et al. *(25)* that indicated that tyrosine residues in the C-terminus of the GHR are involved in the dephosphorylation of JAK2.

Recently, a family of cytokine-inducible inhibitors of signaling proteins, or suppressor of cytokine signaling (SOCS), have been cloned and implicated in a negative feedback loop that regulates cytokine signaling *(68,69)*. These proteins have been shown to inhibit JAK kinases and suppress the tyrosine phosphorylation of STAT proteins *(70)*. Recently, SOCS-3 has been shown to be induced by GH both in vivo and in vitro. Furthermore, expression of this protein leads to a decrease in the GH activation of the Spi 2.1 promoter *(71)*. Therefore, these proteins are likely to play a role in the turning off of the GH signal in other cell systems.

DEFECTS IN GHR SIGNALING

Laron Syndrome

Insight into the role of the GHR and its signaling pathway in whole-body physiology comes from a clinical syndrome known as Laron syndrome (for a review see ref. *72*). In 1966 Laron described this syndrome as due to GHR deficiency. Patients with Laron syndrome typically show decreased binding of GH but normal insulin binding in hepatocytes. The clinical phenotype is the same as seen with GH deficiency but with elevated serum levels of GH. These phenotypes include low IGF-1, growth failure (dwarfism), obesity, and hypoglycemia. As of 1993 there were approx 200 defined cases of Laron syndrome *(72)*.

Molecular techniques have allowed identification and cataloguing of the mutations in the GHR gene that are responsible for Laron syndrome. Most of the defects identified are found in the extracellular domain of the GHR. Thus, these defects influence the binding of GH to its receptor, the first step in GH action. An interesting example of a GHR defect was recently shown by Duquesnoy et al. *(73)*. They described a single-

point mutation in the extracellular domain of the receptor that is necessary for receptor dimerization. When the mutant receptor is expressed in COS-7 cells, it retains GH-binding capability and has a normal subcellular distribution. However, GHR-specific antibodies as well as solution-binding studies demonstrated that the mutant receptor did not form GH-induced homodimers (or heterodimers with the wild-type GH receptor). The fact that patients with this mutation display all the classical features of Laron syndrome supports the critical role of GHR dimerization in its signal transduction.

In approx 20% of the cases of Laron syndrome, the extracellular domain of the GHR is found to be normal. In these cases, the defect must be either in the transmembrane and/or intracellular domains of the GHR, or in the intracellular signaling pathway that is activated by the GHR. For example, two related Laron patients were described by Woods et al. *(74)* to have a homozygous point mutation that resulted in aberrant splicing at exon 8 that results in a GHR protein lacking the transmembrane and intracellular domains, thus demonstrating the importance of the intracellular domain of the GHR in the transduction of the GH signal for its physiological actions.

In addition, it has recently been demonstrated that GH insensitivity may also result from defects in the intracellular signaling pathways that are activated by GH. Studies by Freeth et al. *(75)* have shown that fibroblasts from children with Laron syndrome have no apparent GHR defect but display a decrease in cell proliferation and expression and secretion of IGFBP-3 when compared with normal fibroblasts. These results suggest that this GH insensitivity is due to a defect(s) in the intracellular signaling pathway activated by GH. Further studies with fibroblasts from these Laron patients demonstrated that one patient had a defect in both the MAP kinase and STAT signaling pathways whereas two other siblings seemed to have intact STAT signaling but a possible defect in the MAPK pathway *(75,76)*. Studies are under way to elucidate these defects present in Laron patients and will provide insight into which pathways of signaling described by the in vitro work discussed previously are essential for GH actions in vivo.

Mouse Models

Several mouse models exist that represent defects in either GH secretion, the GHR, or proteins involved in the GH signaling pathway. Two well-studied models of spontaneous GH deficiency are the Ames and Snell dwarf mice *(77,78)*. These mice lack GH, PRL, and thyroid-stimulating hormone owing to hypoplasia or lack of the pituitary cells responsible for secreting these hormones. The Snell mouse contains a mutation in the anterior pituitary transcription factor, Pit-1, which activates the GH and prolactin genes during development and is found in mature lactotrophs, somatotrophs, and thyrotrophs. The Ames mouse contains a mutation of another tissue-specific transcription factor, prophet of Pit-1, that also is involved, upstream of Pit-1, in the development of these cell lineages in the pituitary. These mice display well-defined characteristics of GH deficiency, including the dwarf phenotype, as well as an increase in the mRNA and protein for the growth hormone releasing hormone (GHRH) and a decrease in the mRNA and protein for somatostatin *(79–81)*.

Another genetically transmitted dwarf mouse model is the little (lit/lit) mouse *(82)*. These mice have a mutation in the GHRH receptor protein. Normally, the GHRH receptor, a seven-transmembrane G-protein–linked receptor, signals through an increase in cyclic adenosine monophosphate. However, a point mutation in the gene for the GHRH receptor in lit/lit mice results in a single amino acid substitution in the receptor

Table 1
Models of GHR and STAT5B Knockout Mice

GHR[a]	*STAT5B*[b]
Decreased size and weight	Decrease in body weight gain in males
Increased GH levels	Increase in GH in males
Decreased IGF-1 levels	Decrease in IGF-1 levels
Decreased lactation	Mammary gland development impaired
Decreased litter size	Increase in aborted fetuses (reversed by progesterone)
Decreased organ size	Male-specific liver gene transcription lost
Retarded growth	Less adipose tissue
Delayed sexual maturation	No lipid in some cells

[a]Compiled from ref. *84*.
[b]Compiled from ref. *85*.

protein such that it does not transmit a signal. Defective GHRH receptor signaling at the level of the pituitary results in decreased GH mRNA and secretion and a resulting reduction in serum IGF-1 levels. Studies using this lit/lit mouse model, have indicated that the GH/IGF-1 axis plays a permissive role in the growth of breast cancer cells in mice *(83)*.

The recent development of two additional mouse models has provided a background in which to study the role of the GHR and its signal transduction pathway in a variety of target tissues. A comparison of the phenotypes of these two models can be found in Table 1. One model was developed by targeted disruption of the GHR gene in mice, known as the GHR knockout *(84)*. Southern blot analysis confirms that the GHR gene has been disrupted and that hepatocytes display no GHR on Western blotting and very low levels of specific binding to ^{125}I-GH, demonstrating that these mice have no functional GHR. Table 1 lists the phenotypic characteristics of these mice. This mouse model demonstrates, therefore, the important role of the GHR in the physiological characteristics listed. The authors propose that this mouse provides an additional model of Laron syndrome. This mouse model will facilitate investigation of the mechanisms involved in the physiological defects of Laron syndrome and provide a model to test the effectiveness of treatment paradigms.

A second mouse model developed by Udy et al. *(85)* targeted disruption of the gene for the STAT5b transcription factor. As discussed previously, STAT5b has been shown in in vitro studies and in a rat model to be part of the GHR signaling pathway (*see* "Genes Activated by GH"). The STAT5b knockout model provides additional evidence that STAT5b is the major transducer of the pulsatile GH signal leading to sexually dimorphic expression in the liver. In these mice lacking STAT5b, the expression pattern of a number of liver genes (including several P-450 genes) was more like that seen in normal females as opposed to the usual male-specific pattern. In addition, these mice share some of the same characteristics as those seen in the GHR knockout mouse model discussed previously (*see* Table 1). Comparison of the phenotype of these two mice models in males and females will provide information on which GH effects are transduced by STAT5b. These studies are especially important given the high degree of homology between STAT5a and STAT5b (~94% at the amino acid level). In the STAT5b knockout model, STAT5a is intact and expressed at normal levels. Thus, this

highly homologous protein cannot substitute for the role of STAT5b in transducing GH effects. This result lends further support to the critical importance of the differences between the two proteins, mainly at the C-terminal transactivation domain.

CLINICAL IMPLICATIONS

GH Antagonists

As described under "Receptor Dimerization," and depicted in the model of Fig. 1, the GH molecule has two sites of binding to the GHR, leading to receptor dimerization, a requirement for signaling. This model has led to the development of GH antagonists that bind the receptor as a monomer and thus inhibit signal transduction from the receptor in vitro. Recent studies have also indicated that this antagonism occurs in vivo. In fact, mice that are transgenic for the GH antagonist exhibit a dwarf phenotype *(86)*. Furthermore, when these mice are crossed with those transgenic for wild-type GH, the progeny show varying levels of GH and GH antagonist. The phenotypes of these mice indicate that the GH antagonist can counteract the effects of GH excess in vivo. Increased levels of GH are known to occur in diabetes and have been proposed to be involved in the development of diabetic nephropathy and retinopathy. In two different transgenic mouse models, Kopchick et al. *(87,88)* have demonstrated that GH antagonists protect mice against streptozotocin-induced glomerulosclerosis and ischemia-induced retinal neovascularization. These studies suggest, therefore, that GH antagonists could be used clinically to treat diseases of GH excess, such as acromegaly, complications of diabetes, and potentially breast cancer (see the little mouse model discussed previously).

The studies described by Kopchick et al. *(87,88)*, in addition to other animal models, have led to optimism regarding the use of GH antagonists to treat human diseases of GH excess. In fact, the results of the first clinical trials with GH antagonist are now being reported. In these studies, a recombinant human GH antagonist molecule that was pegylated for stability and that was mutated at 8 amino acids in site 1 (to increase binding) and 1 amino acid in site 2 (to inhibit binding) was used to treat patients with acromegaly. The initial phase of these studies indicates that this molecule given subcutaneously, once a week for 6 wk, can decrease IGF-1 levels in acromegalics *(89)*. These studies are the first of many that will address the potential use of GH antagonist molecules in the treatment of human disease caused by GH excess.

Peptide and Nonpeptide Mimetics

In recent years, several groups have begun the search for small molecules, peptide and nonpeptide, that can act at cytokine receptors to induce signal transduction *(90)*. The availability of such molecules, especially those that are orally active, would greatly advance treatment paradigms. In fact, small peptide agonists for both the EPO and TBO receptors have been developed and characterized *(91–94)*. More important, these small peptide mimetics are able to activate the end points seen with the natural ligand. Most recently, a small, nonpeptide mimetic of granulocyte colony-stimulating factor (G-CSF) has been developed that activates the G-CSF receptor and has been shown to activate the JAK/STAT signaling pathway and stimulate primary bone marrow cells to form granulocyte colonies in vitro *(95)*. These studies provide the framework for the development of small molecule peptide and nonpeptide mimetics for other members

of the cytokine receptor family, including GH. The development of orally active nonpeptide GH mimetics would eliminate the need for daily injections of recombinant human GH, and thus make treatment much more amenable.

SUMMARY

Major advances have been made just in the past 5 years in understanding the mechanisms involved in transducing the signal from the GHR at the membrane of a cell to gene transcription in the nucleus. Understanding cell type–specific signaling by GH is important, especially given the increased use of GH in the clinical setting. Recent years have seen the development of antagonists of the GHR that can be used to treat GH excess, as well as the development of orally active growth hormone secretagogues that can be used to treat GH deficiency. Elucidation of the mechanism of action of growth hormone at the cellular level is necessary in order to understand better the role of these treatment paradigms in whole-body physiology and metabolism.

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2 Insulin Action

Molecular Mechanisms and Determinants of Specificity

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CONTENTS

INTRODUCTION

Insulin is a peptide hormone that plays critical roles in the regulation of growth, differentiation, and metabolism. The physiological importance of insulin is underscored by the fact that the insulin receptor has been evolutionarily conserved and is found in organisms ranging from Drosophila to humans. Furthermore, insulin-dependent diabetes mellitus, a disease characterized by absolute insulin deficiency, was a uniformly fatal condition before the advent of insulin therapy. Like other circulating polypeptide hormones, insulin initiates its biological actions by binding to specific cell-surface receptors. The molecular cloning of the insulin receptor led to the discovery that it belongs to a large family of ligand-activated receptor tyrosine kinases (RTKs) that includes receptors for many other growth factors *(1–5)*. Many of the molecules involved with the transduction of signals from a multitude of RTKs also participate in insulin signaling. One of the central puzzles in the field of signal transduction is understanding how signal specificity is achieved after the interaction of the ligand with its receptor since so many postreceptor events seem to be shared in common by a variety of different RTKs. In this chapter, we briefly review the current understanding of how insulin receptor signaling follows a general paradigm for RTK signal transduction. Particular emphasis is given to signaling pathways related to glucose transport since this is among the most important physiological actions of insulin and is a specialized metabolic function that

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distinguishes the insulin receptor from other RTKs. Finally, we discuss several potential mechanisms for achieving signal specificity that are illustrated by recent studies relevant to insulin signaling.

INSULIN SIGNALING FOLLOWS PARADIGM FOR RTK SIGNAL TRANSDUCTION

As illustrated in Fig. 1, the propagation of information resulting from the binding of insulin to its cell surface receptor follows a general paradigm for RTK signal transduction that ultimately culminates in multiple biological effects, including increased glucose transport, gene and enzyme regulation, and mitogenesis, that are important for the regulation of metabalism and growth.

Ligand Binding and Receptor Dimerization

The first step in initiating signal transduction by an RTK involves the specific binding of a ligand to the extracellular portion of its cognate cell-surface receptor. In the case of monomeric receptors such as the epidermal growth factor (EGF) receptor, ligand binding results in receptor dimerization, a necessary first step in signal transduction *(4,6,7)*. The insulin receptor has a heterotetrameric structure (actually, a dimer of $\alpha\beta$ heterodimers) consisting of two extracellular α-subunits and two transmembrane β-subunits joined by disulfide bonds *(8)*. Thus, even in the absence of ligand, the insulin receptor exists in a dimeric form. The α-subunit of the insulin receptor contains fibronectin III repeats and cysteine-rich domains that are also found in several other RTKs. Insulin binds with high affinity to specific regions of the α-subunit (including the cysteine-rich domain), resulting in a rapid conformational change in the receptor *(9,10)*. In the absence of ligand, the α-subunit of the insulin receptor appears to exert a tonic inhibitory influence on insulin receptor function because insulin receptors that have had the α-subunit removed by trypsin digestion or expression of the cytoplasmic domain of the insulin receptor alone results in constitutive activation of receptor signaling *(11,12)*.

Receptor Autophosphorylation and Activation of Intrinsic Tyrosine Kinase

Ligand binding and receptor dimerization result in activation of the RTK. The kinase region of all RTKs shares substantial homology in both the adenosine triphosphate binding site and the catalytic domain *(4)*. The kinase of one half of the receptor dimer phosphorylates cytoplasmic tyrosine residues on the other half of the receptor dimer. This mutual transphosphorylation event is known as receptor autophosphorylation and results in a large increase in the catalytic activity of the receptor. The β-subunit of the human insulin receptor contains tyrosine residues distal to the catalytic domain at positions 1158, 1162, and 1163 (in the so-called activation loop) that undergo autophosphorylation and are important for enhancing the tyrosine kinase activity of the receptor *(13–15)*.

Tyrosine Phosphorylation of Cellular Substrates and Recruitment of Distal Signaling Molecules

Activation of the RTK leads to tyrosine phosphorylation of cellular substrates that propagate signaling. In addition, receptor autophosphorylation enables the RTK to

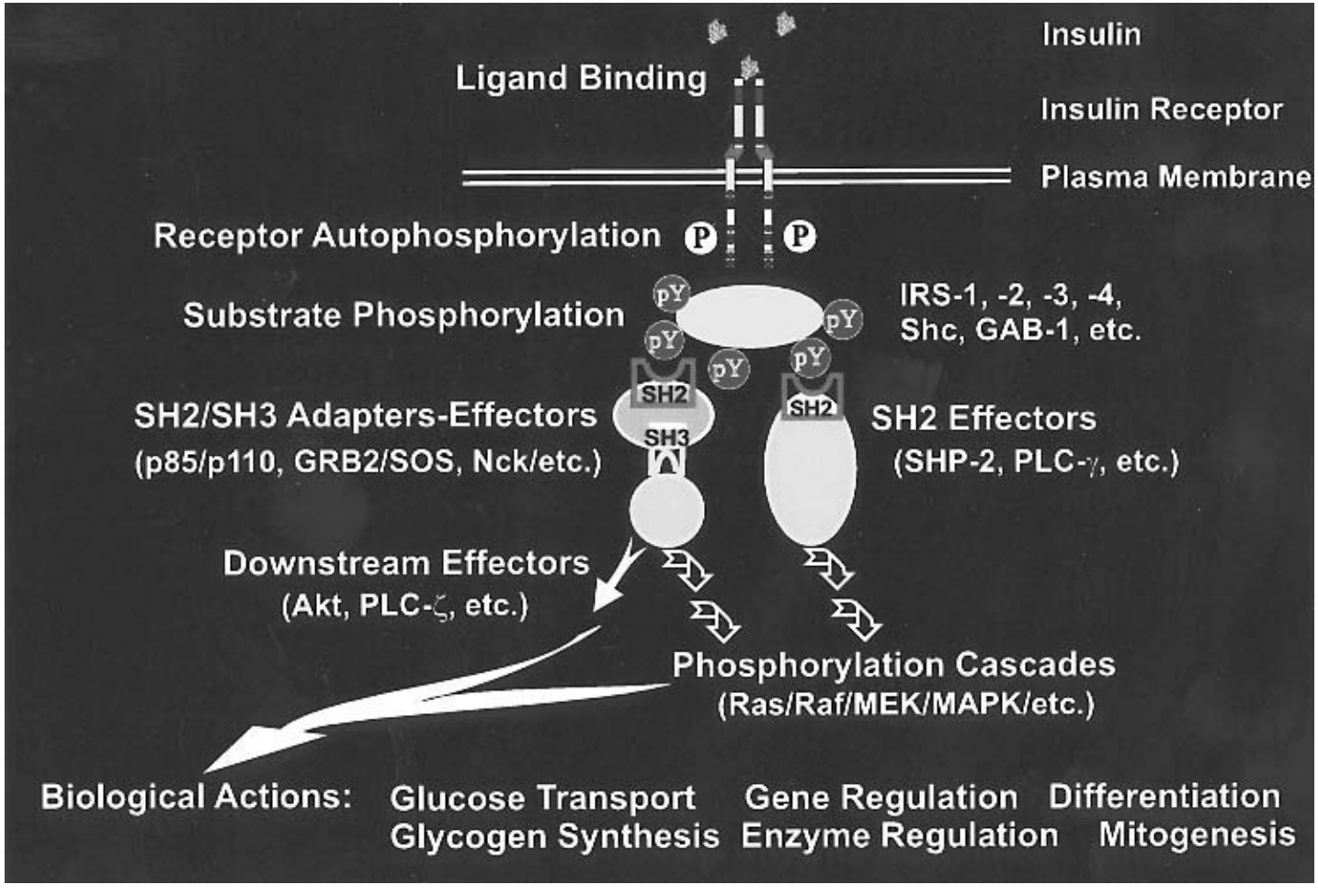

Fig. 1. Insulin signal transduction follows paradigm for RTK signaling.

directly engage signaling molecules via interactions between phosphotyrosine motifs on the receptor and src homology-2 (SH2) domains on downstream molecules. SH2 domains are protein domains of ~100 amino acids that share homology with a noncatalytic region of the src protooncogene product. Many molecules involved with RTK signaling, including src, phosphatidylinositol 3-kinase (PI3K), growth factor receptor-bound protein 2 (GRB-2), SH2-containing phosphatase-2 (SHP-2), GTPase-activating protein (GAP), and phospholipase C-γ (PLC-γ), contain SH2 domains. Motifs defined by the three amino acid residues on the C-terminal side of the phosphotyrosine residue provide specificity for interaction with particular SH2 domains *(16,17)*. In the case of receptors for EGF and platelet-derived growth factor (PDGF), the particular phosphotyrosine sites that engage specific SH2 domains of various signaling molecules have been well mapped *(18,19)*.

Although the autophosphorylated insulin receptor β-subunit is capable of directly interacting with molecules such as PI3K, SHP-2, and GAP *(20–22)*, direct binding of phosphotyrosine motifs on the insulin receptor with SH2 domain-containing molecules does not appear to be the major pathway for insulin signal transduction. Instead, there are substrates of the insulin receptor tyrosine kinase such as insulin receptor substrate-1 (IRS-1), IRS-2, IRS-3, IRS-4, SHC, and GRB-2-associated binder-1 (GAB-1) that provide an interface between the insulin receptor and downstream SH2 domain–containing molecules *(23–29)*. The IRS family of proteins contain a number of conserved regions including a pleckstrin homology (PH) domain and a phosphotyrosine binding domain that are important for the ability of the autophosphorylated insulin receptor to interact with and phosphorylate IRS molecules *(23,30,31)*. In addition, these insulin receptor substrates contain multiple phosphotyrosine motifs that can bind to SH2 domains and may serve as docking molecules that mediate the formation of signaling complexes consisting of several SH2 domain–containing proteins.

Signaling Proteins Containing SH2 and SH3 Domains

Many of the signaling molecules participating in RTK signal transduction pathways contain SH2 and/or SH3 domains that mediate protein-protein interactions. As mentioned previously, SH2 domains interact specifically with phosphorylated tyrosine motifs. SH3 domains bind with high affinity to particular proline-rich sequences *(4)*. Some SH2 domain-containing proteins (e.g., SHP-2, PLC-γ) are effector molecules that possess intrinsic catalytic activity that is regulated or localized by interactions of the SH2 domain of the effectors with phosphotyrosine motifs on other proteins (e.g., IRS-1). Other SH2/SH3 domain-containing proteins (e.g., GRB-2, Nck, and the p85 regulatory subunit of PI3K) are known as adaptor proteins because they have no intrinsic catalytic activity and their function involves forming specific signaling complexes mediated by the simultaneous interactions of multiple SH2/SH3 domains on the adaptor protein with both upstream and downstream signaling molecules. Activation of Ras and PI3K, two major effector pathways common to a number of growth factor receptors including the insulin receptor, fit this latter pattern. For example, GRB-2 is normally prebound to SOS (a guanine nucleotide exchange factor) via interactions of the two SH3 domains of GRB-2 and proline-rich regions of SOS. When phosphotyrosine motifs on IRS-1 and Shc interact with the SH2 domain of GRB-2, activation of the prebound SOS promotes formation of the GTP-bound form of Ras, leading to activation of Ras. Similarly, the p85 regulatory subunit of PI3K is normally preassociated with the p110

catalytic subunit. Insulin stimulation results in the interaction of phosphotyrosine motifs on IRS proteins with SH2 domains on p85, leading to activation of the prebound p110 catalytic subunit (for reviews see refs. *32* and *33*).

Downstream Phosphorylation Cascades

Distal RTK signaling pathways are difficult to dissect cleanly because multiple branching pathways begin to emerge from single effectors. Adding to the complexity, multiple upstream inputs often converge on single branch points. Furthermore, negative feedback mechanisms sometimes exist that lead to downstream signals affecting upstream components. However, it is clear that various serine/threonine phosphorylation cascades contribute to the propagation of signaling from the cell surface to the nucleus. These phosphorylation cascades seem to be common to signaling for many growth factors including insulin. For example, Ras directly activates Raf, a serine/threonine kinase that phosphorylates and activates MEK, which in turn phosphorylates and activates mitogen-activated protein kinase (MAPK), leading to induction and activation of early immediate genes such as the protooncogenes c-*jun* and c-*fos*. Insulin signaling mediated by PI3K pathways also involves downstream serine/threonine kinase cascades. For example, phospholipid products generated by PI3K activate PDK1, a serine/threonine kinase that phosphorylates and activates Akt (another serine/threonine kinase), which in turn phosphorylates and inactivates glycogen synthase kinase-3 (GSK-3) *(34–36)*. This process results in activation of glycogen synthase and the stimulation of glycogen synthesis.

Protein Tyrosine Phosphatases

Since tyrosine phosphorylation is critical to initiating and propagating signaling by RTKs, it is not surprising that dephosphorylation of tyrosine residues by protein tyrosine phosphatases (PTPases) contributes to the regulation of signaling. The number and diversity of PTPases rivals that of the RTKs *(37,38)*. PTPases are generally subdivided into a family of nontransmembrane proteins containing a single catalytic PTPase domain and a family of transmembrane receptor-like PTPases that typically contain tandem PTPase domains. The transmembrane PTPases (also known as receptor-like PTPases) have been further categorized into eight groups based on shared structural features of various extracellular domains *(38)*. The large number of PTPases discovered and characterized to date suggests that each PTPase plays a specific role in modulating signaling by RTKs. PTPases such as SHP-2 contain SH2 domains that confer specificity whereas the receptor-like PTPases have extracellular domains that presumably interact with specific ligands. In addition, subcellular localization of particular PTPases may contribute to their specificity. Although all of the determinants of PTPase specificity are not understood, there is evidence that particular PTPases show selectivity for specific RTKs *(39)*.

In the case of signaling by the insulin receptor, the transmembrane PTPases, PTP-α, PTP-ε, and LAR, have all been implicated as modulators of insulin action *(40–42)*. In particular, LAR has been shown to interact with and dephosphorylate the insulin receptor in intact cells *(43)*. In addition, the expression and level of activity of LAR in insulin targets such as muscle and adipose tissue is increased in insulin-resistant states such as obesity and diabetes *(44,45)*. Among the nontransmembrane PTPases, PTP1B and SHP-2 have both been shown to modulate insulin signaling. PTP1B dephos-

phorylates the insulin receptor both in vitro and in intact cells *(39,46,47)*. In addition, PTP1B regulates both mitogenic and metabolic actions of insulin *(41,48,49)*. In tissue culture models, an increase in the level and activity of PTP1B has been associated with insulin resistance induced by exposure to high glucose levels. In addition, the level and activity of PTP1B in human skeletal muscle is positively correlated with in vivo measures of insulin sensitivity *(50–52)*. Binding of the SH2 domains of SHP-2 to phosphotyrosine motifs on either the insulin receptor or IRS-1 results in activation of SHP-2 PTPase activity *(53,54)*. Interestingly, a number of studies have shown that SHP-2 participates in Ras- and MAPK-dependent pathways as a positive mediator of mitogenic actions of insulin and other growth factors *(55–58)*.

INSULIN SIGNALING PATHWAYS THAT REGULATE GLUCOSE TRANSPORT

A primary metabolic function of insulin that distinguishes it from other growth factors is the promotion of whole-body glucose utilization and disposal. The rate-limiting step in glucose utilization under normal conditions is glucose transport into cells. The insulin-responsive glucose transporter GLUT4 is expressed at high levels almost exclusively in classical insulin targets such as muscle and adipose tissue (for a review see ref. *59*). Insulin stimulates increased glucose transport in these tissues by causing the redistribution of GLUT4 from an intracellular pool to the cell surface, where it acts as a facilitative transporter to enhance entry of glucose into the cell *(60–62)*. This redistribution of GLUT4 is due largely to insulin increasing the rate of exocytosis of GLUT4 (insulin may also have a minor effect in decreasing endocytosis of GLUT4) *(63–65)*.

Although the tissue-specific distribution of GLUT4 and the effects of insulin on the subcellular localization of GLUT4 have been known for some time, elucidation of metabolic insulin signaling pathways has lagged behind other areas of insulin signal transduction for several reasons. First, although muscle and adipose tissue normally express high levels of GLUT4 and are extremely responsive to insulin stimulation, the ability to apply modern molecular methods such as transfection of recombinant DNA to these terminally differentiated tissues has been limited. Second, tissue culture models of muscle and adipose cells that are easier to manipulate (e.g., 3T3-L1 adipocytes, L6 myocytes, or C2C12 cells) do not always faithfully reflect important characteristics of bona fide insulin target cells. For example, the relative levels of expression of IRS-1, -2, and -3 are quite different in primary adipose cells and 3T3-L1 adipocytes *(25,66,67)*. Third, the requisite cellular machinery for appropriate subcellular trafficking of GLUT4 seems to be lacking in commonly used tissue culture cells such as NIH-3T3 fibroblasts, Chinese hamster ovary cells, or COS cells, which do not normally express GLUT4. Therefore, even when recombinant insulin receptors and GLUT4 are stably expressed in these cells, they are much less responsive to insulin than muscle or adipose cells *(68)*.

The recent use of electroporation to transfect adipose cells in primary culture in conjunction with quantitative methods for assessment of cell surface GLUT4 has led to a clearer understanding of metabolic insulin signaling pathways *(41,69–75)*. In addition, transgenic mice that have had key signaling molecules either knocked out or overexpressed have provided valuable insights *(76–80)*. Finally, microinjection or viral

transfection strategies in differentiated 3T3-L1 adipocytes along with semiquantitative methods for assessing cell surface GLUT4 have also been informative *(81–85)*. Figure 2 summarizes some of what is currently known about insulin signaling pathways related to the translocation of GLUT4 in adipose cells.

Since insulin receptor autophosphorylation and enhancement of RTK activity are among the earliest known events in insulin signaling, one might predict that RTK activity is necessary for most, if not all, biological actions of insulin including metabolic actions such as recruitment of GLUT4 to the cell surface. This idea was supported by the identification of kinase-deficient insulin receptor mutants in some patients with syndromes of extreme insulin resistance *(86)*. Direct evidence that insulin RTK activity is important for mediating the effect of insulin to stimulate translocation of GLUT4 in insulin target cells has been obtained using transfected rat adipose cells in primary culture (Fig. 3) *(70)*. Cells overexpressing wild-type insulin receptors showed a marked increase in cell surface GLUT4 in the absence of insulin when compared with control cells transfected with an empty expression vector. In contrast, cells overexpressing a kinase-deficient mutant insulin receptor had an insulin dose-response curve similar to that of the control cells. Taken together, these data suggest that intact RTK activity is necessary to mediate signaling from the insulin receptor to translocation of GLUT4. Furthermore, it is likely that unoccupied insulin receptors have a low level of intrinsic tyrosine kinase activity whose signal is proportional to the amount of receptors expressed. Additional evidence that the insulin RTK is important in metabolic signaling comes from studies on PTPases such as LAR and PTP1B that are known to dephosphorylate the insulin receptor. Both of these PTPases have been implicated in the negative regulation of metabolic signaling by insulin *(39,41,43,45–47,49)*. In particular, overexpression of PTP1B in rat adipose cells leads to a significant decrease in the level of GLUT4 at the cell surface in both the absense and presence of insulin *(41)*. The fact that PTP1B decreases cell surface GLUT4 in the absence of insulin provides further support for the idea that a small signal is generated by the intrinsic tyrosine kinase activity of unoccupied receptors.

Downstream from the insulin RTK, a number of insulin receptor substrates play roles in insulin-stimulated translocation of GLUT4. Overexpression of IRS-1 in transfected rat adipose cells leads to an increase in cell-surface GLUT4 in the absence of insulin similar to that seen with overexpression of the insulin receptor *(71)*. Interestingly, transfection of adipose cells with an antisense ribozyme against IRS-1 results in a decrease in insulin sensitivity without a decrease in maximal responsiveness with respect to translocation of GLUT4 *(71)*. Thus, although IRS-1 is capable of mediating the effect of insulin to stimulate translocation of GLUT4, other parallel pathways are probably involved. Indeed, the fact that transgenic IRS-1 knockout mice are only mildly insulin resistant provides unequivocal evidence that IRS-1 contributes to metabolic actions of insulin but is not absolutely required for insulin-stimulated glucose uptake *(76,77)*. Overexpression of IRS-2 *(74)* and IRS-3 *(143)* in rat adipose cells also leads to translocation of GLUT4 in the absence of insulin, suggesting that these substrates may also contribute to metabolic actions of insulin. Of note, in adipose cells, the time course for the association of IRS-3 with the p85 regulatory subunit of PI3K in response to insulin stimulation is much more rapid than for IRS-1 *(87)*. In addition, the magnitude of the association between IRS-3 and p85 in response to insulin seems to be greater than for IRS-1. Furthermore, in transgenic mice lacking IRS-1, IRS-3 is the insulin

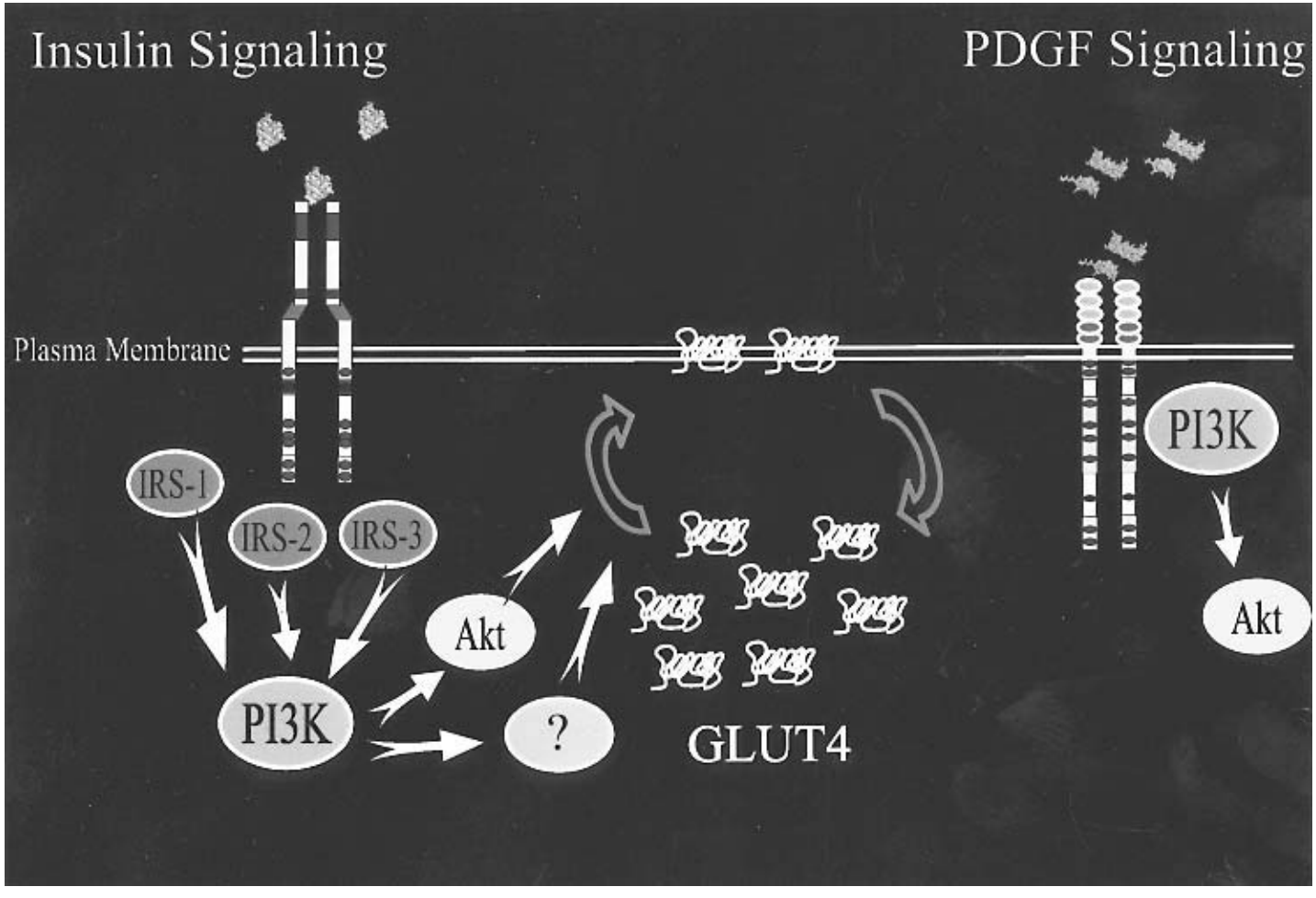

Fig. 2. Insulin signaling pathways that contribute to translocation of GLUT4 in adipose cells. Interestingly, although activation of PI3K is necessary for insulin-stimulated translocation of GLUT4, it does not appear to be sufficient because activation of PI3K by PDGF is without effect on translocation of GLUT4 when PDGF receptors are expressed at physiological levels.

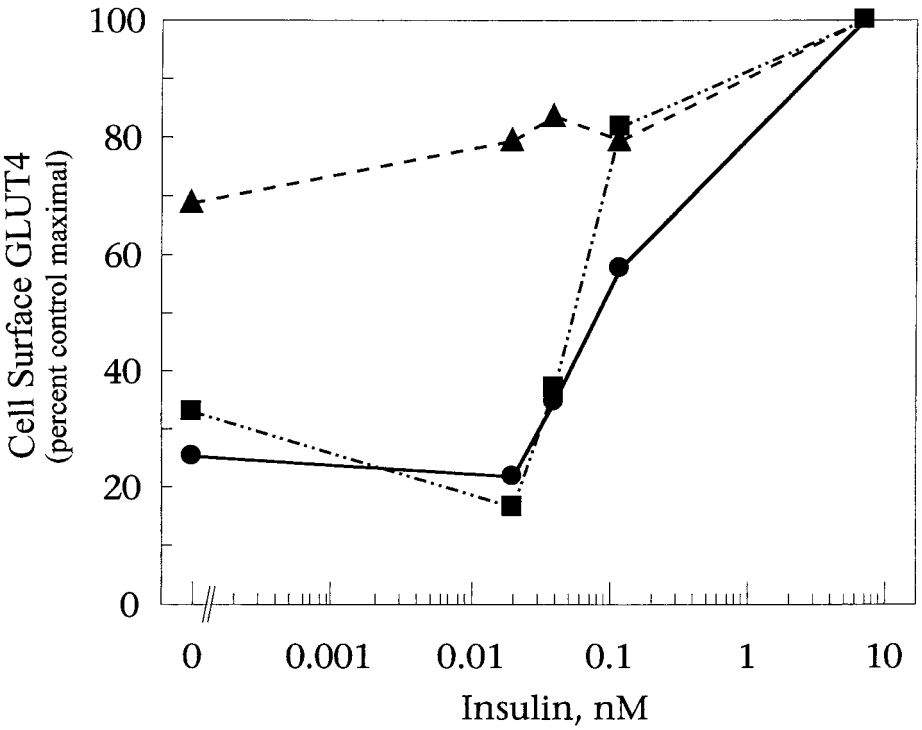

Fig. 3. Insulin RTK activity is important for insulin-stimulated translocation of GLUT4. Rat adipose cells were cotransfected with an epitope-tagged GLUT4 and wild-type human insulin receptors (▲), tyrosine kinase–deficient mutant insulin receptors cells (■), or an empty expression vector (control) (●). Cell-surface concentrations of epitope-tagged GLUT4 are shown as a function of insulin concentration (expressed as a percentage of the maximally stimulated control cells) *(70)*.

receptor substrate in adipose cells responsible for the majority of activation of PI3K in response to insulin *(87,88)*. Since PI3K is necessary for insulin-stimulated glucose transport (*see* the next paragraph), these data suggest that IRS-3 may be a major insulin receptor substrate mediating metabolic actions in vivo.

As already mentioned, two major insulin signaling pathways downstream from the receptor substrates are the PI3K- and the Ras-dependent pathways. Overexpression of constitutively active mutants of either PI3K or Ras in adipose cells leads to massive recruitment of GLUT4 to the cell surface in the absence of insulin *(72,89)*. However, overexpression of recombinant proteins can sometimes lead to effects that do not occur under physiological conditions. Interestingly, when dominant inhibitory mutants were used to knock out either endogenous PI3K or Ras in adipose cells, overexpression of the PI3K mutant resulted in a nearly complete inhibition of insulin-stimulated translocation of GLUT4 (Fig. 4) whereas overexpression of the Ras mutant did not cause a significant change in the insulin dose-response curve *(72)*. Thus, even though constitutively active PI3K and Ras are both capable of stimulating the recruitment of GLUT4 to the cell surface, it appears that only PI3K plays a necessary physiological role in this process. However, PI3K activity per se is not sufficient to cause translocation of GLUT4 because stimulation of PI3K activity in adipose cells using other growth factors such as PDGF does not result in translocation of GLUT4 *(73,90)*.

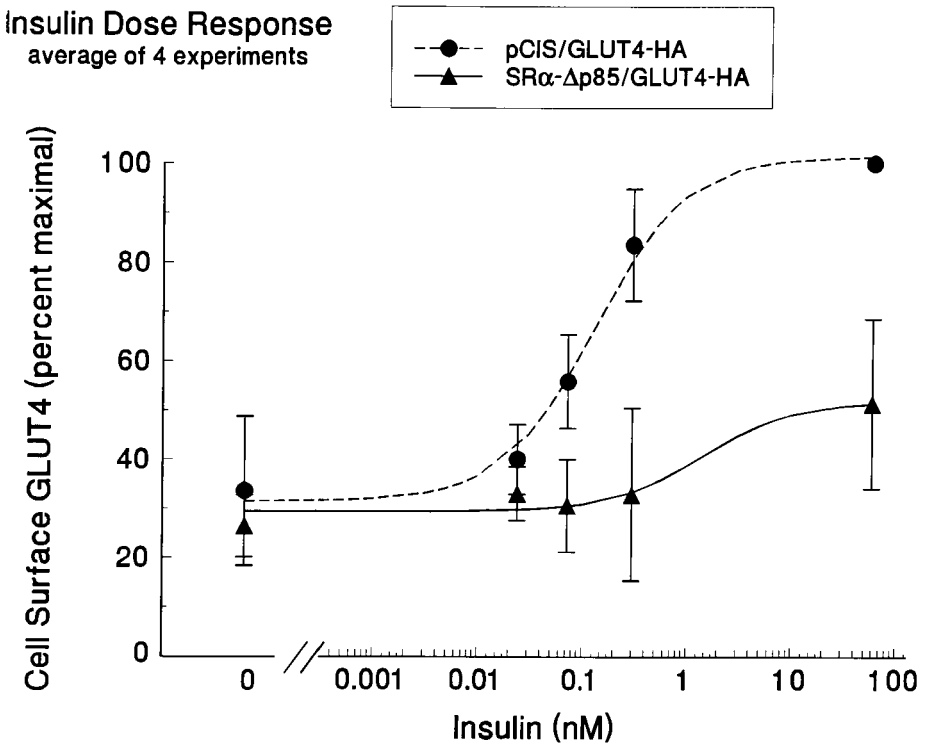

Fig. 4. PI3K is necessary for insulin-stimulated translocation of GLUT4. Rat adipose cells were cotransfected with an epitope-tagged GLUT4 and either a dominant inhibitory mutant of the p85 regulatory subunit of PI3K (▲) or an empty expression vector (control) (●). Cell-surface concentrations of epitope-tagged GLUT4 are shown as a function of insulin concentration (expressed as a percentage of the maximally stimulated control cells) *(72)*. Data represent insulin dose response for an average of four experiments.

There are several effectors downstream of PI3K that may play a role in insulin-stimulated translocation of GLUT4. Akt is a serine/threonine kinase that is activated by insulin via lipid products of PI3K binding to the PH domain of Akt, and phosphorylation of critical serine and threonine residues on Akt by phosphoinositide-dependent kinase-1, another kinase downstream of PI3K that is activated by lipid products of PI3K *(34,91,92)*. Like PI3K and Ras, overexpression of constitutively active mutants of Akt in rat adipose cells or 3T3-L1 adipocytes leads to massive recruitment of GLUT4 to the cell surface *(75,84)*. However, in contrast to PI3K, dominant inhibitory mutants of Akt that are kinase deficient only partially inhibit insulin-stimulated translocation of GLUT4 in adipose cells (Fig. 5). This suggests the possibility that multiple downstream effectors of PI3K contribute to mediating the translocation of GLUT4. For example, the atypical PKC isoform PKC-ζ is a good candidate for another downstream effector of PI3K that may contribute to metabolic signaling by insulin. In 3T3-L1 adipocytes, overexpression of a constitutively active PKC-ζ mutant increased glucose transport whereas overexpression of a dominant inhibitory PKC-ζ mutant decreased insulin-stimulated glucose transport *(93)*.

In addition to the progress being made by tracing signaling pathways starting from

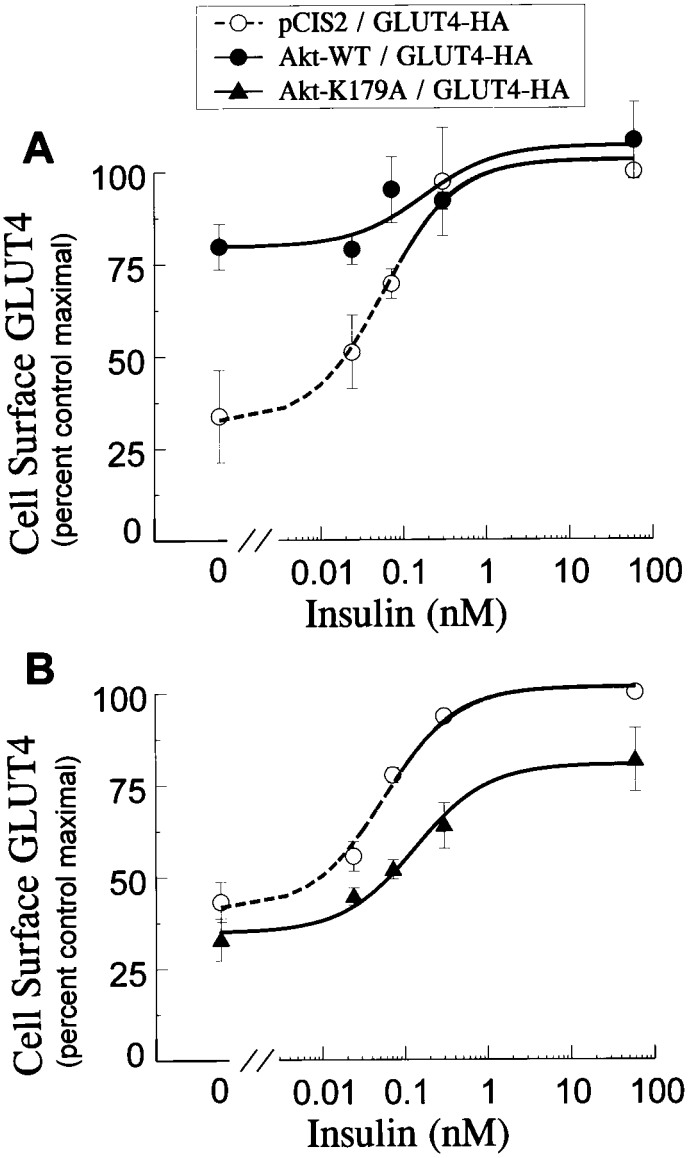

Fig. 5. Physiological role for Akt in insulin-stimulated translocation of GLUT4. Rat adipose cells were cotransfected with an epitope-tagged GLUT4 and wild-type Akt (●), a kinase inactive mutant Akt (▲), or an empty expression vector (control) (○). Cell-surface concentrations of epitope-tagged GLUT4 are shown as a function of insulin concentration (expressed as a percentage of the maximally stimulated control cells) *(75)*.

the insulin receptor, progress has also been made in understanding the molecular mechanisms underlying the subcellular trafficking of GLUT4 from an intracellular compartment to the cell surface in response to insulin. Mechanisms common to vesicular trafficking during regulated exocytosis of synaptic vesicles in neurons also apply to the subcellular localization of GLUT4. In general, vesicle docking and fusion to the plasma membrane is mediated by specific interactions of soluble *N*-ethylmaleimide-

sensitive factor attachment protein receptors (SNAREs) *(94)*. v-SNARE proteins are localized to the vesicle membrane and t-SNARE proteins are localized to the target plasma membrane. There is good evidence that specific isoforms of v-SNARE and t-SNARE molecules are involved with GLUT4 trafficking (for a review see ref. *95*). For example, VAMP2 (a v-SNARE) is localized to GLUT4-containing vesicles in adipose cells and appears to participate in insulin-stimulated exocytosis of GLUT4 *(96–98)*. Syntaxin 4 (a t-SNARE) binds specifically to VAMP2 and is localized to the plasma membrane in muscle and adipose cells. Furthermore, insulin-stimulated translocation of GLUT4 in 3T3-L1 adipocytes can be blocked by using antibodies against Syntaxin 4 or overexpressing the cytoplasmic tail of Syntaxin 4 *(98–100)*. Presumably, the signaling pathways leading from the insulin receptor interface at some point with the vesicular trafficking machinery for GLUT4. An important goal of current investigations related to metabolic actions of insulin is to identify and characterize direct interactions between signaling proteins and trafficking machinery.

MECHANISMS FOR ACHIEVING SPECIFICITY

Although insulin signaling follows a general paradigm for signaling by RTKs and many downstream signaling components are shared in common with other RTK signaling pathways, the biological actions resulting from insulin stimulation such as increased glucose transport are quite specific and distinctive. At each step in the signal transduction pathway, there are opportunities and potential mechanisms for incorporating signal specificity. Here we briefly discuss selected examples that illustrate potential mechanisms that may be used to achieve specificity in insulin signaling.

Specificity at the Receptor Level

The binding affinity between insulin and its receptor is quite high and provides an obvious first determinant of signal specificity. However, insulin is also capable of binding and activating other related receptors such as the insulin-like growth factor-1 (IGF-1) receptor. Similarly, IGF-1 is capable of binding and activating the insulin receptor *(101)*. Furthermore, because the insulin receptor and IGF-1 receptor are homologous, the formation of hybrid receptors with an insulin receptor αβ-subunit joined to an IGF-1 αβ-subunit can occur. These hybrid receptors are capable of undergoing transphosphorylation and may generate unique signals. Since the relative amounts of insulin receptors and IGF-1 receptors differ in particular tissues, the numbers of pure receptors and hybrid receptors may vary from tissue to tissue. Although the binding affinities of insulin and IGF-1 for the heterologous receptor are approx 100-fold less than for their own receptor, the integration of multiple signals at different amplitudes may contribute to the determination of specific effects. For example, in vascular endothelial cells that normally express 10 times as many IGF-1 receptors as insulin receptors, stimulation with insulin at concentrations sufficient to saturate both IGF-1 and insulin receptors results in the production of nitric oxide at a level twice that seen with stimulation by IGF-1 at comparable concentrations *(102)*. Additional evidence that the binding interaction between ligand and receptor affects signaling specificity comes from studies with point mutants of insulin molecules that have been designed to have higher binding affinities for the insulin receptor than the native insulin molecule. For

example, the Asp B10 insulin mutant has a much higher binding affinity than native insulin for the insulin receptor and appears to favor mitogenic rather than metabolic actions of insulin *(103)*. Another feature of insulin binding to its receptor that may affect signal specificity is the fact that insulin binding exhibits negative cooperativity *(104)*. That is, the binding affinity of insulin for its receptor decreases with increasing insulin concentrations. Thus, the dynamics of intracellular signaling events in response to a particular insulin secretory profile may encode some specificity. Finally, integration of signals generated by cross talk between different types of receptors may contribute to the specificity of insulin signaling. For example, in addition to the well-known cross talk that occurs between insulin and IGF-1 at the receptor level, there is evidence for cross talk between insulin and PDGF signaling with respect to interactions between IRS-1 and PI3K *(105)*. Furthermore, recent evidence suggests that activation of G-protein-coupled receptors such as the angiotensin II receptor can influence insulin signaling through interactions with IRS-1 and -2 *(106–108)*.

Specificity at the Receptor Substrate Level

The existence of multiple substrates of the insulin receptor also provides opportunities to incorporate specificity. Members of the IRS family of substrates contain multiple phosphotyrosine docking sites for SH2 domain–containing proteins. The number of these docking sites and the particular SH2 domains with which they interact vary among the different IRS proteins. That is, the combination of downstream signaling molecules engaged by an IRS protein as well as the relative affinities of particular downstream effectors for each substrate are unique for each IRS protein. Thus, tissue-specific differences in the relative expression levels of these IRS substrates may result in formation of distinct signalling complexes in particular tissues and help explain why some actions of insulin predominate in certain tissues *(66,109)*. In addition, in some downstream signaling molecules containing tandem SH2 domains (e.g., SHP-2 and the p85 regulatory subunit of PI3K), the spatial relationship between these SH2 domains provides an additional level of specificity. That is, the geometry of multiple phosphotyrosine motifs on a particular substrate is important for optimal binding and activation of proteins with tandem SH2 domains *(110,111)*. Similarly, the relationship of SH2 and SH3 domains in various adaptor or effector molecules may impose physical constraints on the formation of signaling complexes that are important for signal specificity.

In addition to members of the IRS family, there are other substrates of the insulin receptor that are also expressed in a tissue-specific manner and may contribute to specificity in insulin signaling. For example, there is a family of M_r 120,000 integral membrane glycoproteins that are phosphorylated by the insulin receptor. pp120/HA4 was the first member of this family to be identified as a substrate for the insulin receptor *(112–116)*. Based on the sequence flanking the tyrosine phosphorylation site in pp120/HA4, Najjar et al. *(117)* predicted that the protein would bind to the SH2 domain of SH2-containing phosphotyrosine phosphatases. Subsequently, two other laboratories identified two homologous glycoproteins (SHP substrate-1 *[118]* and signal-regulatory protein [SIRP] *[119]*) that were phosphorylated by the insulin receptor and other tyrosine kinases. Furthermore, the phosphorylated proteins did indeed bind to SHP-1 and SHP-2, and served as substrates for these two phosphotyrosine phosphatases. Moreover, SIRP was demonstrated to exert an inhibitory effect on signaling through RTKs.

Subcellular Compartmentalization of Signaling Complexes

Signal specificity may also be determined by localization of signaling complexes to particular subcellular compartments. For example, in adipose cells, there is evidence that insulin stimulation results in the localization of IRS-1/PI3K complexes to GLUT4-containing vesicles *(120)*. The subcellular targeting of PI3K by insulin may help explain why activation of PI3K by insulin results in translocation of GLUT4 but similar activation of PI3K by PDGF does not *(73,90)*. The fact that PDGF stimulation of adipose cells overexpressing PDGF receptors results in translocation of GLUT4 is consistent with the idea that overexpression of proteins may lead to aberrant localization of signaling molecules in compartments where they would normally be excluded *(73)*.

Another subcellular compartment that may contribute importantly to organizing microdomains of signaling complexes are caveolae (small invaginations in the plasma membrane that contain scaffold-like proteins such as caveolins) *(121,122)*. Caveolae are quite abundant in terminally differentiated cell types such as muscle, endothelial, and adipose cells. Furthermore, growth factor receptors such as PDGF and EGF as well as other signaling proteins such as Ras, MAPK, phosphoinositides, G-proteins, calmodulin, and nitric oxide synthase have all been localized to caveolae (some of these interact directly with caveolin) *(123–126)*. Recently it was shown that all the necessary factors for PDGF-stimulated MAP kinase activation (including the PDGF receptor, Ras, Raf1, Mek1, and Erk2) are localized and functionally active in caveolae *(123)*. Interestingly, in 3T3-L1 cells, insulin stimulates the phosphorylation of caveolin only when the cells are differentiated into adipocytes, but not in the fibroblast form *(127)*. Furthermore, in endothelial cells, the interaction of caveolin with nitric oxide synthase is modulated by tyrosine phosphorylation *(128,129)*. Therefore, it is conceivable that some of the specificity in insulin signaling is determined by the organization of signaling complexes in caveolae or other similar subcellular compartments.

Tissue-Specific Expression of Key Effectors

Specific biological responses to insulin may also be determined, in part, by tissue-specific expression of signaling and effector molecules that are necessary for particular actions of insulin. For example, in the case of insulin-stimulated glucose transport, GLUT4 is the major insulin-responsive glucose transporter that is recruited to the cell surface in response to insulin. Since GLUT4 is predominantly expressed in skeletal muscle and adipose tissue, the effect of insulin to increase glucose transport occurs mostly in these tissues. However, transfecting other cell types (e.g., fibroblasts) with GLUT4 and insulin receptors is not sufficient to make cells as responsive to insulin with respect to glucose transport as classical insulin target cells. Thus, there are presumably additional tissue-specific signaling elements important for insulin-stimulated glucose transport besides the insulin receptor and GLUT4.

Another example of the importance of tissue-specific expression of key effectors is demonstrated by the recent finding that caveolin is tyrosine phosphorylated in response to insulin stimulation only in differentiated 3T3-L1 adipocytes, not in undifferentiated 3T3-L1 fibroblasts *(127)*. The phosphorylation of caveolin can be mediated by the kinase fyn, which is thought to be activated by the binding of phosphorylated c-*cbl* in response to insulin stimulation. Interestingly, although the insulin receptor, c-*cbl*, fyn,

and caveolin are all expressed in both 3T3-L1 fibroblasts and adipocytes, insulin stimulation results in phosphorylation of c-*cbl* only in the differentiated 3T3-L1 adipocyte *(130)*. This implies that the kinase responsible for phosphorylation of c-*cbl* in response to insulin (or some other upstream component) is expressed only in the adipocyte, not the fibroblast form of 3T3-L1 cells, and may explain why caveolin is phosphorylated in response to insulin only in adipocytes.

The existence of multiple isoforms of key signaling molecules may also be important for signal specificity. For example, PI3K is essential for insulin-stimulated glucose transport. However, multiple isoforms and splice variants of both the regulatory p85 and catalytic p110 subunits of PI3K that have differential responses to insulin have been discovered *(131–137)*. Each of these isoforms may generate a distinct pattern of lipid products that have specific roles in signaling. The lipid products of PI3K are known to bind to PH domains of downstream effectors, resulting in activation or regulation of these PH domain–containing molecules. Recently, different lipid products of PI3K were shown to have differential binding affinities for particular PH domains from various signaling molecules *(138)*. Thus, the combination of different isoforms of regulatory and catalytic subunits of PI3K in conjunction with tissue-specific expression and localization to subcellular compartments may result in the generation of a particular profile of lipid products that interact in specific ways with downstream effectors that determines the biological response to insulin stimulation.

Feedback Regulation

The function of end products to dampen signals from one pathway while amplifying signals from others is a common mechanism used in the regulation of enzymatic pathways. It is possible that specificity in RTK signal transduction is also determined, in part, by positive or negative feedback. In the case of insulin signaling, it was recently shown that GSK-3 (a downstream metabolic effector of insulin inactivated by Akt) can phosphorylate IRS-1 on serine/threonine residues and inhibit insulin RTK activity *(139)*. Similarly, PI3K (downstream from IRS-1) has serine/threonine kinase activity in addition to its lipid kinase activity and phosphorylates IRS-1 on serine residues, which may result in modulation of IRS-1 function *(140)*. In addition, there is evidence that PI3K has functional interactions both upstream and downstream from Ras, suggesting another feedback loop that may be involved with insulin signaling *(141,142)*.

Modulation of Signal Frequency and Amplitude

Cellular signals generated by changes in ion fluxes or membrane potential often encode information in the modulation of the signal frequency and amplitude. It is conceivable that the dynamics of signaling by RTKs also encode specific information by modulation of the frequency and amplitude of various phosphorylation cascades. For example, it was recently shown that the time course for association between PI3K and IRS-3 in rat adipose cells in response to insulin stimulation is faster than for IRS-1 *(87)*. Furthermore, in the same study, the amount of PI3K associated with IRS-3 in response to insulin stimulation was also greater than for IRS-1. This difference in the time course and amplitude of PI3K activation may help distinguish signals that are mediated by IRS-1 from those by IRS-3 and ultimately result in different biological effects.

SUMMARY

The molecular mechanisms of insulin action follow a general paradigm for RTK signal transduction. As a result, significant progress has been made in recent years to elucidate the insulin signaling pathways involved with the promotion of glucose uptake and metabolism, one of the most distinctive and important biological actions of insulin. A fundamental challenge for future investigations is to understand how specific biological actions of insulin are determined using signaling molecules that are common to signaling pathways used by many other growth factors and cytokines. Convergence and divergence of multiple branching pathways, subcellular compartmentalization, tissue-specific expression of key effectors, and modulation of signal frequency and amplitude are among the potential mechanisms underlying specificity in insulin signaling.

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3 Ets Transcription Factors

Nuclear Integrators of Signaling Pathways Regulating Endocrine Gene Expression and Carcinogenesis

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CONTENTS

INTRODUCTION

Structure of the Ets Family

The Ets transcription factors represents a novel structural class of transacting proteins that have important roles in development, proliferation, and differentiation of cells and in oncogenic transformation *(1–3)*. At least 40 different Ets sequences have been identified in both vertebrates and invertebrates, representing approx 25 distinct proteins. The Ets gene family has not been detected in plants, fungi, or protozoans *(4)*. The founding member of the Ets family, v-Ets, was discovered some 15 yr ago as a fusion protein with gag and myb in the avian erythroblastosis retrovirus E26 (Ets = E twenty-six specific) *(5,6)*. The family is defined by the highly conserved Ets domain, comprising 84–90 amino acids folded into a novel DNA binding structure, consisting of three alpha helices and a four-stranded antiparallel beta sheet forming a winged helix-turn-helix motif *(7)*. The most highly conserved residues are those located in the hydrophobic core or invariant arginines and lysines that make contacts with specific bases or the phosphate backbone, respectively *(8)*. More divergent sequences are present in loops and turns and are thought to confer binding specificity *(4)*. The DNA binding activity of several Ets factors (Ets-1, Ets-2, SAP-1, Elk, Net, and ERM) is also subject to intramolecular autoinhibition by sequences flanking the Ets domain *(2,4)*. Homologies

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within the Ets domains and their localization divide the Ets superfamily into related classes, as shown in Table 1.

DNA Binding and Transcriptional Regulation

All Ets proteins, with the exception of the dimeric GABP, bind as monomers to DNA sequences containing a core 5′ GGAA/T 3′ motif *(2,4,9)*. Ets factors typically contact 9–15 bases of DNA, and flanking nucleotides, although similar, may dictate binding specificity and affinity of a given Ets binding site (EBS) *(4)* (Table 2). Transcriptional activation domains (TADs) of Ets proteins exhibit no overall sequence homology, but are somewhat related within a given class. Ets factors may contain multiple TADs with proline-glutamine-rich sequences or acidic regions typical of transacting proteins *(1,4,10)*. Certain Ets proteins (e.g., Net/Erp/Sap-2 and ERF) can also act as transcriptional repressors and may function as antagonists of activating Ets factors at a given promoter element *(11–13)*.

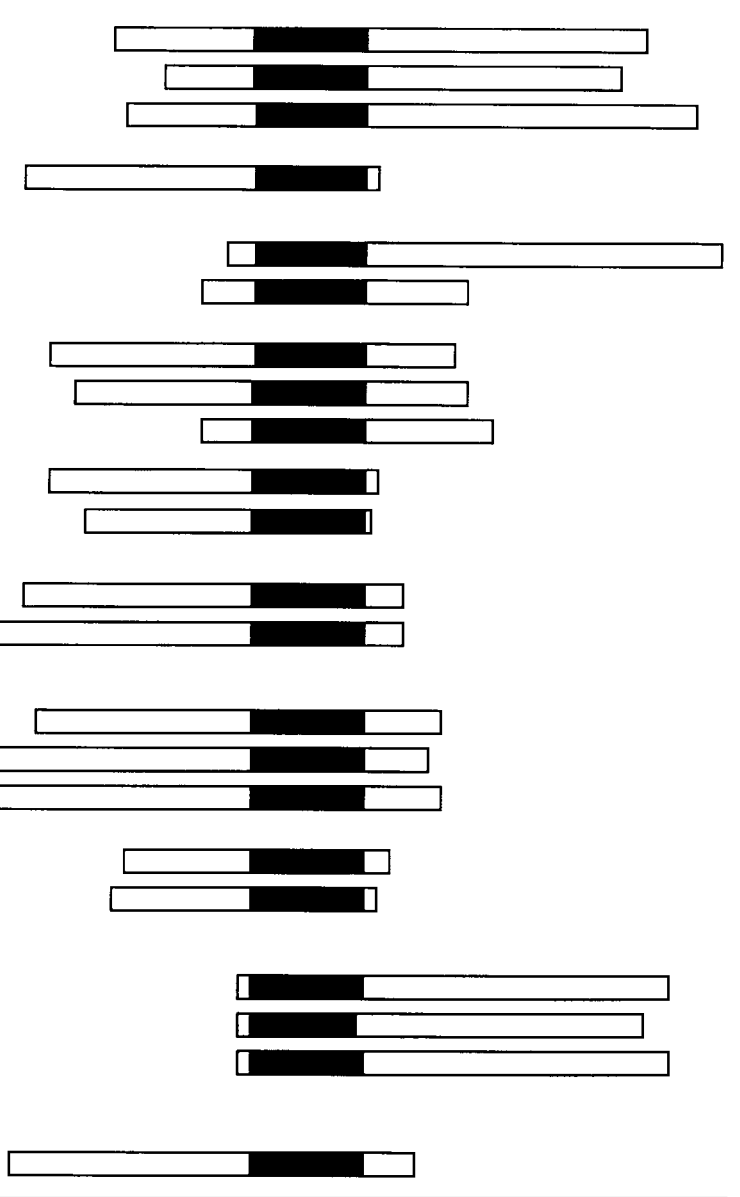
Combinatorial interactions between complex arrays of transcription factors are a central theme in the regulation of gene transcription *(1,2,14)*. The activity of a given promoter is modulated by both protein:DNA and protein:protein interactions that dictate the ability of factors to bind to regulatory elements. Ets family members typically act in concert with other transcription factor partner proteins, resulting in cooperative interactions at composite DNA elements and synergistic transcriptional responses *(1,2,10,14)*. Thus, Ets factors and their partners are often recruited to bipartite binding sites, forming ternary complexes *(14)*. Perhaps the best-known example is that of the Ets protein Elk-1 *(15)*, which is recruited by the serum response factor (SRF) to the serum response element (SRE), present in the promoters of many early response genes *(16)*. Such functional interactions permit highly specific regulation of diverse target genes, beyond the scope of those recognized by either Ets or the partner proteins alone. Additional selectivity may be conferred by differential expression of Ets members and coactivators (or repressors), which may be ubiquitous or tissue specific. Finally, Ets factors of the Ets-1 and TCF subfamily are targets of the growth factor/Ras/mitogen-activated protein kinase (MAPK) signaling cascades. Phosphorylation of these Ets members modulates their transactivation potency and/or cooperative interactions with coactivators *(2,3,17,18)*. Ets transcription factors and their partners have been shown to act as signal integrators, providing molecular mechanisms by which ubiquitous signal transduction pathways can be interpreted in a cell-type-specific fashion *(19,20)*. Thus, Ets transcription factors contain multiple structural and functional domains involved in functional and physical interactions that modulate DNA binding and transcriptional activation or repression. Furthermore, combinatorial interactions of Ets proteins with coactivators (or repressors) and the modification of Ets factors by phosphorylation, in response to extracellular signals, can both enhance specificity and diversity of gene regulation.

THE ROLE OF ETS FACTORS IN ENDOCRINE GENE REGULATION

Recent evidence has implicated Ets family members in the control of neuroendocrine gene expression and its regulation by hormones and growth factors as well as in the development and progression of endocrine-related tumors. In this chapter, we discuss

Table 1
The Ets Gene Family

Subfamily	*Member*	ETS Domain
ELF	Elf-1	
	NERF	
	MEF	
ELG	GABPα	
ERF	ERF	
	PE-1	
ERG	Erg	
	Fli-1	
	FEV	
ESX	ESX (ESE-1)	
	Ehf	
ETS	Ets-1	
	Ets-2	
PEA3	ER81	
	ERM	
	E1AF	
SPI	PU.1 (Spi-1)	
	Spi-B(Spi-2)	
TCFs	Elk-1	
	Net (ERP/SAP-2)	
	SAP-1	
YAN	TEL	



[a]Adapted from refs. *1*, *4*, and *134*.

Table 2
Consensus DNA Binding Sites of Ets Subfamilies[a]

Subfamily	*Consensus binding sequence*	*Reference*
ELF	AACCA**GGA**AGT T C	*199*
ELG	GCC**GGA**AGTN	*200*
ERG	TNGACC**GGA**AGTA A G	*204*
ETS	ACC**GGA**AGCN T T	*201*
PEA3	GGC**GGA**AGTN T	*200*
SPI	AAAAAGA**GGA**AGTA C G G T	*202*
TCFs	ACC**GGA**AGTG A	*203*

[a]Optimal EBSs were selected by random sequences. The conserved core GGA element is shown in bold. Ets subfamilies are as in Table 1. Adapted from ref. *4*.

the role of Ets factors in tissue-specific endocrine gene regulation and the evidence for involvement of Ets proteins in normal and pathological endocrine functions.

Pituitary and Hypothalamus

Several members of the Ets family of transcription factors have been shown to be expressed in the hypothalamus and pituitary during embryogenesis and development in the rat and mouse. The PEA3 group, ERM, ER81, and PEA3, is present in murine endothelial cells during elongation of Rathkes pouch, from which the anterior pituitary is derived, and in adjacent neuroepithelium and cephalic mesenchyme. These Ets proteins may play a role in mediating tissue interactions or differentiation during pituitary/hypothalamic organogenesis *(21)*. c-Ets-1 is also transiently expressed during development of the rat hypothalamic-hypophyseal system, primarily in endothelial cells, and is implicated in vascularization and angiogenesis *(22)*. Other Ets factors detected in pituitary cell lines and/or tumors include GABP *(23)*, Ets-1 or a related factor *(24)*, and the recently described Ehf *(25)*.

Regulation of Prolactin Gene Expression

Study of the role of Ets factors in the regulation of endocrine tissue–specific gene expression has focused primarily on the prolactin (PRL) gene. Members of the Ets family, acting in concert with the pituitary-specific POU homeodomain transcription factor GHF-1/Pit-1 *(26)*, have been implicated in both basal activity and hormone/growth factor stimulation of the PRL promoter *(20,24,27–29)*. PRL gene expression is highly restricted to the somatomammotroph and lactotroph cells of the anterior pituitary and is subject to regulation by a variety of hormones and second messengers *(30,31)*. The rat (r) PRL promoter comprises a distal enhancer (−1710 to −1550), containing an estrogen response element, and a proximal region (–425) promoter

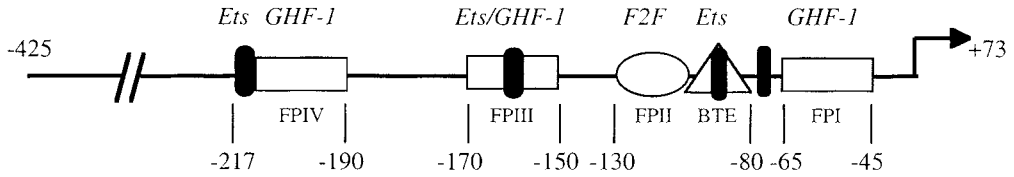


Fig. 1. Structure of the proximal rat PRL promoter. The region between nucleotides –425 and +73 is depicted. Shaded rectangles, GHF-1 binding sites (FPI, FPIII, and FPIV), determined by DNAseI footprinting; solid ovals, Ets binding sites; circle and triangle, the FPII repressor site and the BTE respectively.

(Fig. 1), which is sufficient to confer tissue-specific expression and impart both positive and negative hormonal regulation *(30–34)*.

PRL, growth hormone (GH) and thyroid-stimulating hormone (TSH) β gene expression is regulated by growth hormone factor-1 (GHF-1), which also plays a critical role in the development and differentiation of lactotroph, somatotroph, and thyrotroph cells *(35–37)*. In addition, several hormone response elements have been localized to GHF-1-binding sites on PRL, GH, and TSHβ promoters *(26)*. However, since pituitary somatotrophs, lactotrophs, and thyrotrophs all express GHF-1 but each express a distinct, highly specialized peptide hormone gene *(38)*, factors other than GHF-1 must be involved in the regulation of PRL, GH, and TSHβ expression. Thus, the differential control of cell-type-specific pituitary gene promoters is dependent on combinatorial interactions of GHF-1 with other transcription factors.

Ets Factors Are Required for Basal PRL Promoter Activity

A role for Ets transcription factors in the regulation of basal, lactotroph-specific PRL gene expression was first suggested by the observation that overexpression of a dominant negative Ets construct inhibited PRL promoter activity in GH4 cells *(27,28)*. A similar reduction in basal rPRL promoter activity was seen on expression of the alternative splice isoform GHF-2/Pit-1β, which contains a 26 amino acid insertion within the transcription activation domain conferring distinct functional properties *(27,28,39–41)*. To characterize further the role of Ets factors and GHF-1 in the regulation of PRL gene expression, a transient transfection approach was used to reconstitute rPRL promoter activity in a nonpituitary HeLa cell line, which does not express either GHF-1 or Ets-1 *(27,42)*. The activity of the rPRL promoter in HeLa cells is minimal, typically <1% of that observed in GH4 pituitary cells *(43,44)*. Expression of either GHF-1 or Ets-1 alone results in substantial activation (Table 3), but neither restores full PRL promoter activity, implying that each factor is necessary but not sufficient. Consistent with this hypothesis, coexpression of both Ets-1 and GHF-1 synergistically activates the rPRL promoter, fully reconstituting basal activity comparable to that observed in pituitary GH4 cells (Table 3). Moreover, the Ets/GHF-1 response mapped to a composite Ets-1/GHF-1 binding site (–214 to –190) *(27)*. Subsequent in vitro binding assays, using bacterially expressed proteins, demonstrated a direct, DNA-independent, protein:protein interaction between Ets-1 and GHF-1 *(27)*. By contrast, Ets-2, a related but functionally distinct isoform *(10)*, failed to bind to GHF-1 and had no effect on rPRL promoter activity. Consistent with these observations, the functional and physical interactions of Ets-1 and GHF-1 required protein sequences unique to

Table 3
Reconstitution of rPRL Promoter Activity by Ets-1 and GHF-1[a]

HeLa Nonpituitary cells				*GH4 cells*
Basal	*Ets-1*	*GHF-1*	*Ets-1 + GHF-1*	*Basal*
140 ± 15	17,160 ± 1,680	36,379 ± 8,044	352,902 ± 39,032	348,273 ± 33,477

[a]Promoter activity is expressed as total relative light units normalized to β-galactosidase activity *(24)*. Results are mean ± standard deviation of 18 experiments. Vector (pA3luc) gave minimal (20–30) light units over background in both cell lines.

Ets-1 *(27)*. Similarly, the alternatively spliced GHF-2 failed to synergize with Ets-1 in the reconstitution assay but was able to bind to Ets-1 in vitro. This suggests that the inhibitory effects of GHF-2 on the rPRL promoter, in both GH4 and HeLa cells, may be due to sequestration of Ets-1 in an inactive or inhibitory complex *(27)*. The cooperative effects of Ets-1 and GHF-1 were specific for the rPRL promoter since, in analogous experiments, the ancestrally related and homologous rGH promoter did not exhibit synergistic activation *(27)*. Thus, a selective functional and physical interaction of GHF-1 with Ets-1, acting via a composite Ets-GHF DNA element, is both necessary and sufficient to establish optimal lactotroph-specific PRL promoter activity and may serve to specify appropriate terminally differentiated pituitary cell lineages.

Ets Proteins and Growth Factor Regulation of the PRL Gene

Ets transcription factors have also been implicated as critical nuclear targets of hormone/growth factor signal transduction pathways regulating PRL gene expression. Ras, insulin, insulin-like growth factor-1 (IGF-1), and fibroblast growth factor response elements have been mapped to EBSs in the proximal rPRL promoter. Moreover, Ets members are key components of the molecular mechanisms that permit differential tissue- and promoter-specific transcriptional responses to hormones and growth factors *(19,20,23,24,29,45)*.

p21 Ras

Initial studies on p21 Ras focused on the activation of the rPRL promoter by the guanosine 5′-triphosphate-binding protein p21 Ras *(46,47)*. Ras is a critical component of many signaling pathways and functions as a molecular switch *(48,49)* linking receptor tyrosine kinases (RTK) to the MAPKs signaling cascades *(50–52)*. Signals initiated at transmembrane receptors are transduced via Ras and propagated, by a phosphorylation cascade, to the nucleus, resulting in changes in the activity of specific transcription factors *(17,53,54)*. Distinct signaling components of the Ras pathway may be present in different cell types allowing the signal to be interpreted in a cell-specific manner *(55–57)*. Indeed, cell-specific phenotypic sequelae of Ras activation are exemplified by the differential effects of oncogenic Ras in PC12 pheochromocytoma, TT medullary carcinoma, and FRTL5 thyroid cells, which results in terminal differentiation of the first two cell lines but causes transformation of the last *(58–60)*. Thus, characterization of nuclear effectors of the Ras pathway and determination of the molecular mechanisms by which signals elicit cell-specific responses are important questions in gene regulation and transcription factor biology *(61)*.

Expression of activated V-12Ras in GH4 pituitary cells results in a selective activation of the rPRL promoter but has no effect on the ancestrally related rGH promoter *(24,47)*. Ras activation of rPRL transcription was shown to be mediated via Raf and MAPKs and was inhibited by expression of a dominant negative Ets protein *(47)*. These results suggested a role for an Ets factor in mediating the rPRL promoter Ras response, consistent with the role of Ets factors as nuclear acceptors of the Ras signal in other systems *(2,3)*. In support of this hypothesis, overexpression of Ets-1 or GHF-1, in GH4 cells, synergistically enhanced the rPRL promoter Ras response, optimal activation requiring both factors *(20,24)*. By contrast, Ets-2 had no effect on rPRL promoter activity and expression of the alternatively spliced GHF-2 essentially abrogated Ras activation *(20,28)*. Thus, a selective functional interaction of a pituitary-specific transcription factor, GHF-1, with the protooncogene c-Ets-1 provides a mechanism by which the Ras signaling cascade can be interpreted in a cell-type-specific manner *(20,24)*. Several Ets factors have been shown to be nuclear targets of growth factor signaling pathways acting via the MAPK family *(3,18,62–66)*. MAPK phosphorylation can both positively and negatively regulate the transcriptional activity of Ets proteins *(11–13,65,67,68)* and may also regulate DNA binding and association with obligate partner proteins *(8,69,70)*. Consistent with these observations, the domain of Ets-1 required to mediate the rPRL promoter Ras response contains a consensus MAPK phosphorylation site (PLL**T**^{82}P) *(20)* shown to be critical for Ras-induced enhanced transactivation *(65,67)*. Mutation of this MAPK site abrogates the ability of Ets-1 to enhance Ras activation of the rPRL promoter and eliminates Ras-induced transactivation by GAL4Ets fusion proteins *(24,65)*.

Using a series of site-specific and deletion mutations of the rPRL promoter, the Ras response element (RRE) was mapped to an EBS immediately adjacent to the most distal and lowest-affinity binding site for GHF-1 (FPIV) *(20,24)*, forming a composite element located between −217 and −190 (Fig. 1). Mutation of the GHF-1 binding site of the composite RRE results in marked attenuation of Ras-Raf-induced activity whereas site-specific mutation of the EBS essentially abrogates Ras/Raf activation of the rPRL promoter. These results suggested that, while both *cis* elements contribute to Ras and Raf responses of the rPRL promoter, Ets-1 (or a related factor) is the critical nuclear target of the Ras pathway, whereas GHF-1 binding is necessary but not sufficient to mediate Ras-inducible PRL gene transcription *(20,24)*. The composite RRE is identical to the Ets-1/GHF-1 binding site required to reconstitute basal rPRL promoter activity in HeLa cells. However, this basal, synergistic, functional interaction between Ets-1 and GHF-1 is independent of Ras *(27)*. Based on our results, we propose a model for regulation of rPRL transcription via the composite RRE (Fig. 2), whereby interaction of GHF-1 and Ets-1 serves to establish and maintain basal, lactotroph-specific rPRL promoter activity. Ras induction of rPRL transcription is mediated via MAPK phosphorylation of Ets-1 at a conserved threonine residue. Phosphorylation of Ets-1 enhances its transactivation potency and may also modulate DNA binding or interaction with GHF-1. By contrast, GHF-2 forms an inhibitory complex with Ets-1, attenuating both basal- and Ras-induced promoter activity (Fig. 2). Thus, the functional interaction of Ets-1 with the pituitary-specific GHF-1 (or GHF-2) provides a molecular mechanism by which activation of the general Ras signal transduction pathway can be harnessed to mediate transcriptional regulation of a cell-type-specific gene.

A second more proximal rPRL promoter Ets/GHF-1 composite element (−165 to

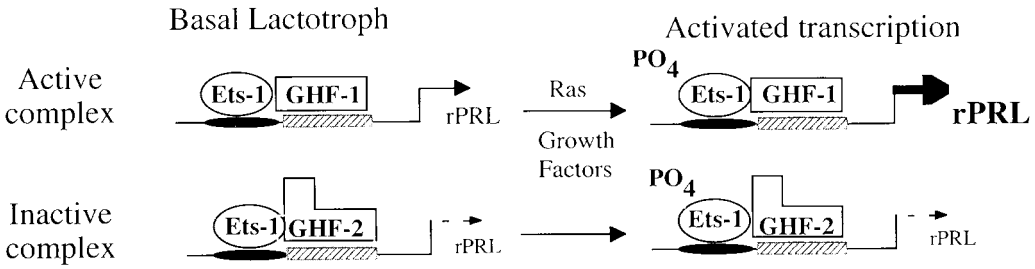


Fig. 2. Model of combinatorial interactions governing PRL gene expression. Interaction of Ets-1 and GHF-1 is necessary to establish basal lactotroph-specific PRL expression. Activation of the Ras signaling pathway leads to MAPK phosphorylation of Ets-1, enhancing its transcription potency and increasing rPRL promoter activity. By contrast, GHF-2 sequesters Ets-1 in an inhibitory complex, attenuating both basal and Ras/MAPK-induced PRL transcription.

−150) has been described (Fig. 1), which is sufficient to confer multihormone and growth factor responses, including Ras, when fused to a minimal heterologous promoter *(29)*. However, mutation or deletion of this element in the context of the intact proximal (−425) rPRL promoter has no effect on the Ras response, suggesting that it may not be physiologically relevant *(24)*. Although composite RREs have been defined in other systems, typically consisting of activator protein-1 (AP-1) or serum response elements in combination with EBSs *(1,3,10,17),* the factors that bind to these sites have been ubiquitously expressed proteins. In the case of the PRL promoter, it is the precise juxtaposition of binding sites for both Ets-1 and the pituitary-specific transcription factor GHF-1 that is necessary for optimal Ras responsiveness. Thus, the rGH promoter, despite being GHF-1 dependent and containing several putative EBSs, is not Ras inducible since the GHF-1 and Ets elements are not found in the appropriate juxtaposition analogous to the rPRL promoter *(20,47)*. Substitution of the distal rGH GHF-1 site with the rPRL composite Ets-1/GHF-1RRE renders the rGH promoter responsive to Ras *(24)*. Hence, the requirement for a tripartite regulatory unit, comprising c-Ets-1, GHF-1, and a composite *cis*-acting DNA element, provides an elegant mechanism by which tissue-specific transcription factors, such as GHF-1, serve as signal integrators for generalized signaling pathways, and by which only a subset of GHF-1-dependent genes are selected to respond to the Ras pathway. In certain respects, this is reminiscent of the SRE in the c-Fos promoter, which requires the interaction of the Ets member, Elk-1, with SRF, in order to achieve a growth factor response *(71,72)*. Thus, in the case of the rPRL gene, GHF-1 appears to function as a "cell-specific SRF" *(24,26)*. Indeed, the GHF-1 binding site within the RRE (FPIV) bears some similarity to the AT-rich palindromic SRE recognition element (CCTAATTAGG) *(73)*. However, expression of the TCF factors Elk or Net in GH4 cells results in a significant inhibition of basal rPRL promoter activity and attenuation of the Ras response. Therefore, unlike Ets-1, neither Elk nor Net is a likely nuclear component of the Ras pathway leading to activation of rPRL transcription *(24)*.

Insulin-Like Growth Factor-1

Recent evidence suggests that the Ras signaling pathway leading to the rPRL promoter is activated by binding of IGF-1 to its cognate receptor *(45)*. Treatment of GH4C1 cells with IGF-1 stimulated rPRL promoter activity in a Ras- and Ets-dependent manner.

However, the IGF response element localized to EBSs between −101 and −76. This apparent discrepancy with previous reports *(20,24)* may reflect alternate utilization of EBSs in truncated promoters *(29)* or may be owing to IGF signaling via multiple pathways both Ras dependent and independent *(45,74)*. Differential regulation of PRL and GH gene expression by IGF-1 and Ras in related pituitary GH4C1 and GH3 rat tumor cell lines has also been documented *(74)*.

Insulin

The rPRL gene is also subject to regulation by insulin, which stimulates promoter activity *(75)* and increases steady-state mRNA levels *(76)*. An insulin response element (IRE) was initially localized in the −106 to −96 region of the rPRL promoter *(77);* however, subsequent analysis implicated more proximal sequences between −97 and −67. This putative IRE overlaps the cyclic adenosine monophosphate (cAMP) response element of the rPRL promoter, but activation of the rPRL promoter by insulin and cAMP are mediated via distinct signaling pathways *(78,79)*. This region of the rPRL promoter contains two EBSs at −96 and −76 (Fig. 1), and expression of a dominant negative Ets construct blocked activation by insulin, suggesting a role for Ets factors in the insulin response *(23)*. IREs containing EBSs have also been identified in the somatostatin and thymidine kinase promoters *(23)*. Further analysis suggested that the Ets factor GABP may mediate insulin induction of the rPRL promoter in response to activation of MAPK *(80)* although the TCFs, Elk-1 and SAP, will also bind to the IRE *(23)*. Moreover, insulin activation of the rPRL promoter was shown to require an intact GHF-1 binding site (−65 to −45) and additional sequences upstream of the EBS at −96 *(78,80),* suggesting that Ets factors may be necessary but not sufficient to mediate insulin stimulation of the PRL gene. Insulin stimulation of PRL transcription was also shown to be independent of Ras *(79)*. However, these studies used a truncated rPRL reporter, lacking a primary RRE, and also required overexpression of the insulin receptor to obtain a response. Thus, the potential role of Ras and the RRE in the rPRL insulin response remains to be determined.

Fibroblast Growth Factors

Fibroblast growth factors (FGFs) are a family of 14 heparin-binding polypeptides that play important roles in growth, differentiation, and development *(81–83)* and have been implicated in the formation and progression of tumors in a variety of issues, including the pituitary *(84–87)*. FGF-2 (basic FGF) and FGF-4 (hst-1) have been shown to activate specifically the rPRL promoter, but not the ancestrally related rGH promoter, in GH4 pituitary cells and to stimulate PRL secretion *(19,86,88)*. This selective activation of the rPRL promoter is reminiscent of the Ras response, in that V-12 Ras activates the rPRL promoter, but not the rGH promoter in GH4 cells *(20,47)*. However, in contrast to other systems *(89–94),* the rPRL FGF response is independent of p21 Ras and is not mediated via Raf-1 kinase *(19)*. Moreover, FGFs fail to stimulate Raf-1 kinase catalytic activity in this system. FGF induction of the rPRL promoter is, however, dependent on MAPK, whose catalytic activity is stimulated by FGFs in GH4 cells *(19)*. Ras-independent signal transduction via tyrosine kinase receptors is not without precedence in the pituitary, since epidermal growth factor (EGF) activation of the rPRL promoter also does not require the Ras/Raf-1 pathway. Indeed, the Ras and EGF pathways are mutually antagonistic *(95,96)*. In addition, Ras-independent activation of

MAPK has been documented in two other pituitary cell systems: thyrotropin-releasing hormone (TRH) signaling in GH3 lactotrophs *(97),* and gonadotropin-releasing hormone signaling in the gonadotrope alpha T3-1 cell line *(98).*

The FGF response of the rPRL promoter maps to two elements (centered at −212 and −96), each of which contain binding sites for members of the Ets family of transcription factors, and of which expression of a dominant-negative Ets factor inhibited FGF induction of the rPRL promoter *(19).* Furthermore, we and others have shown specific binding of Ets factors to both of these elements in vitro *(24,80).* The two FGF response elements (FREs) appear to contribute equally to rPRL promoter activation. Both are required for optimal FGF induction and together represent one of the first examples of physiologically relevant FREs containing EBSs *(19).*

The FRE centered at −212 colocalizes with the composite RRE (discussed previously) that comprises juxtaposed Ets and GHF-1 binding sites (FPIV) (Fig. 1) *(24).* Ras activation of the rPRL promoter is mediated via a functional interaction between Ets-1 and GHF-1 at this element and is enhanced by overexpression of either or both factors *(20).* Mutation of either the EBS or GHF-1 site reduced the FGF response of the rPRL promoter. However, in contrast to Ras activation of rPRL promoter activity, the FGF response was inhibited by overexpression of Ets-1 or GHF-1 *(19).* Thus, an Ets factor(s) and a POU homeodomain protein(s) distinct from Ets-1 and GHF-1, respectively, may mediate the FGF response. The inhibition of FGF activation of the rPRL promoter by Ets-1 and GHF-1 may reflect the formation of nonproductive complexes that block access to the FRE. Moreover, expression of the alternatively spliced isoform GHF-2, which blocks Ras activation of the rPRL promoter *(24),* had no effect on the FGF response *(19).* Taken together, these results imply that, despite targeting a common *cis* element (−207 to −190) in the rPRL promoter, the Ras and FGF pathways utilize distinct nuclear factors to transduce their effects.

The second rPRL promoter FRE (−96) lies within a basal transcription element (BTE) *(43,99)* that has also been implicated in the cAMP, TRH, phorbol ester, and EGF responses *(99–102).* This element is also required for the insulin activation of the rPRL promoter via the Ets factor GABP *(23,80).* The BTE is immediately adjacent to FPII, which binds an unknown factor and exerts a modulatory effect on the BTE to repress PRL transcription in nonpituitary cells *(43).* FPII has also been implicated in EGF activation of the rPRL promoter *(95).* Mutation of either FPII or the EBS within the BTE reduces the rPRL promoter FGF response, suggesting that this region, like the RRE, may also function as a composite response element.

Ets Transcription Factors and Homeodomain Proteins as Nuclear Integrators of Signaling Pathways

Current evidence suggests a central role for members of the Ets family of transcription factors in the regulation of basal lactotroph-specific rPRL gene expression *(27)* and in rPRL promoter regulation in response to Ras *(20,24),* insulin *(80),* and FGFs *(19).* Several Ets factors are nuclear targets of growth factor signaling pathways acting via MAPK *(1,10,18)* and can both positively and negatively regulate transcription *(11,13,20,65,67).* Thus, based on our data, we propose a model by which growth factors can elicit distinct responses, despite acting via common *cis*-acting response elements, by targeting different members of the Ets family (Fig. 3). Moreover, since Ets factors typically act in concert with other transcription factors at composite elements *(1,10,18),*

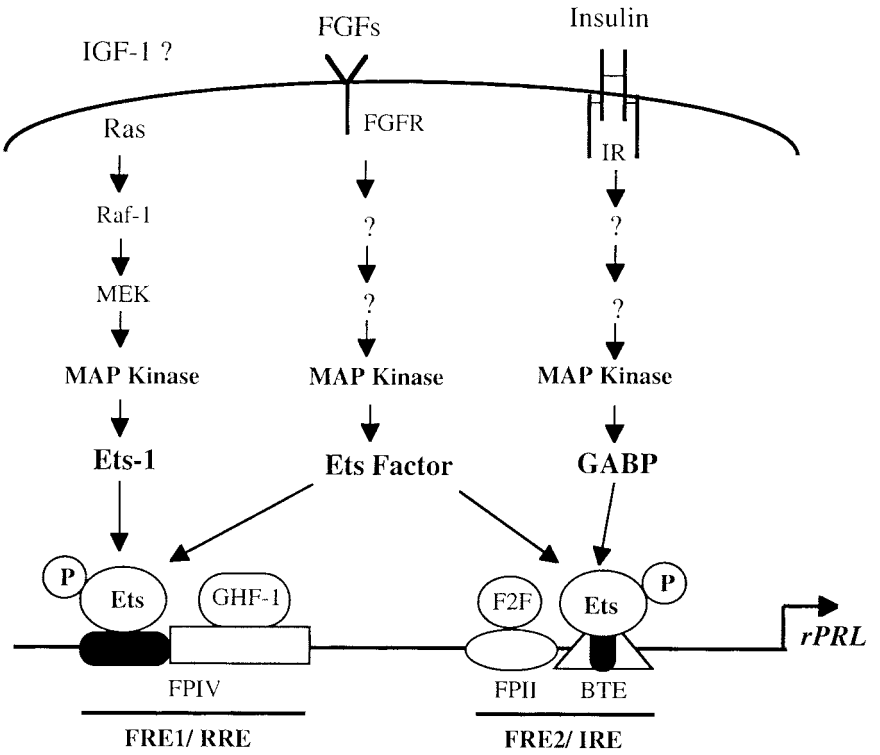


Fig. 3. Integration of PRL promoter growth factor responses by Ets transcription factors. Multiple growth factors have been shown to activate the rPRL promoter via MAPK and Ets transcription factors. FGF induction of PRL transcription requires two composite elements, which have also been identified as RREs IREs. However, despite converging at MAPK, FGFs, Ras, and insulin appear to target distinct Ets factors and partner proteins. The Ras response is mediated by a functional interaction between Ets-1 and GHF-1, activation by insulin requires the Ets factor GABP, and FGF induction of the rPRL promoter is mediated by a yet unidentified Ets protein(s). Thus, different Ets factors serve to coordinate and integrate growth factor signaling pathways, targeting common *cis* elements, to elicit specific transcriptional responses.

further specificity can be conferred by interactions with different coactivators. For example, the rPRL promoter Ras response is mediated via interaction of Ets-1 and the pituitary-specific POU homeodomain protein GHF-1 at the RRE *(20,24),* whereas FGF induction of promoter activity is mediated by distinct Ets members and other cofactors, perhaps homeodomain proteins, that bind to this same composite element (FRE1). Similarly, insulin activation of rPRL promoter activity may be mediated via the Ets factor GABP binding to the BTE *(80).* The FGF response also utilizes this element but may require interactions with coactivators binding at FPII that remain to be identified (Fig. 3). Thus, Ets factors may serve to sort, integrate, and coordinate transcriptional responses to different growth factor signaling pathways, resulting in highly selective regulation of tissue-specific gene expression.

Finally, we *(20,26)* and others *(103)* have proposed a hypothesis whereby homeodomain proteins, such as GHF-1, target signal transduction pathways to selected tissue-specific genes by functionally interacting with a variety of signal-dependent transcription factors, such as Ets-1, AP-1, CRE-binding protein (CREB), thyroid receptor (TR),

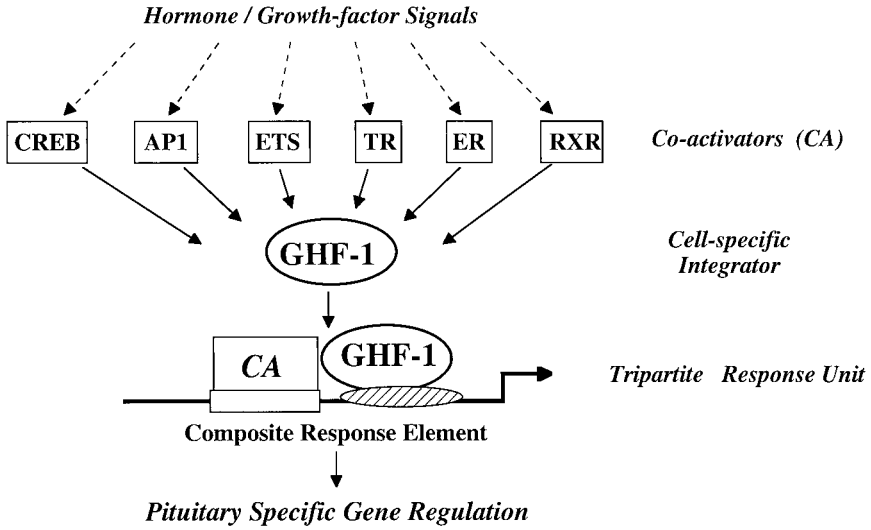


Fig. 4. The homeodomain protein GHF-1 functions as a pituitary-specific signal integrator of hormone/growth factor signals. Extracellular signals are targeted to inducible nuclear coactivators such as CREB, the Jun/Fos family (AP1), Ets transcription factors (ETS), thyroid receptor (TR), estrogen receptor (ER), or retinoid X receptor (RXR). Functional interaction of these signal-dependent coactivators (CA), e.g., Ets-1, with GHF-1, at composite GHF-1/CA DNA binding sites, forms a tripartite transcriptional response unit, which permits highly specific pituitary transcriptional responses to general signaling pathways.

estrogen receptor (ER), or retinoid X receptor, at composite DNA regulatory elements (Fig. 4). Synergistic interactions between GHF-1 and other factors may also determine cell phenotype and regulate proliferation during pituitary organogenesis *(104)*. Several examples of such interactions, in addition to the Ets-1/GHF-1 interaction at the composite RRE, can be found among GHF-1-dependent genes, including GHF-1, GH, PRL, and TSHβ genes. For example, the murine GHF-1 enhancer contains an atypical, cell-specific retinoic acid response element, composed of adjacent GHF-1 and retinoid receptor binding sites, and both GHF-1 and retinoic acid receptor are required to confer retinoid induction of GHF-1 gene transcription *(105)*. Additionally, the coordinate actions of GHF-1 and CREB/ATF-1-related factors, at a cAMP response unit comprising a GHF-1 site flanked by cAMP response elements, are necessary to mediate the effects of cAMP on the human GH gene *(106)*. Similarly, the rGH gene is synergistically activated by GHF-1 and TR via relatively closely spaced DNA binding sites *(107)*, and direct protein-protein interaction between GHF-1 and TR has been demonstrated *(108)*. Cooperation of GHF-1 and ER is required for rPRL distal enhancer activity, and the binding of both factors, at adjacent elements, is required for the estradiol response *(109)*. Finally, an AP-1-like factor functionally cooperates with GHF-1 to mediate forskolin and phorbol-ester activation of the human TSHβ gene *(110)*. In this case, binding sites for GHF-1 and AP-1 are located somewhat farther apart. However, both AP-1 and GHF-1 can induce DNA bending, which may facilitate synergistic interactions *(110)*. Note that in most of these cases, mutation of the GHF-1 binding

site, adjacent to the hormone response element, results in loss of the specific hormonal effect. Thus, in many GHF-1-dependent promoters, the inductive effects of extracellular signals require binding of both GHF-1 and the signal-dependent coactivator at composite DNA binding sites. Specifically, in the context of our model (Fig. 4), we hypothesize that GHF-1 either recruits Ets-1 or stabilizes its binding to the adjacent EBS, and that the actual Ras/Raf response is transduced via a MAPK phosphorylation of c-Ets-1 at threonine 82 *(24,65)*. Hence, GHF-1 functions as a cell-specific integrator of hormonal and growth factor signaling, resulting in distinct patterns of GHF-1-dependent gene expression in pituitary development, differentiation, and proliferation.

Ets Factors in Reproductive Endocrinology

Normal reproductive function is dependent on the secretion of the gonadotropic hormones, luteinizing hormone (LH), and follicle-stimulating hormone (FSH), from the anterior pituitary. These gonadotropins are heterodimeric proteins consisting of a common α-subunit with distinct β-subunits conferring physiological specificity *(111)*. The synthesis and secretion of LH and FSH are differentially regulated by pulsatile release of gonadotropin-releasing hormone (GnRH) from the hypothalamus *(112,113)*. The signal transduction pathways that mediate differential transcriptional responses to GnRH have not been fully defined. GnRH stimulation of the α glycoprotein subunit promoter in α-T3-1 cells is dependent on GnRH-induced activation of MAPK *(114)*, and the DNA sequences required to mediate GnRH responses in the human, mouse, and rat α-subunit genes include EBSs *(114,115)*. Moreover, expression of a dominant negative Ets construct, or mutation of the EBS within the GnRH response element, inhibits GnRH responsiveness. Thus, an Ets factor appears to be a critical nuclear component of GnRH induction of α-subunit transcription mediated via MAPK.

Both Ets-2 and ER81 have been shown to bind to the GnRH response element; however, the Ets member(s) that functionally contribute to α-subunit transcriptional regulation remain to be identified *(114)*. The LHα- and β-subunit genes are differentially regulated by GnRH according to its pulsatile secretion frequency and amplitude *(113)*. Recent evidence indicates that GnRH induction of the α-subunit gene is mediated via protein kinase C and MAPK targeting an Ets factor at the GnRH response element, whereas activation of the LHβ gene is dependent on calcium influx *(114–116)*. The LHβ promoter lacks consensus Ets sites within the GnRH-responsive region, providing a further example of Ets factors playing a critical role in mediating differential transcriptional responses to signal transduction pathways activated by a single ligand/receptor interaction *(115)*.

Ets factors have been implicated in mammalian testicular development and regulation of the spermatogenic pathway. Specifically, Ets response elements may mediate the transcriptional switch between isoforms of the glycolytic enzyme phosphoglycerate kinase (PGK), from the somatic cell type PGK-1 to the testis-specific PGK-2, in spermatocytes during meiosis. An Ets factor designated TAP-1 is thought to inhibit PGK-1 transcription, where stimulating that of PGK-2 *(117–119)*. In addition, the Ets factor Elk-1 may play a role in FSH-mediated differentiation and maintenance of Sertoli cells, which form the seminiferous tubules and establish the microenvironment necessary for spermatogenesis *(120)*. Finally, androgen-induced expression of the PEA3 Ets protein in the epididymis may regulate transcription of the glutathione peroxidase

(GPX) type 5 gene *(121)*. GPX5 is postulated to protect mammalian sperm membranes from the deleterious effects of lipid peroxidation by detoxification of free radicals and reactive oxygen species, which can lead to reduced fertility *(122)*.

Ets proteins have been implicated in the control of mammary cell–specific gene expression *(123)* and activation of milk protein genes, such as the whey acidic protein, both in the developing mammary gland and during pregnancy *(124)*, and may also be important in oogenesis. The Ets-1 gene is expressed in human trophoblast endothelial cells in the first trimester of pregnancy. Regulation of metalloproteinase genes by Ets-1 may be involved in angiogenesis, during development of the villous tree, and may mediate invasion of the endometrium and maternal vessels by trophoblastic cells, which is essential for normal fetal development *(125)*. Ets-1 and Ets-2 are expressed in theca cells of the adult mouse ovary *(126)*, and Ets-2 is required for meiotic maturation of xenopus oocytes *(127)*. Knockout of the TEL Ets gene in mice results in defective yolk sac angiogenesis and regional, intraembryonic apoptosis, suggesting a role for TEL in development and maintenance of the vascular network in the yolk sac and in survival of mesenchymal cells and neuronal tissues *(128)*. Similarly, targeted mutation of Ets-2 is also embryonically lethal and indicates that Ets-2 is essential for placental function by mediating growth factor activation of trophoblast matrix metalloproteinase genes *(129)*. Thus, modulation of key target genes by Ets transcription factors appears to be important in gametogenesis, fertility, pregnancy, and early embryonic development.

Ets Factors in Diabetes

There is no direct evidence linking Ets factors and diabetes. However, genetic analysis, by restriction fragment length polymorphism, suggests an association of the Ets-1 gene with insulin-dependent diabetes mellitus (IDDM) in the Japanese but not Caucasian populations *(130,131)*. In addition, the rat RT6 mono-ADP-ribosyltransferase gene promoter exhibits EBSs *(132)*. Defects in RT6 expression have been linked to increased susceptibility to IDDM in a rat model *(133)*. However, the physiological significance and role of Ets factors, if any, in the regulation of RT6 and development of IDDM has not been determined.

ETS TRANSCRIPTION FACTORS IN ENDOCRINE CARCINOGENESIS

Members of the Ets family of transcription factors may contribute to the development and progression of cancer by several mechanisms. First, in Ewing tumors and certain types of leukemia, Ets factors are fused to other genes as a result of chromosomal translocations resulting in formation of chimeric oncogenes *(134,135)*. Second, Ets proteins are important regulators of genes involved in the degradation and remodeling of extracellular matrix and in angiogenesis. Thus, expression of Ets factors in tumors is linked to invasion and metastasis *(2,134,136)*. Third, Ets members are critical nuclear targets of several hormone and growth factor/oncogene signal transduction pathways, deregulation or constitutive activation of which are associated with tumorigenesis *(2,3,53,137–139)*.

Ets transcription factors may also have an indirect effect on tumor invasion and metastasis, by regulating the expression of parathyroid hormone–related protein

(PTHrP) *(134)*. First identified as the principal agent responsible for humoral hypercalcemia of malignancy, PTHrP is found in most tissues and is thought to play a role in the development, differentiation, and proliferation of endocrine and other organs *(140)*. PTHrP is, like Ets factors, expressed in aggressive tumors, including breast, pituitary, ovary, and prostate, and is associated with the malignant metastatic phenotype *(134,140)*. Systemic PTHrP may be responsible for osteotropism of metastases and osteoclastic bone resorption in breast carcinomas *(140,141)*. Reduction of PTHrP levels, by antisense expression in the rat, inhibited the progression and metastasis of a malignant pituitary (mGH3) tumor *(142)*.

The major human PTHrP promoter (P2) is thought to be regulated by Ets-1, in cooperation with the transcription factor Sp1 *(143,144)*. Similar Ets-1 and Sp1 binding sites are also found in the murine PTHrP gene *(145)*. Moreover, PTHrP expression, like that of Ets-1, is induced in endothelial and vascular smooth muscle cells by mitogenic agents and is implicated in angiogenesis *(134,146–150)*. Thus, PTHrP gene expression in a variety of tissues and tumors may be an important target of Ets-1 transcriptional regulation *(134)*.

Pituitary Cancer

We and others have implicated Ets-1, or a related factor, in the Ras signal transduction pathway in pituitary somatomammotrophs leading to activation of the rPRL gene *(20,24,29,45,47)*. Mutations in the Ras gene have been identified in a number of human cancers *(151),* but appear to be uncommon in pituitary adenomas. However, a glycine-to-valine (V-12) activating Ras mutation was detected in a recurrent prolactinoma, which proved to be a highly aggressive, invasive, and ultimately lethal tumor, with some features of a malignant neoplasm *(152)*.

Ets transcription factors have also been shown to be a critical nuclear target of FGF signaling in the pituitary *(19)*. FGF-2 has been found in human pituitary tumors *(84)* and stimulates prolactin secretion from cultured human pituitary adenomas *(85)*. Furthermore, elevated levels of immunoreactive FGF-2 are present in patients with multiple endocrine neoplasia type-1 *(87,153)*. Pituitary lactotroph adenomas also express distinct FGF receptor insoforms and subtypes compared to the normal pituitary *(154)*.

FGF-4, first identified as a transforming gene in human stomach cancer *(155),* was subsequently isolated from human prolactinomas tested for the presence of DNA transforming sequences *(156)*. In addition, FGF-4 has been shown to induce PRL secretion and gene transcription in rat pituitary cells *(19,86),* and rats injected with GH4 cells stably transfected with FGF-4 expression vectors developed highly aggressive, prolactin-secreting tumors *(86)*. Thus, FGFs, signaling via Ets proteins, may be directly involved in the development, progression, and metastasis of pituitary tumors.

Interestingly, a novel Ets family member, Ehf, was recently cloned from a mouse pituitary somatotroph tumor cDNA library, based on its differential expression *(25)*. Ehf is most closely related to the epithelial specific ESE-1/ESX isolated from breast and pancreatic cancers *(157,158)* and may play a role in tumorigenesis in the pituitary *(25)*. However, Ehf, like ESE-1/ESX, lacks consensus MAPK phosphorylation sites typical of growth factor-inducible Ets transcription factors such as Ets-1 *(2,3,17,159)*. Thus, the function of Ehf in the basal and hormone/growth factor regulation of pituitary-

specific gene expression, differentiation of somatotroph and lactotroph lineages, and development of pituitary tumors remains to be established.

Breast Cancer

Several lines of evidence suggest a role for Ets factors in breast cancer. The Ets family members ER81, ERM, and PEA3 are normally transcribed at low levels in mammary tissue *(160)* but are overexpressed in breast cancer cell lines *(161,162)* and tumors *(163,164)*. Expression of ERM and ER81 in breast cancer cells exhibited an inverse correlation with levels of estrogen receptors and progesterone receptors *(162)*. Additionally, human PEA3 (E1AF) was identified in a screen for human breast cancer–associated genes in the BRCA1 region *(165)*. PEA3 binding sites are found in several genes that encode matrix-degrading proteoglycolytic enzymes such as urokinase type plasminogen activator, the matrix metalloproteinases MMP9 (type IV collagenase), MMP3, MMP10, and MMP11 (stromelysins 1, 2, and 3) and matrilysin *(166,167)*. Unregulated expression of these enzymes is typically associated with tumor metastasis *(166,168,169)*. As such, PEA3 (or related Ets factors) may be a key regulator of the invasive or metastatic phenotype. Overexpression of human PEA3 (E1AF) in MCF-7 breast cancer cells confers an invasive, motile phenotype accompanied by an increase in collagenase (MMP9) gene expression *(170)*. MMP9 expression also induced metastatic activity in rat embryo cells *(171)*.

PEA3 is also upregulated in the majority of her2/neu/erbB-2 oncogene-positive breast cancers. Her2/neu is a transmembrane RTK, similar to the EGF receptor, and is implicated in tumorigenesis in a variety of tissues including breast, ovary, stomach, colon, kidney, and bladder *(172)*. Her2/neu is overexpressed in 20–30% of all breast tumors and is associated with increased metastasis, decreased efficacy of hormone and chemotherapy and poor prognosis *(173,174)*. Elevated levels of her2/neu in breast cancers are a result of both gene amplification and transcriptional upregulation *(161,175)*. Ets factors, including PEA3, have been shown to bind to a critical regulatory element in the her2/neu promoter *(161,176)* and, conversely, have also been identified as downstream targets of the her2/neu oncogene *(177)*. Hence, PEA3 is thought to be activated by her2/neu, via Ras-dependent stimulation of the MAPK and Jun kinase pathways *(175)*. PEA3 contains eight potential MAPK sites and is phosphorylated by both MAPK and Jun kinase, leading to enhancement of its transactivation potency *(178)*. Interestingly, PEA3 also autoregulates its own expression by binding to Ets elements within its promoter *(176)*. Thus, elevated levels of PEA3 in her2/neu-positive breast tumors may be due to her2/neu-mediated increases in PEA3 transcriptional activity *(175,176)*. Furthermore, since PEA3 also activates the her2/neu promoter, a positive feedback loop is established, resulting in overexpression of both her2/neu and PEA3. Therefore, PEA3 may be a critical factor in the molecular mechanism(s) by which alteration in her2/neu expression leads to breast tumorigenesis *(161,176)*. PEA3 overexpression in her2/neu-negative tumors may be due to other related oncogenes, such as erbB-1 and erbB-3, which also contain cognate Ets response elements in their promoters *(176)*.

Expression of Ets-1, urokinase type plasminogen activator (uPA), and stromal proteases in mammary epithelia or breast carcinoma cell lines also correlates with invasiveness and neoplastic scattering *(179,180)*. Furthermore, a dominant negative Ets-1 protein inhibited uPA activity, cell migration, and invasion in murine cell lines derived from normal or cancerous mammary tissue *(181)*. Similarly, introduction of a transdominant

Ets-2 construct abolished anchorage-independent growth, and macrophage colony-stimulating factor stimulated invasion by BT20 breast carcinoma cells, suggesting a role for Ets-1 and Ets-2 in regulation of growth and invasiveness of neoplastic mammary epithelial cells *(181,182)*.

A search for other factors regulating her2/neu expression in human breast cancers identified a novel, epithelial-specific Ets factor, ESX *(157)*. This protein, which represents a new subfamily of Ets members, was also isolated from a pancreatic cancer cDNA library and termed ESE-1 *(158)*. Fluorescence *in situ* hybridization analysis localized ESX/ESE-1 on chromosome 1q32, a region amplified in approx 50% of early breast cancers *(157,183)*, which also contains the gene for the TCF Ets factor SAP1 *(184)*. ESX is inducible by heregulin, the ligand for her2/neu, and is overexpressed in breast cancer cell lines, which exhibit her2/neu amplification. Increased ESX expression is detectable in the earliest stages of breast cancer, termed ductal carcinoma *in situ*, and may, like PEA3, both result from and contribute to her2/neu overexpression in breast tumorigenesis *(157)*. However, elevated ESX levels are attributed primarily to multiple copies of the 1q32 locus, and ESX lacks consensus MAPK phosphorylation sites analogous to those in PEA3. Thus, the mechanism of heregulin induction of ESX in breast cancer cells remains to be established *(157)*.

Cervical Cancer

Cervical cancer is frequently associated with human papillomavirus (HPV) type 16 infection and integration at the 21q22.2–22.3 locus, a region encoding the Ets family members Erg and Ets-2. Elevated levels of Erg and Ets-2, associated with the development of cervical carcinoma, are thought to result from HPV-induced translocations and alterations in chromosomal structure at this locus *(185)*. Differential Erg mRNA transcripts were also observed in cervical carcinoma cell lines. Thus, Erg and Ets-2 transcription factors may be important targets in HPV-mediated cervical carcinogenesis *(185)*.

Prostate Cancer

Prostate cancer is the most common tumor arising in men *(186)*, yet the molecular and genetic events underlying its development and progression are not fully understood. An analysis of differential gene expression between normal and cancerous tissue derived from prostate glands revealed elevated expression of Ets-2, which may therefore play a role in tumor progression *(187)*. Ets factors are also implicated in the transcriptional regulation of maspin, a tumor-suppressing serpin, expressed in breast and prostate epithelia, whose transcription is differentially regulated in normal and malignant tissue *(188)*. Maspin expression is subject to regulation by an inhibitory, androgen receptor, hormone response element and a stimulatory EBS. Loss of maspin expression during prostate tumor progression may be attributable to lack of Ets regulation of the maspin gene owing to inactivation of the Ets response element in cancerous cells *(189)*.

Pancreatic Cancer

Ets-1 may be involved in tumorigenesis in the pancreas. Elevated levels of Ets-1 have been detected in pancreatic carcinomas, and Ets-1 expression correlated with the degree of differentiation of adenocarcinomas *(190)*. However, despite the putative role

of Ets-1 in cell migration and metastatic invasion via control of genes involved in extracellular matrix degradation and remodeling *(2,136),* Ets-1 expression in pancreatic adenocarcinoma has not been linked to tumor size, metastasis, or prognosis *(190)*.

Ovarian and Testicular Cancer

Ovarian cancer is frequently linked to loss of heterozygosity of the chromosomal region 12p12.3–13.1, which includes the gene encoding the Ets transcription factor TEL *(191)*. Fusion proteins of TEL domains with growth factors or other oncogenes, owing to chromosomal translocations, have been linked to certain types of human leukemia *(192–195)*. However, no translocations, fusions, or mutations of TEL have been detected in ovarian carcinoma samples to date *(191)*. Expression of Ets-2 has been detected in ovarian cancer cell lines exhibiting enhanced activity of the Ras/MAPK signaling pathway *(196)*. MAPK phosphorylation and activation of Ets-2 in these cells has been linked to induction of the matrix metalloproteinase uPA. Thus, Ets-2 may be a critical nuclear target of the Ras signal transduction pathway, which is activated independently of mutations in the Ras gene, in ovarian cancer *(196)*.

Finally, Down syndrome–afflicted males exhibit a higher incidence of testicular germ cell tumors, which may be partially attributable to increased levels of Ets-2 *(197)*. Ets-2 overexpression has also been implicated in the cranial and cervical skeletal abnormalities occurring in Down syndrome *(198)* and may be linked to abnormal ovarian follicle development and histology observed in this condition *(126)*.

SUMMARY

Members of the Ets family of transcription factors are emerging as critical regulators of both basal and hormone/growth factor–stimulated neuroendocrine gene transcription and cell-type ontogeny. Ets factors and their target genes have also been implicated in the development, progression, and metastasis of several endocrine tumors. The ability of Ets members to interact with partner proteins, such as GHF-1, at composite *cis* elements provides a mechanism to permit highly selective, distinct regulation of closely related genes such as PRL and GH. Moreover, cooperation of Ets proteins with cofactors greatly expands the repertoire of target genes. Certain Ets factors (Ets-1, Ets-2, GABP, and the TCFs) are also key nuclear components of the MAPK signaling pathways, which may modulate their transcriptional activity, DNA binding, and interactions with partner proteins. The Ets family includes both transcriptional activators and repressors, which share a common core binding sequence (GGA); thus, binding of distinct Ets proteins allows diverse responses to be mediated via a common *cis* element, such as the FGF, insulin and Ras response elements of the rPRL promoter. Further flexibility and diversity is conferred by the potential interaction of Ets factors with distinct protein partners, such as the alternatively spliced homeodomain proteins GHF-1 and GHF-2. Hence, Ets factors serve both to target hormone and growth factor signals to tissue-specific endocrine promoters and to integrate and coordinate transcriptional responses to multiple inductive signals.

The spectrum of endocrine genes and functions subject to regulation by members of the Ets family of transcription factors will undoubtedly expand in the near future.

Furthermore, by virtue of their role in tissue-specific signal transduction, angiogenesis, and metastasis, Ets factors represent novel potential targets for therapeutic intervention in breast cancer and other endocrine tumors.

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4 Pit-1 Expression, Regulation, and Modulation of Multiple Pituitary Genes

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Contents

INTRODUCTION

Anterior pituitary development is a highly complex process whereby five distinct, mature cell types arise in a precise spatial and temporal pattern *(1–3)*. Three of these cell types, somatotropes, lactotropes, and thyrotropes, are dependent on expression of the pituitary-specific transcription factor Pit-1/GHF-1 *(4)*. In the mouse, the pituitary anlage is first detectable at d 12.5 postcoitum (12.5 p.c.) *(5)*. Pit-1 transcripts are detectable throughout the anterior pituitary at d 13.5 p.c. Interestingly, Pit-1 protein is not detectable until d 15.5 p.c. This long lag time between Pit-1 RNA and protein expression indicates that the precise temporal control of Pit-1 expression is under translational as well as transcriptional control. Other homeobox proteins in other species appear to have similar dual control expression. Rapidly following expression of Pit-1 protein at d 15.5 p.c., transcripts for growth hormone (GH), prolactin (PRL), and the thyroid-stimulating hormone β subunit (TSHβ) are identified. The major expression of PRL, however, is not observed until after d 17.5 p.c. A second population of thyrotropes was identified in the rostral tip of the anterior pituitary by Lin and colleagues *(6)*.

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These thyrotropes appear at embryonic d 12.5 (E12.5) long before any detectable expression of Pit-1 (E14.5). By postpartum d 0.5, these rostral thyrotropes are no longer detectable, and only the Pit-1-dependent caudomedial thyrotropes remain. The physiological significance of these early Pit-1-independent rostral tip thyrotropes is unknown.

Long before the identification of Pit-1, rat anterior pituitary development had been studied in detail by immunohistochemistry *(7–9)*. ACTH protein first appears at d 14–15 p.c., whereas TSH-staining cells first appeared in the posterior half of the pituitary at d 16 p.c. (rostral tip?) followed by the predominant cells in the anterior half of the gland. Luteinizing hormone and follicle-stimulating hormone staining appeared on d 17 and 19 p.c., respectively, and GH was detectable on d 18 p.c. PRL-staining cells were detectable only in the newborn rats. More recently, Simmons et al. *(10)* used *in situ* hybridization to show that all five cell types contained Pit-1 transcripts, whereas only somatotropes, lactotropes, and thyrotropes expressed Pit-1 protein. Pit-1 transcripts were first detected in the anterior pituitary on E15.5–16.5, 1 to 2 d after the first detectable expression of the THSβ subunit, perhaps paralleling the Pit-1-independent population of thyrotropes seen in the mouse. Expression of GH and PRL are observed on d E17.5, long after Pit-1 is detectable.

Finally, Pit-1 developmental expression has been studied in the human fetus by Puy and Asa *(11)*. They performed both *in situ* hybridization and immunohistochemistry on human fetal pituitaries between 6 and 19 wk of gestation and at term. Both Pit-1 RNA and protein were detectable at the earliest developmental time point, 6 wk. Pit-1 protein expression rapidly increased at 8 to 9 wk. Unlike the mouse and rat, in which expression of Pit-1 was rapidly followed by expression of the Pit-1-dependent hormones, protein expression of GH PRL, and TSHβ was not observed until gestational wk 17–19 in the human fetus. Expression of these hormones was not observed in the 10- to 12-wk fetuses, long after Pit-1 protein was detectable. There were no fetal pituitaries studied between 12- and 17 wk of gestation. These data suggest that although Pit-1 is an important factor in the development of mature somatotropes, thyrotropes, and lactotropes, this factor alone is not sufficient for terminal differentiation of these cell types, since Pit-1 is expressed in the human fetal pituitary long before these hormones.

Pit-1 pituitary mRNA has been identified in several species. The original descriptions of this transcription factor were in rat, mouse, and human pituitaries *(12–15)*. More recently, Pit-1 has been isolated from turkey pituitary, where PRL plays a critical role in egg laying and incubation *(16)*. Pit-1 has also been isolated from Atlantic salmon and rainbow trout, in which GH, PRL, and somatolactin are involved in osmoregulation and adaptation from a freshwater to a saltwater environment *(17,18)*. Immunoreactive Pit-1 has also been identified in receptosecretory cells of the prochordate lancelets, indicating the conservation of this important transcription factor throughout evolution *(19)*.

PIT-1 EXPRESSION IN THE ANTERIOR PITUITARY

Expression of Pit-1 in the anterior pituitary is complex and different in various species. Pit-1 mRNA is expressed in all five cell types (somatotropes, lactotropes, thyrotropes, corticotropes, and gonadotropes) in mouse and porcine pituitaries, whereas protein expression is limited to somatotropes, lactotropes, and thyrotropes, in which

this transcription factor is required for specific hormone expression *(10,20)*. These data suggest that regulation of Pit-1 expression occurs at both the transcriptional and translational levels in these species. Data from human studies, however, are different *(11)*. Expression of both RNA and protein in fetal and adult human pituitaries is restricted to the somatotropes, lactotropes, and thyrotropes, suggesting that regulation of Pit-1 expression is primarily at the level of transcription in humans. Although the physiological importance of different mechanisms governing expression of Pit-1 in different species is unclear, these differences may provide a model for mechanisms underlying control of gene expression. Our laboratory has been investigating a murine model of thyrotrope gene expression. TtT-97 cells, a hyperplastic thyrotrope model grown in hypothyroid mice, expresses both Pit-1 RNA and protein as well as both subunits of TSH, mimicking normal thyrotropes. The αTSH cell line, which is thyrotrope derived, also expresses Pit-1 RNA, but lacks expression of Pit-1 protein, suggesting an aberration in posttranscriptional control of Pit-1 gene expression *(21)*. This cell line also lacks expression of the critical TSHβ subunit gene, which is dependent on Pit-1 for expression, yet retains expression of the common α-subunit, which does not appear to require the presence of Pit-1 for expression. Comparison of these cell types may provide a model for posttranscriptional control of Pit-1 gene expression in rodents.

PIT-1 STRUCTURE AND DNA BINDING

Pit-1 contains three basic modular domains: the N-terminal domain, the POU-specific domain, and the POU homeodomain, which has similar sequence with other homeodomain transcription factors. The N-terminal domain contains activation function regions, and the POU-specific domain and POU homeodomain together comprise the DNA-binding region *(22,23)*. Pit-1 interacts with target genes through a core consensus TATNCAT region with variations in the TSHβ gene promoter A(A/T)(A/T)AATNCAT and the GH and PRL gene promoters A(A/T)(A/T)TATNCAT. In vitro transcription assays using the PRL promoter show that Pit-1 appears to activate gene transcription through enhancement and stabilization of RNA polymerase II transcription complex assembly *(24)*. Mutational analysis of the DNA-binding region and high-resolution X-ray analysis of Pit-1 have provided insight into the exact nature of Pit-1/DNA interaction *(25,26)*.

Liang and colleagues *(25)* performed random mutagenesis of the POU-specific domain and POU homeodomain, and then assessed function in a yeast system as well as in in vitro DNA binding. Multiple single amino acid mutations were identified throughout the region that impaired DNA binding with a majority of mutations occurring in one of the four POU-specific α-helices or one of the three POU homeodomain α-helices. No mutations appeared to affect DNA binding in the extreme carboxyl terminal of the protein (aa 270–291). Interestingly, the most common human mutation of Pit-1 identified is at amino acid 271, which does not affect DNA binding but severely alters function in a dominant negative manner. Two mutations in the linker region between helix 4 of the POU-specific domain and helix 1 of the POU homeodomain increased promoter activity in yeast, suggesting an enhanced binding. Jacobson and colleagues subsequently reported the crystal structure of the Pit-1 POU domains bound to DNA as a homodimer *(27)*. The POU-specific domain and POU homeodomain were unexpectedly found to bind to perpendicular faces of the DNA rather than opposite

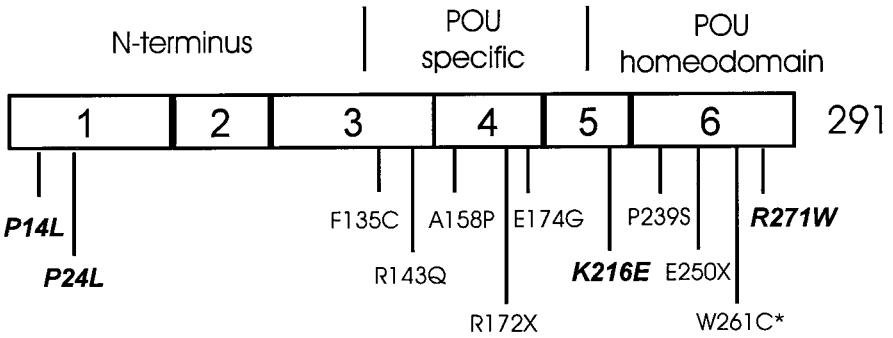

Fig. 1. Location of human Pit-1 mutations. Shown schematically are the relative positions and amino acid changes for several different mutations in the human Pit-1 gene. Dominant mutations are depicted in bold italic and recessive mutations are in regular type. The asterisk denotes the Pit-1 mutation present in the Snell dwarf mouse. The full-length 291 amino acid protein is shown divided into its 6 coding exons, numbered 1–6, and the relative location of the N-terminus and POU-specific domains and the POU homeodomain.

sides, as was found with Oct-1 *(28)*. The dimerization domain appeared to be formed between the POU-specific domain of one monomer and the POU homeodomain of the other monomer, rather than the POU-specific domain of both monomers as predicted *(23)*. The α-helices of the POU-specific domain make contacts exclusively in the major groove of the DNA, while the contacts of the POU homeodomain are made in both the major and minor grooves. Furthermore, these contacts result in much less DNA bending (7°) than seen with the Oct-1 monomer (30°), providing an understanding of the differential effects of these closely related factors on similar DNA elements.

PIT-1 MUTATIONS

The absolute requirement of Pit-1 for development of somatotropes, lactotropes, and thyrotropes has been emphasized by naturally occurring mutations of Pit-1 in mice and humans. Snell and Jackson dwarf mice were found to have a point mutation and gene rearrangement of Pit-1, respectively, both resulting in a lack of functional Pit-1 protein *(29,30)*. These animals are, as a result, small with hypoplastic pituitaries lacking somatotropes, lactotropes, and thyrotropes. Many human Pit-1 mutations have been subsequently identified causing selective hypopituitarism (somatotropes, lactotropes, and thyrotropes) in either a dominant or recessive pattern, depending on the location of the mutation (Fig. 1) *(4,31–34)*.

A third dwarf mouse strain has been identified, the Ames dwarf mouse, with a similar phenotype to the Snell and Jackson mice *(35)*. These mice fail to express Pit-1 later in ontogeny, but lack a Pit-1 mutation. A genetic defect, referred to as the Ames dwarf (*df*), is located on chromosome 11, and has recently been identified by positional cloning *(36)*. The *df* gene encodes a 223 amino acid homeodomain protein called Prophet of Pit-1 (Prop-1) since it is expressed prior to Pit-1 and thus "prophecies its coming." The Pit-1-deficient phenotype seen in the Ames dwarf is caused by a point mutation in Prop-1 leading to a failure to activate Pit-1 gene expression. Prop-1 is expressed early (d E10.5) in the mouse anterior pituitary, well before Pit-1 is expressed

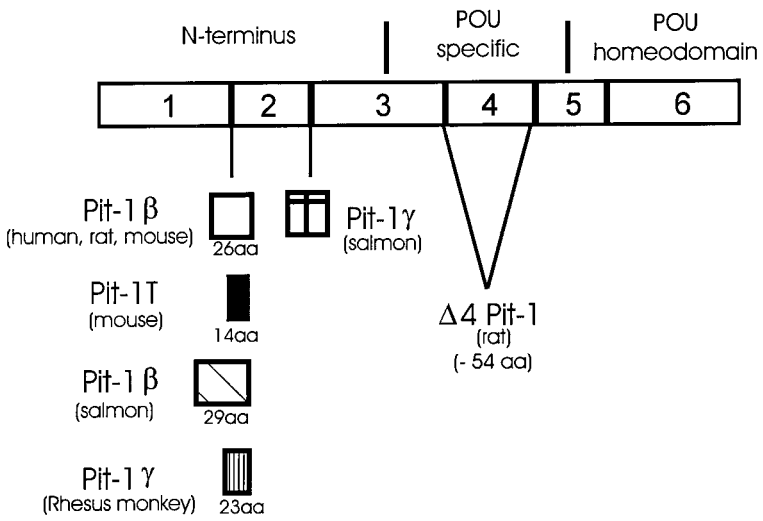

Fig. 2. Splice variant forms of Pit-1. Shown schematically are the relative positions and lengths of several Pit-1 variant proteins from several different species produced by alternative exon splicing of the primary transcript. The protein is divided into its 6 coding exons, numbered 1–6, and the relative location of the N-terminus and POU-specific domains and the POU homeodomain are shown at the top of the figure.

at d E13.5–14.5. This would suggest that Prop-1 is unable to activate Pit-1 gene expression between d E10.5 and E13.5. Another paired-like homeodomain factor, Rpx, is temporally and spatially coexpressed with Prop-1 at d E10.5 and can form heterodimers with Prop-1 on cognate DNA elements *(36,37)*. Coexpression of these two factors showed that Rpx could interfere with gene activation by Prop-1, suggesting that Pit-1 gene expression may be silenced by the presence of Rpx early in pituitary development. The expression of Rpx rapidly decreases and is absent by d E 13.5 just prior to Pit-1 expression, indicating the complex interaction of multiple factors controlling Pit-1 gene expression and, ultimately, Pit-1-dependent hormone gene expression. The human Prop-1 gene is located on chromosome 5q. Since the discovery of Prop-1, three reports have identified mutations in the Prop-1 gene in children with combined pituitary hormone deficiency who did not have Pit-1 mutations *(38–40)*. These mutations were either homozygous or compound heterozygotes, suggesting a lack of DNA binding and not the dominant negative mutations seen in some cases of Pit-1 mutations.

PIT-1 VARIANTS

Since the initial discovery of Pit-1, a number of splice variants have been identified in different species. Although the physiological importance of these isoforms is still unclear, many functional studies have provided insight into the action of Pit-1 and these variants on the target gene promoters (GH, PRL, TSHβ). The first variant of Pit-1 was identified simultaneously by three groups in 1992 and was called Pit-1β, GHF-2, and Pit-1a *(41–43)*. Pit-1β contains an additional 26 amino acids in the N-terminal activation domain generated by alternative RNA splicing between exons 1 and 2 (Fig. 2). Pit-1β RNA appeared to be relatively more abundant than the protein, leading

investigators to speculate that Pit-1β protein may be less stable than Pit-1 protein. The Pit-1β variant appears to function quite differently from Pit-1. Using gene transfer experiments, all three groups showed that Pit-1β had little effect on the activity of the PRL promoter or the activity of its own Pit-1 promoter. This variant did, however, efficiently stimulate activity of the GH promoter. Our own studies show that the Pit-1β variant is unable to stimulate TSHβ promoter activity. Taken together, these data suggest that the differential action of Pit-1β on the GH and PRL promoters may regulate the relative distribution of the two hormones in somatolactotropes, although this has not been proven in vivo. In a recent study, Diamond and Gutierrez-Hartmann *(44)* showed that Pit-1β inhibits basal activity of the PRL promoter in Pit-1 expressing GH4 lactotropes, but that this variant had a differential impact on signaling pathways in these cells. Pit-1β appeared to inhibit Ras-mediated stimulation of the PRL promoter, but this variant augmented protein kinase A–mediated stimulation of this same promoter. These investigators went on to replace the 26 amino acid insert of Pit-1β with five different 26 amino acid inserts and showed that the function of Pit-1β is sequence specific, suggesting that the 26 amino acid β-specific domain acts as a molecular switch to integrate different signaling pathways in the lactotrope.

Our group subsequently identified a second variant of Pit-1, called Pit-1T for its isolation from a pituitary thyrotrope–derived tumor *(45)*. Pit-1T contains an additional 14 amino acids in the N-terminal region generated by alternate splicing between exons 1 and 2 (Fig. 2). The Pit-1T insert is contained entirely within the carboxy terminal region of the Pit-1β insert, suggesting that alternative RNA splicing in this region is important for generating different Pit-1 variants with, perhaps, different functional activity. Pit-1T protein is expressed in thyrotropes, and like Pit-1β, it is expressed at much lower levels than Pit-1. When cotransfected into GH3 cells, which express both Pit-1 and Pit-1β but not Pit-1T, this thyrotrope-specific isoform stimulates TSHβ promoter activity, but not GH nor PRL promoter activity *(46)*. We have further shown that Pit-1T strongly stimulates TSHβ promoter activity in nonpituitary HeLa cells, which lack all Pit-1 isoforms, whereas Pit-1 has a modest stimulatory effect and Pit-1β does not stimulate this promoter, suggesting that Pit-1T has both thyrotrope-specific expression and TSHβ promoter–specific activity.

A third Pit-1 variant was identified by two different groups *(47,48)*. One group performed in vivo transplantation of GH3 cells, which express PRL and GH, into female Wistar-Furth rats. The resultant tumors expressed only GH, suggesting regression of PRL expression in this in vivo model. When the tumor cells were placed back into culture, PRL mRNA rapidly reappeared. Levels of Pit-1 protein were similar between the in vitro and in vivo models, but a smaller protein (approx 27 kDa) was identified with Pit-1 antibody only in the in vivo tumor cells. These investigators subsequently characterized this Pit-1 variant and showed that it lacked exon 4 (Pit-1Δ4) (Fig. 2). Exon 4 encodes 54 amino acids of the DNA-binding POU-specific domain. This group further showed that introduction of Pit-1Δ4 into Pit-1- and PRL-expressing GH3 cells resulted in an inhibition of PRL promoter activity and that this variant could interfere with Pit-1 stimulation of the PRL promoter in heterologous Rat-1 cells *(49)*. The precise role of this variant in normal pituitary development and somatolactotrope function is currently unclear, but Pit-1Δ4 may, like Pit-1β, play a role in differential expression of GH and PRL in somatolactotropes. Finally, other splice variants of Pit-1 have been identified in rhesus monkey pituitary *(50),* salmon pituitary *(18),* and turkey pituitary

(16,51). Although the exact physiological role of the different isoforms in the different species is unclear, their further study should provide insight into mechanisms of differential RNA splicing in different cells, as well as an understanding of the structural requirements of Pit-1, its variants, and protein partners on the differential expression of GH, PRL, and TSH in the anterior pituitary gland.

MODULATION OF PITUITARY GENES BY PIT-1

Pit-1 protein is present in and required for the differentiation of pituitary somatotropes, lactotropes, and thyrotropes *(10,30)*. Since the genes that are dependent on Pit-1 in each cell type are not generally expressed by the other cell types, activation of cell-restricted genes by Pit-1 must require additional cell-specific factors *(10)*. An obvious exception to this cell restriction is the Pit-1 gene itself, as well as the β2 thyroid hormone receptor isoform that is expressed in somatotropes and thyrotropes but not lactotropes *(52)*. Therefore, Pit-1 is essential but not sufficient for the completion of the differentiated program that leads to the cell-restricted expression of the GH, PRL, and TSHβ subunit target genes that are expressed only in somatotropes, lactotropes, and thyrotropes, respectively. The next sections review what is currently known regarding Pit-1-dependent genes within the pituitary gland and the role played by additional factors to impart cell specificity.

The Pit-1 Gene

The Pit-1 gene has been cloned and characterized from rats and humans by several laboratories *(53–55)*. Since Pit-1 protein cannot initially activate its own promoter during early ontogeny of the pituitary, an additional factor was sought to fulfill this critical role. Insights have been obtained using the Ames dwarf (df) mouse as a model. The Ames dwarf is deficient in thyrotropes, somatotropes, and lactotropes, but unlike the case for the Snell dwarf, the mutation did not map to the Pit-1 gene and a low percentage of each cell type could be detected *(56)*. Recently, using the method of positional cloning, the Rosenfeld laboratory identified the mutated locus to be within the Prop-1 gene *(36),* which precedes Pit-1 expression during development and binds to sites in an early enhancer of the Pit-1 gene *(57)*. However, Prop-1 by itself cannot activate the enhancer, suggesting an indirect effect or the participation of additional factors *(58)*. In the mouse, Prop-1 expression occurs transiently during pituitary development. Its initial appearance was detected by d E10–10.5, was maximal by d E12, and was markedly decreased after d E14.5. Concomitant with the lowered levels of Prop-1, a switch occurs in Pit-1 gene expression to a sustained autoregulatory mechanism involving binding sites for Pit-1 protein.

Within the proximal rat Pit-1 gene, two high-affinity binding sites for Pit-1 have been characterized *(53,54)*. DNase I protection analysis demonstrated that the more distal site is a stimulatory element and is found at position −38 to −70, where it is adjacent to the binding site for an additional factor present in partially purified GC cell nuclear extracts *(54)*. Interestingly, the more proximal site is within the 5′ untranslated region of the gene at position +4 to +34. It acts to negatively autoregulate expression since a mutation within this site results in a five- to sixfold increase in expression of Pit-1 promoter reporter constructs when transfected into Pit-1-replete GC pituitary cells or CV-1 cells cotransfected with Pit-1 *(53)*. At least part of the mechanism

of its repression appears to involve alteration of transcriptional elongation in the presence of both Pit-1 binding sites, as demonstrated by in vitro transcription assays *(59)*. However, this site acts as a negative element only in context since moving it upstream of the −36 to +34 PRL promoter construct allows it to act as a transcriptional stimulator *(53)*. Other negative elements may also exist in the Pit-1 gene. For example, thyroid hormone (TH) has been reported to inhibit basal and antagonize cyclic adenosine monophosphate (cAMP)-regulated expression in GH4C1 cells by a novel TR-mediated mechanism involving transcriptional interference with regulatory elements such as the cAMP response element (CRE) or the autoregulatory function of Pit-1 *(60)*. The presence of Pit-1 binding sites appears to be conserved in other species because several sites resembling a Pit-1 consensus sequence are present in the human Pit-1 gene at –457, and −179 and −110 bp upstream of the initiator methionine codon *(55)*.

The Pit-1 gene is also regulated by environmental cues that modulate cAMP levels and the activity of the CRE-binding protein (CREB). Two CRE octamer sites occur in the rat gene at positions −157/–150 and –200/–193, and purified CREB has been shown to protect these regions from DNase I cleavage *(54)*. One of the two CREB binding sites is absent in the human Pit-1 gene *(61)*. However, it appears that CREB itself does not activate the Pit-1 promoter; rather, the effect requires an interaction with additional pituitary-specific factors *(53,54)*.

Several distally located and functional Pit-1 binding sites as well as other important transcription factor complexes were deduced from the results of Pit-1 promoter targeting studies in transgenic mice *(62)*. A *lacZ* reporter transgene was expressed at high levels in the pituitary using 14.8 kb of 5′ flanking Pit-1 DNA whereas constructs shorter than 10.2 kb failed to express the transgene. These studies defined an enhancer sequence of about 700 bp that is positioned more than 10 kb upstream of the transcriptional start site. The enhancer exhibits position and orientation independence and can confer enhanced pituitary-specific activity to heterologous tk and SV40 promoters *(62)*. Within the 700-bp enhancer are five binding sites for Pit-1; of these, three appear to be functional since selective mutations of them result in lowered promoter activity. The data also demonstrate that the three functional Pit-1 sites account for most of the regulation of the Pit-1 gene by Pit-1 protein in vivo. In addition, a pituitary-specific element distinct from Pit-1 was mapped to the distal enhancer.

The enhancer also conferred hormonal responses to both vitamin D_3 and retinoic acid (RA). The promoter/enhancer was stimulated 15-fold by 1,25 dihydroxyvitamin D_3, whereas no effect was found with thyroid hormone, estrogen, or glucocorticoids. Thus, a potent D3 response element, termed the RDE, was defined and appears primarily to mediate hormonal regulation by vitamin D_3. The enhancer/promoter could also be stimulated 16-fold by addition of all *trans* (RA) or 9-*cis* RA, thus defining a novel RA response element complex element within a region termed the Pit-1-dependent RA response element (PRE). The major RA response element in this location is absolutely dependent on Pit-1 and retinoic acid receptor (RAR) for induction by RA. In this case, Pit-1 appears to function as a coregulator of the RAR, and the distance between the two sites is critically important. The PRE in the Pit-1 gene may permit weak cooperative binding of RAR even in the absence of a nearby retinoid X receptor (RXR) binding element, although it presumably works in concert with RXR and perhaps other unidentified factors. The enhancer is conserved in both sequence and function in mice, rats,

and hamsters, thus emphasizing its functional importance *(63)*. In summary, for the Pit-1 gene a number of complex regulatory elements exist both within proximal promoter sequences and in the distal enhancer and indicate that multiple mechanisms operate to regulate its expression in a subset of pituitary cells.

Growth Hormone

Pit-1 is required for GH gene expression since its absence in rodents and humans results in GH deficiency leading to dwarfism *(1,30,31)*. Two sites that bind Pit-1 within the proximal rat and human promoter regions have been shown to be critical for activation of the GH promoter in transient transfections of cultured pituitary-derived cells *(64)* and transgenic mice *(65,66)*. Furthermore, disruption of binding to these sites by site-directed mutagenesis results in the inability of Pit-1 to stimulate the rat GH promoter in nonpituitary cells *(67)* as well as in in vitro transcription assays in extracts of HeLa cells *(68)*.

A highly conserved element located between the Pit-1 sites was shown to bind a novel protein termed Zn-15 that comprised 15 zinc finger–like motifs *(69)*. Mutation of this element decreased expression of a GH promoter reporter fusion transgene >100-fold in transgenic mice. Coexpression of Zn-15 with Pit-1 in heterologous CV-1 cells resulted in a synergistic activation of the GH promoter, which was lost when Zn-15 binding to the element was disrupted by mutation. The N-terminal 855 amino acid residues of Zn-15 that contain eight of the zinc finger domains appear to be dispensable for the synergy with Pit-1. The amino terminal half was transcriptionally inert, as was the isolated DNA binding domain, which is in concordance with a model in which the carboxyl terminus of Zn-15 including the DNA binding domain contains sufficient information for both transactivation and synergistic interaction with Pit-1. By contrast, an N-terminal truncation of Pit-1 that still contained both the POU-specific domain and POU homeodomain was unable to synergize functionally with Zn-15, as was a second mutant that contained a single proline for alanine substitution in the POU domain. This latter mutation was described in humans and shown to cause hereditary dwarfism *(61)*.

In the presence of TH, TRS and Pit-1 synergistically activate the GH promoter, which is further increased by stimulation of both protein kinases A and C *(67)*, suggesting a role for Pit-1 in mediating hormonal effects on GH expression. The participation of intracellular kinases may point to a role for Pit-1 in transducing extracellular signals such as the stimulation of GH by hypothalamic GH-releasing hormone, which is thought to act by increasing intracellular cAMP levels *(70)*. The exact mechanism whereby TH enhances the effect of Pit-1 on GH gene transcription is not known, although it was shown by in vivo dimethyl sulfate footprinting to induce occupancy of both the TH response element and Pit-1 sites *(71)*. Functional synergy between Pit-1 and TR on the GH promoter has been shown to be mediated by the AF-2 domain of TR and residues 72–100 of Pit-1 *(72)*, which were shown to mediate the inhibitory effect of the AF-2 interacting nuclear receptor coactivator RIP 140 *(73)*. The CAAT/enhancer-binding protein isoform was also shown to synergize with Pit-1 on the GH promoter, but it must interact at a different region of Pit-1 than TR because deletion of residues 72–125 had only a marginal effect on synergy *(74)*. Other factors have been shown to inhibit binding of Pit-1 to the proximal GH promoter. This can lead to either a

decrease in GH expression, as seen with activin treatment *(75),* or a potentiation of GH promoter activity by displacement of Pit-1 from one of the two sites by the ubiquitous transcription factor Sp1 *(76,77).*

Prolactin

PRL gene expression in lactotrope cells is under the control of both a proximal promoter sequence between −422 and +33 and a distal enhancer region between −1831 and −1530. Both the proximal and distal areas are required for high-level pituitary-specific expression and contain high-affinity binding sites for Pit-1 *(78).* Within the proximal region, three of four Pit-1 consensus motifs were demonstrated by clustered point mutation analysis to be critical for PRL promoter activity in transient transfections of GH3 pituitary tumor cells *(79).* The distal enhancer region, which contains four Pit-1 sites, contributes >90% of PRL gene activation in cultured pituitary cells and transgenic mice *(78,80).* Pit-1 had little effect in nonpituitary cells on PRL promoter activity unless activated estrogen receptor (ER) was also present *(81).* Disruption of ER binding to an imperfect palindromic estrogen response element (ERE) adjacent to one of the distal area Pit-1 sites abolished the synergistic transactivation by Pit-1 and ER *(81).* It was also shown that loss of Pit-1 binding at the ER adjacent site affected the synergy whereas alteration of the other three distal Pit-1 sites had no effect on the synergy with ER. However, another report suggested that mutation of combinations of the other Pit-1 sites does not affect ER synergy *(82).* The Pit-1/ER synergy requires both Taf-1 and Taf-2 domains of ER and is dependent on two of three tyrosine residues within the N-terminal activation domain of Pit-1. Interestingly, mutation of these tyrosines had no effect on Pit-1 activation of the GH promoter *(81).*

Lactotrope-specific PRL gene expression was also shown to be dependent on a proximal element immediately upstream of the most 5′ proximal Pit-1 site *(83,84).* This site was shown to bind Ets transcription factor family members and to be part of a larger composite Ets-1/Pit-1 binding site that also mediated a cell-specific response to signals initiated at transmembrane receptors and transduced via the Ras pathway to the cell nucleus *(83)* (*see* Chapter 3). Further evidence for a role for Ets factors in PRL gene expression was the observation that overexpression of a dominant negative form of Ets-1 inhibited PRL promoter activity in GH4 cells *(85),* and, in addition, coexpression of Ets-1 resulted in a synergistic effect with Pit-1 on the PRL promoter in HeLa cells *(85).* The cooperativity of Ets-1 and Pit-1 was specific for the PRL promotor because a synergistic effect of Ets-1 with Pit-1 was not observed with the GH promoter *(85).*

Other homeodomain family members have also been shown to synergize with Pit-1 on the PRL promoter. The POU-domain proteins Pit-1 and the more widely expressed Oct-1 interact to form a heteromeric complex and cooperate to induce reporter expression directed by the proximal PRL promoter *(86).* Gel shift analysis revealed that both proteins can simultaneously occupy and form a complex on the most proximal Pit-1 site and that this element when fused to a minimal PRL promoter is sufficient to direct the functional synergy *(86).* The pituitary-restricted LIM-homeodomain protein pLIM also synergizes with Pit-1 on the PRL promoter. It was shown by cotransfection of an N-terminal truncation that the cooperative effect with Pit-1 was dependent on the presence of the LIM domain *(87).* However the pLIM synergy with Pit-1 was not

confined to the PRL promoter because it was also observed with the Pit-1 and TSHβ promoters *(87)*.

Pit-1 also plays a pivotal role in regulation of PRL gene expression by several hormones. Regulation by thyrotropin-releasing hormone (TRH) and epidermal growth factor (EGF), which activate through different signaling pathways, involves three of the proximal Pit-1 sites as well as sequences among them *(79,88)*. This same area is also involved in the response of the PRL promoter to cAMP but does not bind CREB or AP-1. The distal enhancer also contains, in addition to the region mediating the response to estrogen *(89)*, different elements that confer responsiveness to cAMP, TRH, and EGF *(90)*. This suggests that a common mechanism may underlie the similar responses exhibited by the distal and proximal regions of the rat PRL gene. Finally, inhibition of PRL expression by glucocorticoids localized to the proximal promoter region that contains the Pit-1 sites but no discernible glucocorticoid response element (GRE) *(91)*. Cotransfection experiments with Pit-1 and glucocorticoid receptor (GR) mutants show that PRL promoter inhibition is seen only when Pit-1 is present and that the DNA-binding domain of GR is not required *(91)*. A direct physical interaction of GR with Pit-1 was demonstrated, suggesting that the inhibition is achieved by GR inhibiting the binding of Pit-1 to the proximal binding sites.

TSHβ Subunit

Two distinct populations of thyrotrope cells containing TSHβ transcripts have been detected in the anterior pituitary during development and in the adult. In the rodent, Pit-1-independent thyrotropes form first in the rostral tip of the gland following the expression of thyrotrope embryonic factor *(92)*, and are reported to be a transient population that exists only in the fetal pituitary, whereas the Pit-1-dependent population arises later in the caudiomedial area in the fetal gland and persists in the adult *(5,6,10)*. Colocalization of TSHβ and Pit-1 has also been reported during midgestation in the human fetal pituitary *(11)*. Absence of Pit-1 in the Snell dwarf mouse results in the lack of thyrotropes, somatotropes, and lactotropes, suggesting the importance of Pit-1 for proper differentiation and proliferation/survival of these three cell types *(6,29,30)*. However, a Pit-1-independent population of thyrotropes has also been detected in the adult within the pars tuberalis (PT) of several mammalian species including sheep *(93)*, rats *(94)*, and hamsters *(95)*. Whereas the TSHβ transcripts are indistinguishable from those present in the pars distalis, the PT cells lack detectable Pit-1, as well as T3 and TRH receptors, and are not regulated by classical hormone treatment *(93)*. These data demonstrate that in vivo Pit-1 is not absolutely required for TSHβ gene expression in minority populations within the rostral tip and PT, but that it is a necessary and permissive determinant of thyrotrope development for the abundant thyrotrope lineage within the pars distalis.

In mouse pituitaries and TtT-97 thyrotropic tumors, four different Pit-1 transcripts of 3.2, 2.6, 2.4, and 1.9 kb, which may reflect multiple polyA addition sites, can be detected by Northern blot analysis *(21)*. Western and Southwestern analyses of nuclear extracts from TtT-97 cells reveal 33,000- and 31,000-Dalton forms of Pit-1 *(21)* that are likely derived by alternate usage of two initiator methionine codons (M1 and M27), as was reported in the rat *(96)*. In addition, two additional splice variants of Pit-1 are present at lower levels in mouse thyrotropes. Pit-1β, first reported in rat somatolactotrope

cells *(41–43),* contains an additional 26 amino acids in the transactivation domain between exons 1 and 2 as a result of an alternative 3′ splice acceptor site 78 bp upstream of that used in native Pit-1 *(46)*. In addition, a thyrotrope-specific splice variant termed Pit-1T contains a 14 amino acid in-frame insertion between exons 1 and 2 that is encoded by the 3′-most 42 nucleotides of Pit-1β *(45)*. Its presence in mouse thyrotropes was confirmed by RNase protection assays, and the protein was shown to be present at low levels in TtT-97 cells using a specific antibody. As of yet, Pit-1T has not been cloned and characterized from other species and may be a variant present only in mouse thyrotropes.

Thyrotrope-specific promoter activity maps to the proximal 270 bp of the 5′ flanking region of the mouse TSHβ gene *(97,98)*. Within this broad region, DNase I protection studies have identified four distinct areas that bind nuclear proteins present in mouse TSHβ-expressing thyrotropic tumors: D1 (−253 to −222), D2 (−196 to −176), P1 (−133 to −100), and P2 (−86 to −64) *(21,98)*. Bacterially derived Pit-1 can bind to three of these protected regions at the D1, P1, and P2 regions of the mouse gene *(99)*. Similar interactions have been shown for both the human and rat TSHβ genes. A proximal promoter fragment extending from −128 to −61 of the human TSHβ gene formed five specific protein-DNA complexes with mouse thyrotropic tumor extracts and two complexes with in vitro translated Pit-1 *(100)*. All but one of the tumor-derived complexes and both Pit-1-derived complexes were competed by an excess of a fragment containing a Pit-1 consensus site derived from the rGH gene. Biotinylated hTSHβ fragments from −122 to −101, −107 to −86, and −76 to −55 demonstrated binding to Pit-1 in an avidin-biotin DNA-binding assay with the most upstream area demonstrating the highest binding affinity. In the rat TSHβ gene, Pit-1 translated in vitro bound to three areas with varying affinities at positions −274 to −258 (A region, k_D = 360 n*M*), −336 to −326, B region, k_D = 125 n*M*), and −402 to −384 (C region, k_D = 38 n*M*) by gel mobility shift analysis *(101)*. Thus, both proximal and more distal sites on the TSHβ gene can bind Pit-1 and reflect its importance in both basal and hormone-regulated expression of the gene.

Several lines of evidence point to the functional importance of Pit-1 for TSHβ gene expression:

1. Multiple Pit-1 binding sites have been localized on the TSHβ gene in several species.
2. Mutations of some of these sites lead to a marked reduction in promoter activity in GH3 cells or TtT-97 thyrotropes *(46,99)*.
3. A dominant negative form of Pit-1 interferes with wild-type activity in a dose-dependent manner *(102)*.

However, a number of studies have failed to show a significant Pit-1 stimulation of mouse TSHβ promoter activity in both thyrotrope-derived cells and heterologous cells *(21,45,46,103),* whereas others have demonstrated a modest stimulatory effect on the human and rat promoters *(101,104)*. Taken as a whole, these studies suggest that Pit-1 is necessary, but not sufficient, for basal TSH activation.

Within the functionally important P1 element of the mouse gene, a clear difference was noted by DNase I footprinting analysis using TtT-97 nuclear extracts when compared with those obtained by recombinant Pit-1 alone. Specifically, TtT-97 nuclear extracts produced a larger footprint than recombinant Pit-1 that extended proximally an additional 14 bp to position −86 *(99)*. Scanning mutagenesis followed by footprinting

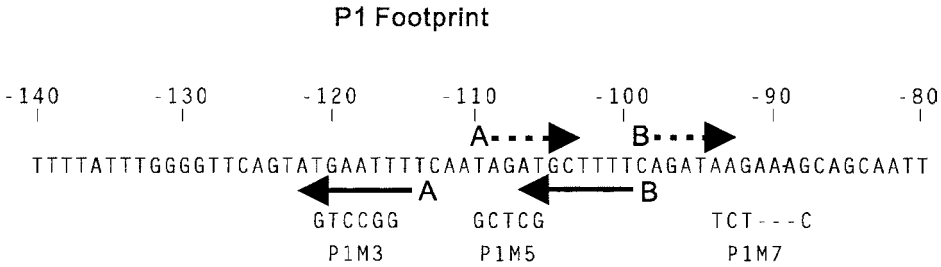

Fig. 3. Schematic of the P1 region of the mouse TSHβ promoter protected from DNase I digestion by TtT-97 thyrotropic extracts showing the location of consensus binding sites for Pit-1 and GATA-2. Also shown are the location of three mutants that abrogate binding of either Pit-1 alone (P1M3), GATA-2 alone (P1M7), or both factors (P1M5).

and functional analysis revealed that two distinct proteins, Pit-1 and a 50-kDa protein, could bind to this region. The 50-kDa protein was determined to be a GATA family member (GATA-2) *(105)*. Within this region are consensus sites for Pit-1 interaction at positions −122 to −115 (Pit-1 A) and −107 to −100 (Pit-1 B) arranged as a direct repeat separated by 7 bp and two GATA consensus sites at −109 to −104 (GATA-2 A) and −98 to −93 (GATA-2 B) arranged as a direct repeat separated by 5 bp (Fig. 3). Gel mobility studies demonstrated that either Pit-1 or GATA-2 alone could form a single distinct complex with a fragment from −144 to −74. However, when both proteins were combined, a novel more abundant complex appeared that migrated more slowly on the gel. This newly formed band is consistent with both factors binding to the same DNA molecule as a ternary complex (Fig. 4). This ternary protein-DNA complex

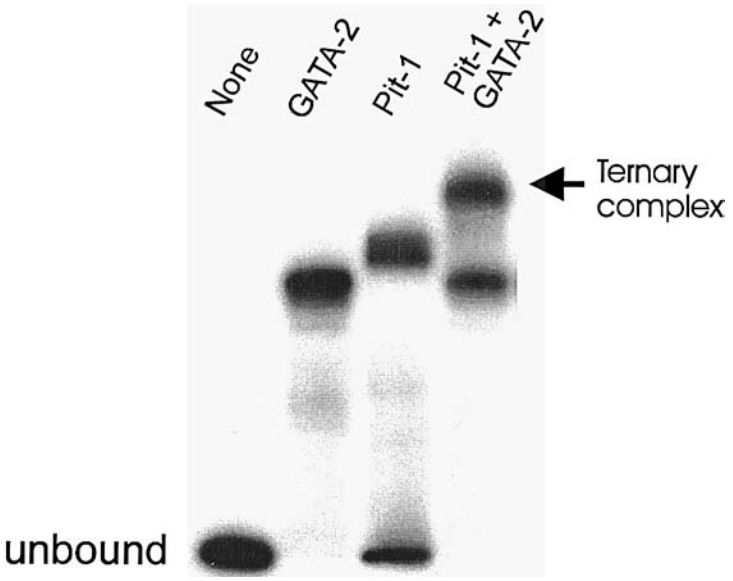

Fig. 4. Formation of an additional complex when GATA-2 and Pit-1 are combined on the P1 region of the mTSHβ promoter. Arrow indicates the position of a more slowly migrating ternary complex. The radiolabeled probe is a −144 to −74 fragment of the mouse TSHβ promoter.

required the participation of Pit-1, GATA-2, and the wild-type P1 duplex DNA. Elimination of the binding of either factor by mutation of the Pit-1 or GATA-2 binding sites within the P1 region resulted in loss of the slowly migrating complex. The functional consequences of transfecting an GATA-2 expression vector, in the presence or absence of Pit-1, on an mTSHβ promoter activity in CV1 cells that lack both factors was investigated. GATA-2 or Pit-1 alone failed to significantly stimulate the mTSHβ promoter. However, the combination of GATA-2 and Pit-1 consistently stimulated promoter activity an average of 8.5-fold. This synergistic effect suggested a possible direct protein-protein interaction between the two factors as the mechanism responsible for the functional cooperativity, and, indeed, it was shown that Pit-1 and GATA2 can physically interact in the absence of DNA by GST pulldown experiments *(106)*.

In summary, two transcription factors, Pit-1 and GATA-2, acting together are necessary for basal activation of the TSHβ gene. Enhanced stimulatory activity requires both protein-DNA and protein-protein interactions at the P1 element. Thus, activation of a highly restricted gene in thyrotropes is due, in part, to the combinatorial synergism between a POU homeodomain and zinc finger transcription factor, neither of which is unique to the thyrotrope cell.

Several studies have demonstrated the participation of Pit-1 in the stimulation of the TSHβ gene by hormonal regulation by TRH and cAMP/phorbol esters. On the rat TSHβ gene, elements conferring hormonal responsiveness to TRH, cAMP, or protein kinase C mapped to the region between −520 and −204 *(107)*. Within this region are three Pit-1 sites termed TSH A, B, and C. The A and C regions by themselves could mediate TRH- and PMA-stimulated responses (phorbol ester) when fused to a heterologous TK promoter *(108)*, and the effect could be abolished by mutations to disrupt the consensus Pit-1 sites. Additionally, the C region could mediate basal TSHβ promoter activation by Pit-1 *(101)*. Similar studies were performed with the −128/+8 region of the human TSHβ gene in which cotransfected Pit-1 was able to restore responsiveness by forskolin and 8 bromo cAMP in heterologous 293 human kidney cells or in GH3 cells *(100,109)*. The stimulatory effect by TRH, phorbol esters, or cAMP analogs were mediated by both a Pit-1 site in the proximal 128 bp of 5′ flanking DNA and an activator protein-like factor binding to an element around the transcriptional start site *(110)*. However, both factors do not form heterodimers nor does each factor modify the binding of the other. Thus, the TSHβ gene contains a unique set of Pit-1 binding sites that can mediate both TRH and cAMP responsiveness.

Other Pit-1-Dependent Genes

Several other genes that are also expressed in other tissues appear to be dependent on Pit-1 for their expression in the pituitary. The thyroid receptor β2 isoform, which exhibits high mRNA levels in thyrotrope- and somatotrope-derived cells *(111,112)* but is expressed at low levels in other tissues *(113)*, contains multiple Pit-1 binding sites within the 5′ region adjacent to the putative start site of translation *(114)*. Of the nine sites of Pit-1 interaction that are present, only two, when mutated, appear to decrease promoter activity in transient transfections of cultured thyrotrope and somatotrope cells *(115)*. These same mutations also affected the ability of cotransfected Pit-1 to stimulate TRβ2 promoter activity in αTSH cells, a thyrotrope-derived cell line that lacks both endogenous Pit-1 and TRβ2 *(115)*. It is currently not known whether Pit-1 interacts with other factors to achieve the differential pituitary cell expression. Interestingly,

however, different promoter areas support activity in the different cell types and extracts derived from cultured thyrotrope, and somatotropes appear to generate differential patterns of protection of the promoter area in DNase footprinting assays *(115).*

Recently the human gene for growth hormone–releasing hormone (GHRH) receptor, which is expressed in the renal medulla as well as pituitary somatotropes, has been cloned *(116),* and its 5′ flanking region contains several Pit-1 motifs. The basal activity of GHRH receptor promoter fusion constructs in GH4 cells and the ability of a recombinant Pit-1 to stimulate promoter constructs in COS-7 cells were both reduced when a region containing the most proximal Pit-1 motif was deleted, suggesting a possible role for Pit-1 in the expression of this pituitary-expressed gene.

SUMMARY

The POU-homeodomain transcription factor Pit-1 is required for differentiation of the pituitary cell types that produce GH, PRL, and TSH. Expression of Pit-1 in the anterior pituitary is complex and differs between species. Pit-1 transcripts are detected in all five cell types (somatotropes, lactotropes, thyrotropes, corticotropes, and gonadotropes) in mouse and porcine pituitaries, and protein expression is limited to somatotropes, lactotropes, and thyrotropes in which it precedes the appearance of and is required for specific hormone expression. Selective hypopituitarism caused by a loss of Pit-1 function by naturally occurring mutations in mice and humans underscores its importance for the development of the three dependent cell types. Structural studies based on informative mutational analysis have shed light on the modular nature of Pit-1 and have shown that it contains an N-terminal activation region as well as a DNA-binding domain, composed of both the POU-specific domain and the POU-homeodomain, that recognizes a core consensus sequence (TATNCAT) in the promoters of target genes. Variant isoforms of Pit-1 owing to splicing differences have been reported in several species, and a specific variant that contains a 14 amino acid insert in the activation domain (PIT-1T) was detected only in thyrotrope-derived cells. Pit-1 is required to activate genes that are specific to a particular pituitary cell and as such different mechanisms involving accessory factors have been invoked to account for the differential action of Pit-1 on these genes. Thus, Pit-1 synergizes with the zinc finger proteins Zn-15 and GATA-2 on the GH and TSHβ subunit promoters, respectively, whereas its action on PRL appears to require an ETS-related factor. In conclusion, Pit-1 plays a diverse role in the pituitary gland. It is required for both the emergence and maintenance of specific cell lineages, the latter function being accomplished by interaction with different cell-restricted conspirators.

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5 Subnuclear Trafficking of Glucocorticoid Receptors

General Mechanisms and Specific Recruitment to a Unique Target Site by Tethering to a DNA-Bound POU Domain Protein

Uma R. Chandran, PhD
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CONTENTS

INTRODUCTION

All members of the steroid hormone/nuclear receptor superfamily function as transcriptional regulatory proteins and have the capacity to interact specifically with select target genes *(1)*. Although many mechanistic aspects of the nuclear receptor–regulated transcription have been elucidated over the past 15 yr *(2)*, our understanding of how this process is efficiently orchestrated in a crowded nucleus remains limited. How do receptors locate their target sites within native chromatin? Are nuclear receptors free to "diffuse" throughout the nucleus in search of high-affinity sites, or is their trafficking restricted through a distinct set of subnuclear compartments? Which factors regulate

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receptor trafficking within the nucleus? This chapter focuses initially on global mechanisms of steroid receptor subcellular trafficking with a particular emphasis on nucleocytoplasmic shuttling and nuclear export of the glucocorticoid receptor (GR). Subsequently, our discussion shifts to a novel mechanism of GR transrepression in which GR recruitment to a unique target site is mediated not by specific DNA binding, but by site-specific tethering of the receptor to a DNA-bound POU domain transcription factor.

NUCLEOCYTOPLASMIC SHUTTLING OF STEROID RECEPTORS

Steroid receptors belong to a class of proteins that have the capacity to shuttle between the nuclear and cytoplasmic compartments *(3–6)*. This property of steroid receptors, although postulated to exist as early as 1972 *(7,8),* was not definitively established until the utilization of sophisticated cell biological techniques nearly 20 yr later *(3–6)*. This largely ignored aspect of steroid receptor function contributed to discrepancies that persisted for many years regarding the subcellular distribution of unliganded steroid receptors (see ref. *9* for historical perspective). Although conflicting reports of unliganded steroid receptor localization had been attributed to differences in fixation and/or antibody preparations, such arguments are irrelevant, given the nucleocytoplasmic shuttling capacity of steroid receptors.

Unliganded (and liganded) steroid receptors are not confined to either the nuclear or cytoplasmic compartment, but, rather, cycle between the nucleus and cytoplasm utilizing the bidirectional transport capacity of the nuclear pore complex (NPC) *(10–12)*. As discussed more thoroughly in a recent review *(9),* the relative overall rates of receptor import into and export from the nucleus is likely to be composed of multiple discrete steps, any one of which may be rate limiting under certain conditions. The accumulation of receptors within any given compartment is therefore governed by the precise step in receptor nucleocytoplasmic shuttling that is rate limiting. Thus, receptors preferentially accumulate within the cytoplasm when nuclear import is rate limiting, whereas a limitation in the rate of nuclear export leads to predominant nuclear localization of receptors.

Transient heterokaryon assays were instrumental in establishing the nucleocytoplasmic properties of steroid receptors *(3–6)*. Although it is difficult to obtain precise kinetic measurements of receptor import or export with these assays, hormone binding did not appear to affect the nucleocytoplasmic shuttling of the progesterone receptor (PR) and estrogen receptors (ER) *(4,5)*. It is difficult to assess hormone effects on nuclear export of GRs given the fact that unliganded GRs do not typically accumulate within nuclei to an appreciable extent *(13–15)*. Nuclear export of steroid receptors appears to be a relatively slow process ($t_{1/2}$ ~ 2–4 h) and is rate limiting for both PR and ER irrespective of their hormone occupancy status *(4,5)*.

It is well established that hormone binding leads to the high-affinity binding of steroid receptors to chromatin, whereas unliganded receptors are only loosely associated with nuclei *(16–18)*. Thus, the ability of steroid receptors to export from nuclei in hormone-treated cells implies that receptor association with chromatin must be dynamic. In a pioneering study of GR recycling *(19),* the kinetics of hormone dissociation from GR was postulated to be directly correlated with the kinetics of receptor recycling. Unfortunately, the relationship between hormone dissociation and chromatin release

of GR was not addressed in that study *(19)*. More recent analysis of the effects of GR on chromatin remodeling more directly reveal a transient association of receptors with chromatin *(20)*. What is the impact of transient associations of steroid receptors with chromatin on their nuclear export? We hypothesize that receptors become accessible to the nuclear export machinery only when liberated from high-affinity interactions with chromatin. Thus, even in cells exposed to hormone, receptors must have some capacity to release from chromatin, owing to either hormone dissociation or turnover of the RNA polymerase II preinitiation complex *(2)*. Since steroid receptors may remain associated with the preinitiation complex for multiple rounds of transcription *(21),* it is unclear exactly how receptor processing within the nucleus (which can lead to nuclear export or targeted degradation) is coupled to its participation in regulated transcription.

STEROID RECEPTOR INTERACTIONS WITH THE NUCLEAR MATRIX

Steroid receptors have long been known to have some association with the nuclear matrix and, in fact, were the first transcription factors found to bind to the nuclear matrix *(22)*. Both the ligand-binding domain (LBD) and DNA-binding domain (DBD) appear to contribute to nuclear matrix binding of steroid receptors *(23,24)*. Furthermore, we have shown that receptor targeting to the matrix does not represent a terminal step in trafficking, or processing, of nuclear receptors since they appear to exchange dynamically between the nuclear matrix and the soluble phase of the nucleoplasm *(24)*. A transient association with the nuclear matrix is likely to apply to many transcription factors that differentially partition between soluble and insoluble nuclear fractions *(25–27)*. Adenosine triphosphate (ATP) appears to be required for the release of steroid receptors from the matrix but not for their binding to the matrix *(24)*. The ATP dependence of steroid receptor release from the nuclear matrix has rendered it difficult to establish whether more distal steps in nuclear receptor nuclear export (e.g., movement through the NPC) are energy dependent.

IN VITRO NUCLEAR EXPORT OF GR

Hormone withdrawal, which leads to a rapid release of bulk GRs from chromatin, is not associated with rapid nuclear export of receptors *(28)*. In this case, unliganded nuclear GRs accumulate within a low-affinity nuclear compartment that could represent a novel nuclear export staging area *(28)*. Differential biochemical extractions established that this low-affinity compartment was not associated with the nuclear matrix or chromatin. Thus, the association of GR with chromatin or the nuclear matrix is not solely responsible for its relatively slow nuclear export. Chromatin-released GRs, which are competent to export from nuclei in vitro, do not collect at the nucleoplasmic face of the NPC *(28)*. This observation suggests that delivery of receptors to the NPC limits the rate of receptor nuclear export.

What mechanisms do steroid receptors utilize to gain access to the nuclear export machinery? Nuclear export of chromatin-released GR in vitro is stimulated by group VI-A transition metal oxyanions such as molybdate and tungstate *(28)*. This in vitro stimulation of nuclear export requires ATP, but not guanosine 5′-triphosphate, hydrolysis and is dependent on the prior release of receptors from high-affinity interactions with chromatin. Furthermore, receptors accumulate at the NPC in this system if the transport-

ing function of the NPC is blocked by wheat-germ agglutinin in the presence of molybdate *(28)*. Thus, we hypothesize that metal oxyanions accelerate the trafficking of receptors from a nuclear export staging area to the NPC.

Other shuttling proteins are likewise stimulated to export from nuclei in vitro by metal oxyanions *(28)*, implying that effects of these compounds might be directed at components of the nuclear export machinery that are preferentially utilized by shuttling proteins. We have not assessed whether RNA export is likewise accelerated by metal oxyanions in vitro. The mechanism of stimulated export in this system appears to involve a tyrosine phosphorylation event since metal oxyanion effects on in vitro GR nuclear export were blocked by tyrosine kinase inhibitors *(28)*. The precise targets of metal oxyanion effects that contribute to accelerated nuclear export in vitro have not been identified.

NUCLEAR EXPORT SIGNAL SEQUENCES

How are receptors targeted to the NPC for nuclear export? Three distinct nuclear export signal sequences (NESs) have been identified, two of which are unique to proteins of the hnRNP family *(29)*. A more common NES that has been identified in a variety of shuttling proteins comprises a leucine-rich sequence *(30,31)*. Recent studies have identified a class of proteins called exportins that are receptors for leucine-rich NESs *(32–34)*. Different members of the exportin family may prefer unique NES-containing substrates *(35)*. A large number of proteins in the database possess matches to this type of NES, making it unlikely that the presence of this sequence alone is sufficient for nuclear export *(35)*. The rat GR possesses a leucine-rich sequence with a good match to a prototypical NES just carboxyl terminal to its DBD. However, the DBD alone of rat GR was shown to export in transient heterokaryon arrays *(6)*, suggesting that this leucine-rich segment is not absolutely required for GR nuclear export. Even if this or other leucine-rich segments of steroid receptors function when isolated as NESs, it will be important to establish that these segments function as NESs in their native context. The relatively slow kinetics of steroid receptor nuclear export also imply that these proteins do not utilize leucine-rich NES. The nuclear export of proteins that utilize leucine-rich NESs is typically much more rapid than the export of steroid receptors *(29)*.

The NESs of hnRNP proteins are distinguished from leucine-rich NESs by their ability to function as nuclear import signal sequences *(36)*. hnRNPs do not contain the prototypical basic amino acid NLS and utilize a unique member of the importin-β family, (i.e., transportin) for nuclear import *(29)*. Transportin recognizes what has been termed the M9 NLS of hnRNP A1, which is also the signal sequence that targets hnRNP A1 for nuclear export *(36)*. Basic amino acid NLSs, which have been shown to exist within all steroid receptors, do not appear to possess NES activity *(36)*, although this remains controversial *(37)*.

ROLE OF MOLECULAR CHAPERONES IN SUBCELLULAR TRAFFICKING OF STEROID RECEPTORS

Using both in vivo and in vitro approaches, we have shown that nuclear import of GRs is influenced by receptor interactions with the 90-kDa heat-shock protein, hsp90 *(38,39)*. These results were predicted from earlier studies of Picard and Yamamoto

(13) who had observed a constitutive nuclear localization of carboxyl-terminal-deleted GRs. Hsp90 associates with GR through its carboxyl-terminal LBD *(40)*. Although, hsp90 can be engineered to cotransport with GRs into nuclei *(41)*, stabilization of native GR/hsp90 complexes in vivo impairs receptor nuclear import *(39)*. Thus, cytoplasmic-to-nuclear transport of steroid receptors may require the appropriate assembly and disassembly of multichaperone complexes that dynamically associate with the receptors *(42)*. Furthermore, steroid receptors may only acquire the competence to interact with the nuclear import machinery once a specific heteromeric assembly has been formed.

GR-associated molecular chaperones, such as hsp90 and the 52/54-kDa immunophilin, FKBP52/54, have been hypothesized to participate in the directed movement of steroid receptors to the NPC along a cytoplasmic scaffold *(43)*. However, this view is not supported by the lack of effects of cytoskeletal-disrupting agents on nuclear import of PR *(44)*. A different molecular chaperone, the 70-kDa heat-shock protein hsp70, had been proposed to assist in steroid receptor nuclear import based primarily on its demonstrated role in nuclear import of other karyophiles *(45,46)*. However, hsp70 was found not to be required for hormone-dependent nuclear import of GR in vitro *(38)*, implying that a requirement for hsp70 in nuclear import may not be universal *(38)*.

Could molecular chaperones participate in subnuclear trafficking of steroid receptors? Although many heat-shock proteins were initially thought to reside exclusively within the cytoplasm, significant levels of these molecular chaperones also exist within the nucleus *(47,48)*. The accumulation of hsp70 within nucleoli of heat-shocked cells serves an important protective function, particularly for the maintenance of ribosome structure and biogenesis *(49)*. Furthermore, nucleolar hsp70 protects the mRNA export pathway in yeast from irreversible damage under conditions of thermal stress *(50)*.

In addition to these protective functions, heat-shock proteins may affect the functioning of transcription factors within the nucleus of non-stressed cells. For example, hsp90 has been shown to affect the in vitro DNA-binding activity of the basic-loop-helix transcription factors MyoD and E12 *(51,52)*. Likewise, hsp70 was found to stimulate specific DNA-binding activity of ER *(53)*. The stimulatory effect of hsp70 on in vitro DNA binding does not appear to extend to other members of the steroid receptor family *(54)*. It is unclear whether these apparently conflicting results reflect a fundamental difference in the role of hsp70 in DNA binding of these different receptors or the use of different receptor preparations and assay systems to detect hsp70 effects on DNA binding. GRs purified from recombinant baculovirus-infected Sf9 cells are tightly associated with hsp70 and yet maintain specific in vitro DNA-binding and transcriptional activation activities *(55)*. Whether the association of hsp70 with GR is required for maximal DNA binding and/or transactivation activity of GR has not been established.

The possibility that heat-shock proteins have an impact on nuclear functions of steroid receptors has also been suggested by in vivo experiments in mammalian cells and yeast. GR-mediated transactivation is potentiated in transfected mammalian cells that are subjected to thermal or chemical stress *(56,57)*. The mechanism responsible for this heat-shock potentiation effect (HSPE) has not been established, although it appears to involve some factor (or factors) that is induced on heat shock *(58)*. The possibility that some heat-shock proteins are involved in HSPE has not been definitively examined.

Hormone-dependent transactivation activity of GR *(59)* and androgen receptor *(60)* is compromised in yeast strains possessing mutations in the DnaJ-homolog, Ydj-1.

DnaJ proteins possess inherent chaperone activity *(61)* but also associate with hsp70 and stimulate its ATPase activity *(62)*. Mutations in Ydj-1 did not affect the constitutive transactivation activity of LBD-deleted receptors *(59,60),* implicating a role for the Ydj-1 protein in some aspect of hormone-dependent signaling. This is consistent with the functioning of chaperones in assembly and maintenance of an active hormone-binding conformation of the LBD *(43,63)*. Since steroid receptor LBDs encode transactivation *(64)* and nuclear matrix–binding domains *(23,24),* effects of Ydj-1 on receptor transactivation may also be exerted within the nucleus.

Recent analysis of a rat GR DBD point mutant adds further support to the notion that molecular chaperone effects on steroid receptor function extend to the nucleus. An LBD-truncated GR that possesses a point mutation at a conserved arginine in the second zinc finger (i.e., R496) of the receptor DBD exhibited aberrant subnuclear trafficking *(65)*. Interestingly, mistargeting of this mutant GR was corrected on overexpression of a human homolog of the DnaJ family of molecular chaperones (i.e., HSDJ-2) *(65)*. HSDJ-2 was also found to correct transactivation and transrepression defects associated with the R496 mutation of rat GR, implying that this chaperone, or its associated partners, might serve a more general role in folding of nuclear receptors than previously appreciated. Additional studies will be necessary to evaluate fully the impact of molecular chaperones on targeting and transcriptional regulatory functions of steroid receptors and other nuclear proteins.

TARGETING OF GR TO UNIQUE TARGET SITES WITHIN THE GENOME: A NOVEL MECHANISM OF RECEPTOR TETHERING TO A DNA-BOUND POU DOMAIN TRANSCRIPTION FACTOR

In addition to the targeting of bulk receptors to chromatin, receptor interactions with unique target sites within chromatin initiate a process of chromatin modification that ultimately allows access of factors required to bring about efficient transactivation *(66)*. However, there appears to be no single unifying mechanism that fully accounts for site-specific recruitment of steroid receptors under conditions of transcriptional repression. Unlike the retinoic acid receptors (RARs) and thyroid hormone receptors (TRs), which can silence gene transcription when unliganded *(1),* GRs repress transcription in the presence of ligand *(67)* and, in some cases, in the presence of antagonist *(68)*. Although silencing of transcription by RARs and TRs is mediated by receptor interactions with corepressors *(69),* it is unclear whether corepressors function in steroid receptor transrepression.

In general, GRs activate transcription when bound as dimers to consensus glucocorticoid response elements (GREs). However, mutant GRs unable to dimerize are still capable of mediating transcriptional repression *(68)*. In fact, GRs repress transcription via a number of different mechanisms that require either direct DNA binding by the receptor to negative GREs (nGREs) *(70)* or the interaction of receptors with other transcription factors *(71–73)* and/or coactivators *(74)* in the apparent absence of direct receptor DNA binding. The activity of several transcriptional factors is negatively affected by GR, including c-*fos* and c-*jun (71–73),* GATA-1 *(75),* Nurr 77 *(76),* NF-κB *(77),* and Oct-1 *(78)*. Thus, glucocorticoids are involved in cross talk with various signal transduction pathways that utilize diverse transcription factors. In cases in which transcriptional repression is brought about by direct interactions between GRs and other

transcription factors in solution, it is difficult to envisage how the selectivity of this process is attained.

Our laboratory has demonstrated that glucocorticoids repress transcription of the gonadotropin-releasing hormone (GnRH) gene in the GT1–7 hypothalamic cell line *(79)*. This repression is mediated via two elements—the distal and proximal nGREs—neither of which binds GR directly. Neither the distal nor proximal nGRE possesses a consensus or composite nGRE binding site. However, gel mobility shift assays demonstrate that GR is present in the nuclear protein complex formed at this site in vitro *(79)*. We have found that Oct-1, a member of the POU-domain family of transcription factors, binds directly to the distal nGRE; however, the identity of the protein (or proteins) that specifically interacts with the proximal nGRE remains unknown *(79)*.

Electrophoretic gel shift assays with crude GT1–7 cell nuclear extracts showed that GR was included in an Oct-1-containing multiprotein complex formed on the distal nGRE in vitro *(79)*. Recently, we found that purified GR interacts with purified Oct-1 bound to the GnRH distal nGRE *(80)*. Thus, GRs may be directed to the GnRH gene to bring about transcriptional repression by direct interactions with a DNA-bound transcription factor. This mechanism of GR-mediated transcriptional repression is distinct from those established with other transcription factors in which direct interactions with GR had been observed only in solution *(71–73,75–78)*. Furthermore, this observation provides a novel example in which the transcriptional repression property of GR is targeted to a specific genomic site by tethering of the receptor to a specifically bound transcription factor.

Both positive and negative effects of GR on Oct-1-directed transcription have been observed. Simultaneous direct binding of GR and Oct-1 to separate sites is required for activation of the mouse mammary tumor virus promoter by GR *(81)*, but there is no evidence for a direct interaction between DNA-bound GR and Oct-1. Glucocorticoid repression of histone H2b gene transcription is apparently brought about by the association of GR with Oct-1 in solution *(78)*, which eliminates Oct-1 binding to the histone H2b promoter. Note that the Oct-1-binding site in this case does not appear to be cooccupied by GR and Oct-1 *(78)*, as we have shown for the GnRH distal nGRE *(80)*. The interaction between Oct-1 and GR in solution requires the receptor DBD *(78)*. The identification of GR domains involved in interactions with DNA-bound Oct-1 at the GnRH distal nGRE remains to be determined.

How do we account for the clear distinctions between GR repression of transcription of histone H2b that involves GR/Oct-1 interactions in solution *(78)* versus repression of GnRH gene transcription in which GR associates with DNA-bound Oct-1 *(80)*? The relatively weak binding of Oct-1 to the distal nGRE appears to be an essential feature of the mechanism of glucocorticoid repression. When the distal nGRE is mutated to increase Oct-1-binding affinity, glucocorticoid repression in transfected GT1–7 cells is hampered *(80)*. Furthermore, GR does not associate as effectively in vitro with tightly bound Oct-1 at the Oct-1 consensus site nGRE *(80)*. We hypothesize that DNA-bound Oct-1 may adopt different conformations depending on the precise nature of its recognition sequence. Interactions between DNA-bound Oct-1 and different coactivators or accessory factors has been shown to be dictated by the precise DNA contacts made by Oct-1. For example, the ability of B-cell-specific coactivator, OCA-B, to activate transcription from Oct-1-bound promoters is selective and occurs only at some octamer sites and not others *(82)*. This observation, therefore, provides another level of control

of steroid receptor targeting, in that not all DNA-bound Oct-1 will be able to recruit GRs efficiently.

SUMMARY

Numerous mechanisms account for selectivity of steroid hormone action. Specific DNA binding by receptors may not be the only mechanism that accounts for selective targeting of receptors to appropriate target sites in vivo. Receptors may be directed to unique target sites by their interactions with DNA-bound transcription factors. These protein-protein interactions may furthermore be influenced by factors (i.e., precise DNA-binding site, molecular chaperones) that impact the conformation adopted by DNA-bound transcription factors. Superimposed on these direct or indirect high-affinity interactions of receptors with their target sites may be unique subnuclear trafficking pathways that facilitate receptor scanning of the genome. Future developments in the nuclear receptor field are likely to yield important insights into the molecular mechanisms that bring about selective transcriptional regulatory effects of these receptors in physiologically relevant settings.

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6 Thyroid Hormone Receptors and Their Multiple Transcriptional Roles

Paul M. Yen, MD

Contents

INTRODUCTION

Thyroid hormone (TH) (thyroxine L-T_4, triiodothyronine L-T_3) has myriad effects on cellular growth, development, and metabolism. TH exerts its major effects at the genomic level, although there are examples of nongenomic action in the cytoplasm, plasma membrane, and mitochondrion. Early observations clearly established that TH could bind to nuclear sites and stimulate transcription and translation of new proteins *(1–3)*. These important early observations and subsequent work from many laboratories led to a general model for genomic TH action. As seen in Fig. 1, circulating free TH enters the cell by passive diffusion and, in some tissues, is converted from T_4 to the more biologically potent hormone T_3. TH then enters the nucleus and binds to nuclear receptors (TRs) with high degrees of specificity and affinity. TRs have been shown to be intimately associated with chromatin, and ligand binding to TRs stimulates transcription of target genes and subsequent protein synthesis in a concentration- and time-dependent manner. Although the critical role for TRs in TH action was established early, the identification and characterization of TRs remained elusive until just over a decade ago.

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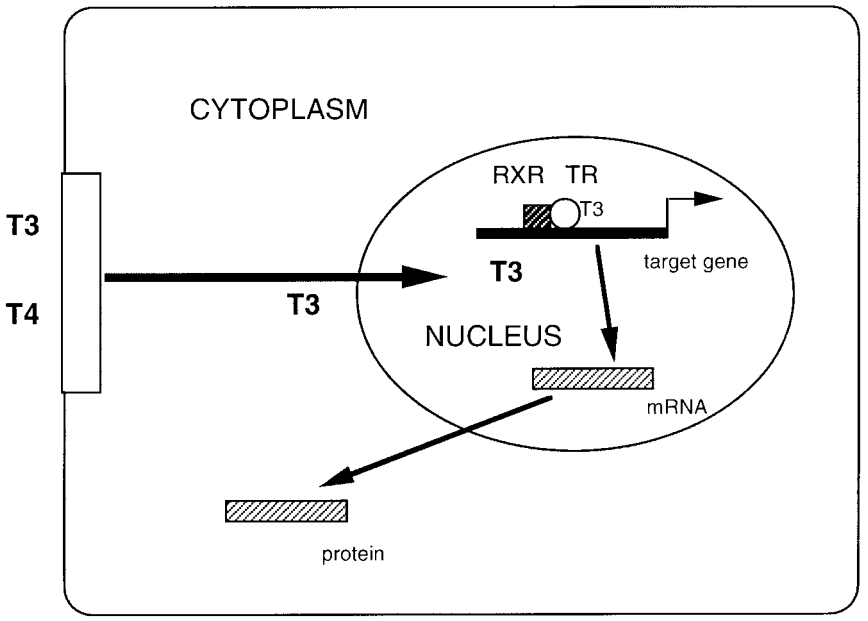

Fig. 1. General model for thyroid hormone action in the nucleus.

TRS AND THEIR STRUCTURE

In 1986 two groups independently cloned cDNAs encoding TRs from embryonal chicken and human placental cDNA libraries *(4,5)*. This ground-breaking work provided the important first step toward understanding the role of TRs at a molecular level. Several important observations stemmed from these and other early studies. First, it was apparent that TRs were the cellular homologs of a previously described viral oncogene product, v-erbA, that caused erythroblastosis in chicks. Second, TRs were members of a large superfamily of nuclear hormone receptors including the steroid hormone receptor, vitamin D receptor (VDR), and retinoic acid receptor (RAR). This was surprising since these receptors bound structurally different ligands. This homology not only occurred at the amino acid level but also in the domain structure of the superfamily of nuclear hormone receptors (Fig. 2).

Similar to its related family members, TRs contain a central DNA-binding domain (DBD) with two zinc finger motifs and a carboxy-terminal ligand-binding domain

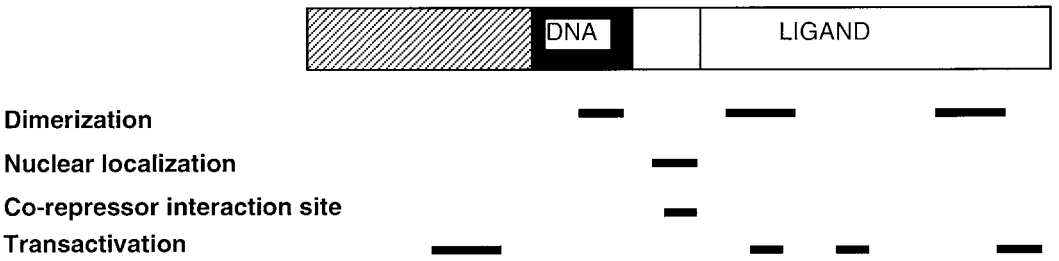

Fig. 2. General organization of major TR domains and functional subregions.

(LBD) *(6,7)*. Within the DBD, the first zinc finger contains a "P-box" region similar to that of estrogen receptor, retinoic acid receptor (RAR), and retinoid X receptor (RXR). This critical region is important for sequence-specific recognition of hormone response elements by different members of the nuclear hormone superfamily. The LBD is required for TH binding but also possesses subregions for transactivation and dimerization. Recent X-ray crystallographic studies of liganded TR suggest that TH is embedded in a hydrophobic pocket surrounded by discontinuous stretches of the LBD *(8)*. At the extreme carboxy terminus is a subregion important for ligand-dependent transcriptional activation function-2 (AF-2) *(9–11)*. This subregion is highly conserved among nuclear hormone receptors. Furthermore, X-ray crystallographic studies of RAR have suggested that this region may undergo major conformational changes on ligand binding *(12)*. It likely serves as a major contact surface for interaction with ligand-dependent coactivators (*see* below). In the LBD there are at least nine hydrophobic, heptad repeats that potentially may be involved in TR homo- and heterodimerization *(13,14)*. Mutations in the ninth heptad region, which abrogated TR heterodimerization, have suggested that this may be a particularly important region for dimerization *(15,16)*. Similar to observations for other nuclear receptors, the amino-terminal region may contain a constitutive ligand-independent AF-1 transactivation domain *(17,18)*. In analogy with steroid hormone receptors, this region also could participate in cell- or target-gene specificity. The hinge region between the DBD and TH-binding domain likely contains a nuclear localization motif common among nuclear hormone receptors *(19)*. However, unlike steroid hormone receptors, which associate with cytoplasmic heat-shock proteins in the absence of ligand, TRs are localized predominantly in the nucleus and bind DNA even in the absence of ligand. Furthermore, recent work has suggested that critical residues within the hinge region may be important for interaction with corepressors to mediate repression of basal transcription in the absence of ligand ([*20,21]; see* "Basal Repression by Unliganded TR").

MULTIPLE TR ISOFORMS

There are at least two genes encoding TRs, α and β, located on human chromosomes 17 and 3, respectively *(6)*. These genes encode TRs (TRs α-1, β-1, β-2) that bind T_3 with similar affinity (reported K_ds between 10^{-10} and 10^{-11} *M*) and mediate TH-regulated gene expression. These TR isoforms range from 400 to slightly more than 500 amino acids in size *(6,22)* among mammalian species, and contain highly conserved DBDs and LBDs (Fig. 3). The TRα gene generates two mature mRNAs by alternative splicing that encode two proteins: TRα-1 and c-erbA α-2 (*see* Chapter 7). These proteins are identical to amino acid residues 1–370 in rat, but their respective sequences diverge markedly thereafter (Fig. 2). Consequently, c-erbA α-2 is unable to bind TH because it contains a 122 amino acid carboxyterminus that replaces a region in TRα-1 that is critical for TH binding. In cotransfection experiments, c-erbA α-2 blocks the transcriptional activity of TRs. Thus, the TRα gene represents one of the first mammalian examples in which multiple mRNAs generated by alternative splicing–encoded proteins may be antagonistic to each other. Another interesting feature of the TRα gene is the employment of the opposite strand to encode a gene product, rev-erbA. Rev-erbA mRNA contains a 269-nucleotide stretch that is complementary to the c-erbA α2 mRNA owing to transcription from the opposite DNA strand used to generate TRα-1 and

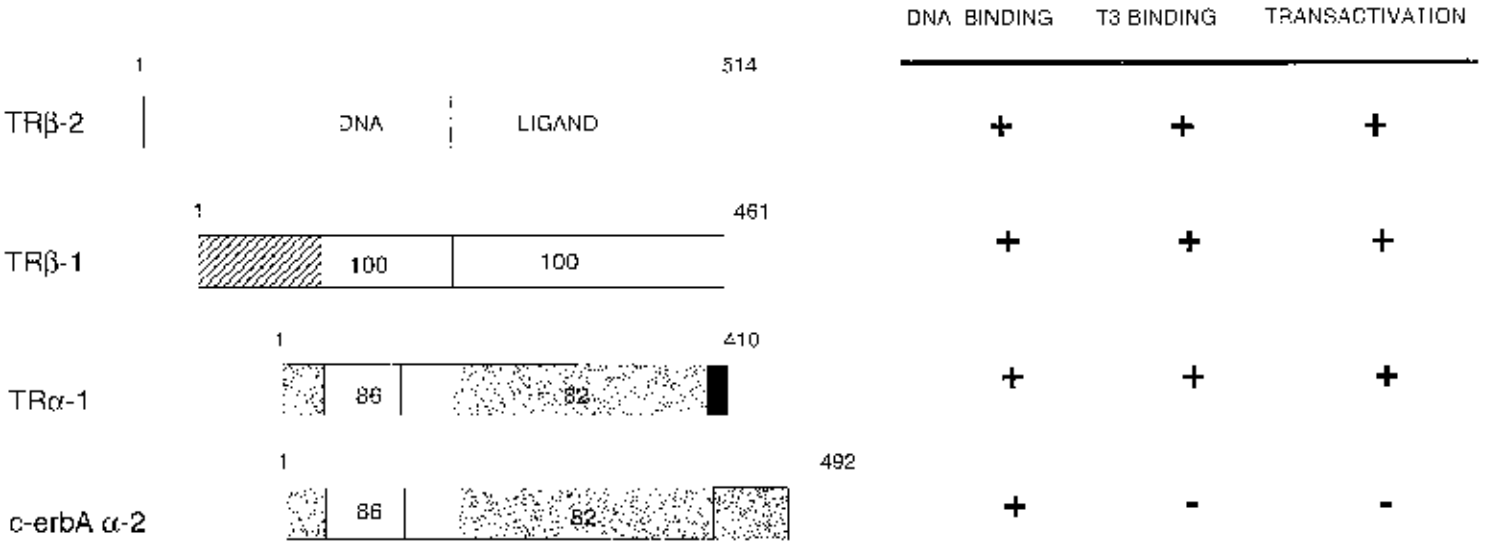

Fig. 3. Comparison of amino acid homologies and their functional properties among TR isoform. Length of receptors is indicated just above receptor diagrams and percentage of amino acid homology with TRβ-2 is included in the receptor diagrams.

c-erbA α2 *(23,24)*. This protein also is a member of the nuclear hormone receptor superfamily and has no known ligand. It is expressed in adipocytes and muscle cells and can bind to TH response elements (TREs) and retinoic acid response elements (RAREs) *(25,26)*.

The TRβ gene encodes two TRs, TRβ-1 and TRβ-2, that likely are generated by alternative promoter use or RNA splicing *(27,28)*. These receptors differ in their respective amino-terminal regions but otherwise are identical. They appear to have similar TH-binding affinity and transcriptional activity. Whereas TRα-1 and TRβ-1 are expressed in almost all tissues, TRβ-2 is selectively expressed in the anterior pituitary gland and specific areas of the hypothalamus as well as the developing brain and inner ear *(29–31)*.

The specific roles of these isoforms are poorly understood, and as suggested by knockout mouse studies, there may be a certain degree of redundancy *(32–34)*. Recent studies suggest that TRβ-2 may be involved in the negative regulation of anterior pituitary genes such as thyroid stimulating hormone (TSH) *(35)* and that TRβ may play important roles in brain and inner-ear development (*[29,30,32]; see* also Chapter 7). There also appear to be important differences in the pattern of expression of these isoforms in development since TRα-1 mRNA is expressed early in brain development whereas TRβ-1 mRNA is induced shortly before and after birth *(36)*.

The regulation of the TR mRNAs is isoform- and cell-type dependent. T_3 decreases TRβ-2 mRNA, modestly decreases TRα-1 mRNA, and slightly increases rat TRβ-1 mRNA in the intact rat pituitary *(37)*. The net result, however, is a 30% decrease in total T_3 binding in the T_3-treated rat pituitary. In other rat tissues, T_3 slightly decreases TRα-1 and c-erb α2 mRNA except in the brain, where c-erbA α-2 levels are unaffected. TRβ-1 mRNA is minimally affected in nonpituitary tissues. Interestingly, in patients with nonthyroidal illness who had decreased free T_3 and T_4 serum levels, TRα and TRβ mRNAs were increased in peripheral mononuclear cells and liver biopsy specimens *(38)*. Thus, induction of TR expression may compensate for decreased circulating TH levels in some of these patients.

TH RESPONSE ELEMENTS

TRs are ligand-dependent transcription factors that bind to distinct DNA sequences generally in the promoter region of target genes. TRs generally positively regulate target genes by stimulating gene transcription in the presence of TH; however, they also can negatively regulate transcription in several cases (e.g., TSHβ, α-glycoprotein subunit, and thyrotropin-releasing hormone). Chapter 8 focuses on such negative regulation; therefore, in this chapter, I focus on the positive regulation of target genes.

In vitro binding and functional analyses of TREs from positively regulated target genes have demonstrated that TREs generally contain a hexamer half-site sequence of AGGT(C/A)A arranged with two or more repeats *(39)*. Thus, similar to steroid hormone receptors, TRs bind to TREs as dimers. However, unlike steroid hormone receptors that bind to two well-conserved half-sites arranged as palindromes, TRs bind to TREs that have considerable variation in their primary nucleotide sequence as well as the number, spacing, and orientation of their half-sites *(6,7,39,40)*. In particular, TRs have been shown to bind TREs in which half-sites are arranged as direct repeats, inverted palindromes, and palindromes (Fig. 4). Of the known 20–30 natural positive TREs that have been characterized, most are arranged as a direct repeat, followed by inverted palindrome, and then palindrome.

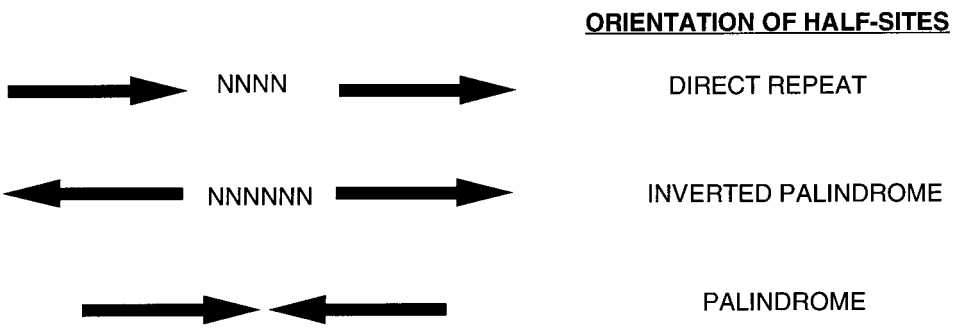

Fig. 4. Half-site orientations and optimal nucleotide spacing between half-sites. N refers to nucleotides and arrows indicate the direction of half-sites on the sense strand.

In simple TREs containing two half-sites, it has been shown that TRs preferentially bind to direct repeats, inverted palindromes, and palindromes separated by four, six, and zero nucleotides, respectively *(6,7,39,40)*. Several studies have shown that flanking sequences, the primary half-site sequence, and even the length of the half-site sequence itself can be variable. In the latter situation, octamer and decamer half-sites may function as well or better than the hexamer half-site, and at least in the case of the octamer half-site, a single half-site may be sufficient to mediate TH-stimulated transcription *(41,42)*. Although this diversity, even promiscuity, of DNA-binding sites for TRs may make predicting TREs difficult, it is a mechanism that potentially allows TH to regulate differentially a wide range of target genes on the basis of differing binding affinities to TREs. This ability to bind to such a wide range of TREs is greatly facilitated by the ability of TR to form heterodimers with RXR (*see* "TR Complex Binding to TREs"), allowing TRs to bind to TREs that contain degenerate half-site sequences, variable spacing of half-sites, and even a hybrid TRE/glucocorticoid response element *(6,7,39,40,43)*.

The palindrome and inverted palindrome TREs have symmetrical arrangements of half-sites whereas direct-repeat TREs have a 5′ to 3′ polarity relative to the minimal promoter. Thus, it is possible that the direct-repeat TRE may specify the orientation of TR/RXR heterodimer binding to the TRE. Recent studies strongly suggest that TR may bind to the downstream half-site and RXR to the upstream half-site when TR/RXR heterodimer binds a direct repeat of half-sites separated by four nucleotides (DR4) *(44–46)*. Furthermore, the orientation of such heterodimers relative to the minimal promoter, and hence transcriptional machinery, may be important since reversing the direction of the direct repeat relative to the minimal promoter decreases TH-dependent transcriptional activity *(46)*. These findings suggest that the proper orientation of TR/RXR heterodimers on TREs may be important for interaction with coactivators that link the liganded heterodimer with the transcriptional machinery (*see* following section). In further support of this notion, recent data suggest that some TREs are active only in the appropriate minimal promoter context, and thus do not function as simple enhancer sequences that function independently of orientation, position, and promoter *(47,48)*.

TR COMPLEX BINDING TO TRES

TRs bind to TREs as monomers, dimers, and heterodimers in vitro. However, the physiological relevance and roles of these putative complexes in transcriptional regula-

tion are only partially understood. TRβ-1 has a greater tendency than TRα-1 to bind as a homodimer to several different TREs, whereas TRα-1 binds more tightly as a monomer on some TREs, suggesting that these two TR isoforms may have different dimerization potentials *(49)*. Furthermore, the arrangement of half-sites also influences TR complex formation, because TR homodimers tend to form more readily on inverted palindromes whereas TR/RXR heterodimers form better on direct repeats *(45,50)*. A major difference between TRs and steroid hormone receptors is the TR's ability to bind to its hormone response element in the absence of ligand. As discussed subsequently, this has implications for the role of unliganded receptors in mediating repression of basal transcription.

It previously was observed that TR formed complexes with proteins from liver and pituitary nuclear extracts and that total DNA binding could be augmented by such interactions with TR auxiliary proteins (TRAPs) *(51,52)*. These findings were initially difficult to reconcile with the observations that steroid hormone receptors tended to bind as homodimers. However, several laboratories showed that RXR could heterodimerize with TR and RAR receptor, and augment their DNA binding and, in some cases, enhance their transcriptional activity (reviewed in refs. *6,7,* and *40*). These studies provided strong evidence that TRs could form heterodimers with TRAPs. Indeed, studies using anti-RXR antibodies showed that the major endogenous TRAPs are RXRs or related proteins *(53)*. Since there are at least three major isoforms of RXR, it is possible that different TR/RXR isoform complexes may have differential affinities for TREs and/or abilities to transactivate target genes (perhaps by differential recruitment or interaction with cofactors involved in transcription). The possibility for dual ligand activation of the heterodimer partner of TR introduces another potential layer of regulation because RXRs bind to 9-*cis* RA. In this connection, the addition of both 9-*cis* RA and T_3 augmented transcription via a rat growth hormone TRE-containing reporter *(54)*.

Further support of the role of the TR/RXR heterodimer came from the observation that T_3 binding to TR homodimers caused rapid dissociation from TREs (direct repeats and inverted palindrome) whereas it had a minimal effect on overall TR/RXR heterodimer binding *(55–57)*. T_3 binding did cause a small increase in heterodimer mobility on electrophoretic mobility shift assay, likely owing to ligand-induced conformational changes. These data stand in contrast to steroid hormone binding to their receptors, in which dissociation of heat-shock proteins and conformational changes promote homodimer binding to hormone response elements. These data argue strongly that liganded TR/RXR heterodimers remain bound to TREs, and hence are able to mediate transcription. The TR homodimers dissociate from TREs and thus are not available to participate in transcriptional activation. On the other hand, both TR homo- and heterodimers bind to TREs in the absence of ligand and therefore could be involved in repression of basal transcription by TRs (*see* "Basal Repression by Unliganded TR" and Fig. 5).

The formation of TR/RXR heterodimers may depend on the heptad repeats located in the LBD. In particular, the ninth heptad repeat seems to be particularly important for heterodimerization since artificial and natural TRβ-1 ninth heptad mutants from patients with resistance to thyroid hormone (RTH) have impaired heterodimerization with RXR *(15,16,58,59)*. Indeed, the crystal structure of the TRα-1 LBD demonstrates that there is a hydrophobic surface in this region that could serve as a potential dimerization interface. The region between amino acids 280 and 300 of TRβ-1 also appears to be an important region for dimerization since deletion or mutation of this

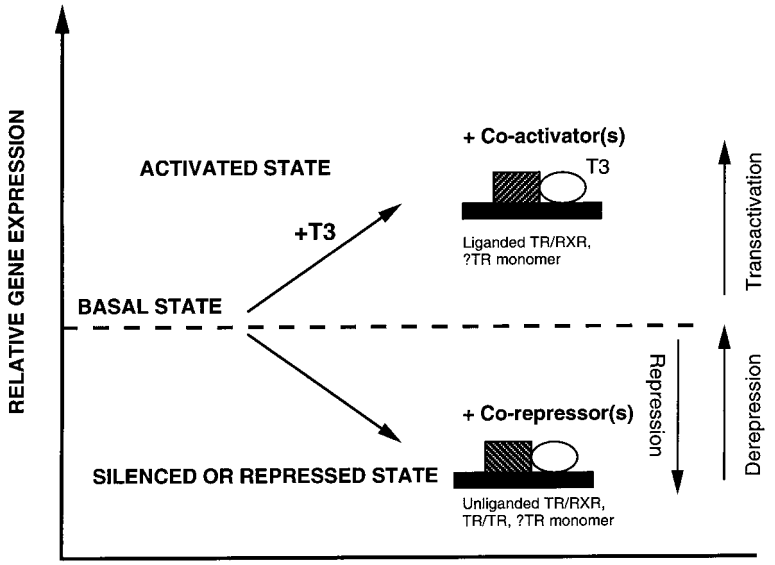

Fig. 5. Model for repression, derepression, and transcriptional activation by TR.

region abrogates heterodimer formation *(60)*. A natural mutant from a patient with RTH at amino acid 316 of the LBD also displayed decreased homodimer formation *(61)*. These results suggest that there may be multiple dimerization regions in the TR LBD. Additionally, X-ray crystal analysis of TR/RXR DBD heterodimer formed on a direct repeat with a gap of four nucleotides showed that there may be several contact points within the TR DBD and the second zinc finger of RXR *(62)*. These interactions may be important for dictating binding to direct repeats of a specific spacing (e.g., direct repeat with a gap of four nucleotides). Recently, it also has been shown that ligand promotes dimerization of TR and RXR in solution before binding to DNA *(63,64)*. Thus, ligand may promote the formation of heterodimers, which then can be recruited to TREs and, in turn, activate transcription. In addition, DNA binding promotes heterodimer formation as well as modulates the conformation of the TR/RXR complex *(65,66)*.

OTHER MODULATORS OF TR ACTION: RECEPTOR CROSS TALK AND PHOSPHORYLATION

Although RXRs appear to be the major heterodimer partner for TRs, there are examples of TR heterodimerization with RAR, VDR, and perioxisome proliferator-activated receptor on specific hormone response elements, suggesting that in some instances, heterodimerization with these receptors can modulate each other's transcriptional activity *(67–69)*. In other cases, TRs can bind readily to direct repeats separated by three and five nucleotides that are vitamin D response element and RARE, respectively *(70)*. TRs also can bind to the palindromic estrogen response element *(71)*. In these cases, TR is transcriptionally inactive or minimally active on these elements, and is able to block the transcriptional activity of the cognate receptor by competing for binding to the hormone response element. The possibility of receptor cross talk among nuclear receptors thus adds further complexity to this system *(72)*.

Phosphorylation has been shown to affect the DNA binding of several other transcription factors such as the glucocorticoid receptor; CREB; and c-Myb, c-Jun, and Max homodimers *(73)*. Several groups have shown that chick TRα-1 and human TRβ-1 can be phosphorylated in vivo and in vitro *(74–76)*. Additionally, in vitro phosphorylation of TRβ-1 enhanced binding to DNA, although the phosphorylation sites have not been determined. Also, phosphorylation alters the stability of TRβ-1 in certain tissues *(77)*. Furthermore, an increased phosphorylation state of TR correlated with enhanced transcriptional activity in cotransfection studies *(78)*. These results suggest that phosphorylation is another mechanism, besides TH binding, that can selectively affect TR complex binding to TREs and potentially modulate transcription. The in vivo phosphorylation sites and their functional significance, as well as the kinase pathways involved in TR phosphorylation, remain to be elucidated.

BASAL REPRESSION BY UNLIGANDED TR

Steroid hormone receptors are transcriptionally inactive in the absence of ligand. By contrast, unliganded TRs bind to TREs and can modulate transcription of certain target genes (Fig. 5). About 10 years ago, a few laboratories showed that unliganded TR repressed basal transcription of reporter plasmids containing positively regulated TREs *(79–81)*. This basal repression depended on TR binding to TREs as mutations in TRβ-1 DBD or the primary sequence of the TRE abrogated basal repression *(59,81)*. Recently, TRs have been shown to interact directly with TFIIB, a key component of the basal transcription machinery, in the absence of ligand *(82–85)*. Moreover, unliganded TRs can repress basal transcription in an in vitro transcription system *(86)*. These findings suggested that unliganded TR interactions with the basal transcriptional machinery could repress basal transcription. This action could be physiologically relevant in hypothyroid states and possibly in early development before the fetal thyroid gland is able to synthesize TH.

Several laboratories recently used the yeast two-hybrid system to clone proteins that interacted with TR and RAR only in the absence of their cognate ligands *(20,21,87,88)*. These proteins repressed basal transcription by TR and RAR, as well as via chimeric GAL4 proteins. They were named TH- and RAR-associated corepressors. One of these proteins, the nuclear receptor corepressor (N-CoR), was a 270-kDa protein that did not have significant homology with any other previously described protein *(21)*. It had two transferable repression domains and a carboxyterminal α-helical interaction domain. Additionally, it contained a putative zinc finger region, suggesting that N-CoR might bind to DNA. Recently, a truncated version of N-CoR, N-CoRI, which is missing the repressor region, was identified, and it may represent an alternative-splice variant of N-CoR *(89)*. This protein blocked basal repression by N-CoR and thus may serve as a natural antagonist for N-CoR. Another corepressor, the silencing mediator for RAR and TR (SMRT [silencing mediator for retinoid and thyroid hormone receptors]), is a 168-kDa protein that has some homology with N-CoR and an N-CoR splice variant, RIP-13 *(20,87,88)*. SMRT also repressed basal transcription in cotransfection studies. The hinge region of TR is important for interactions with these corepressors, because mutations in this region decreased interactions with corepressors and aborgated basal repression without affecting transcriptional activation *(20,21,90)*. Interestingly, rev-erbA contains two aminoterminal subregions that interact with N-CoR and are required

for basal repression, suggesting that there may be different corepressor interaction sites among nuclear hormone receptors *(91)*. Finally, a 16-kDa corepressor was recently identified that has similar functional properties as these other corepressors, but it does not share sequence homology with them *(92)*. It is expressed in most adult tissues, although it also is induced during myocyte and adipocyte differentiation. Moreover, this protein can interact with N-CoR and SMRT in vitro *(92)*. Currently, it is not known whether these different corepressors can form a corepressor complex or may have cell- or gene-specific roles.

Several groups recently showed that corepressors can complex with another putative corepressor, mSin3, and histone deacetylase *(93,94)*. These findings raise the interesting notion that local histone deactylation may play a critical role in basal repression by altering the local chromatin structure. Moreover, this mechanism of basal repression may be employed by other transcription factors such as Mad/Max heterodimers *(93,94)*.

LIGAND-DEPENDENT TRANSCRIPTIONAL ACTIVATION

For many years, there has been active interest in how TH stimulates transcription. As mentioned previously, many factors can potentially modulate TH-mediated transcription, including multiple TR isoforms, TR complexes, heterodimerization partners, the nature of TREs, and TR phosphorylation state *(95)*. It is likely that these factors influence liganded TR interactions with the basal transcriptional machinery either directly or via bridging proteins called coactivators. Several groups showed that unliganded TRs interacted directly with the general transcription factor, TFIIB *(82,84–86)*. Moreover, the interaction seemed to be ligand dependent, suggesting that changes in TR interactions with general transcription factors could play a role in derepression and transcriptional activation *(86,96)*. In support of this possibility, TR also has been shown to interact with TAFII 30 and TFIIB *(97,98)*. In addition, Petty and coworkers *(99)* have shown that TRβ interacts with several *Drosophila* transcription-activating factors (TAFs), particularly $TAF_{II}110$, and that the latter can augment TH-dependent transcription in cotransfection studies.

Recent studies using far-Western and coimmunoprecipitation approaches showed that liganded TR may interact with multiple nuclear proteins that potentially can form a transcriptionally active complex *(83,86,100)*. These observations raised the possibility that proteins that are not part of the basal promoter complex may be involved in ligand-mediated transcription *(83,100,101)*. In this connection, studies using cotransfection and in vitro transcription systems demonstrated the importance of specific minimal promoters in mediating TH-mediated transcription *(47,102,103)*. These studies suggested that adaptor proteins, or coactivators, may bridge the liganded TR complex with components of the minimal promoter. Recently, several groups identified proteins that interact with TR in a ligand-dependent manner and also participate in estrogen-dependent transcriptional activation *(98,104,105)*. Moreover, interaction with some of these proteins involved interaction with an intact AF-2 region located in the carboxyterminal part of the LBD. This region has high homology among many members of the nuclear hormone receptor family and has been shown to be important for ligand-dependent transcription for several receptors *(9,96,106,107)*.

O'Malley and coworkers *(108)* used a yeast two-hybrid system to clone a putative factor called steroid receptor coactivator-1 (SRC-1) that interacted with liganded proges-

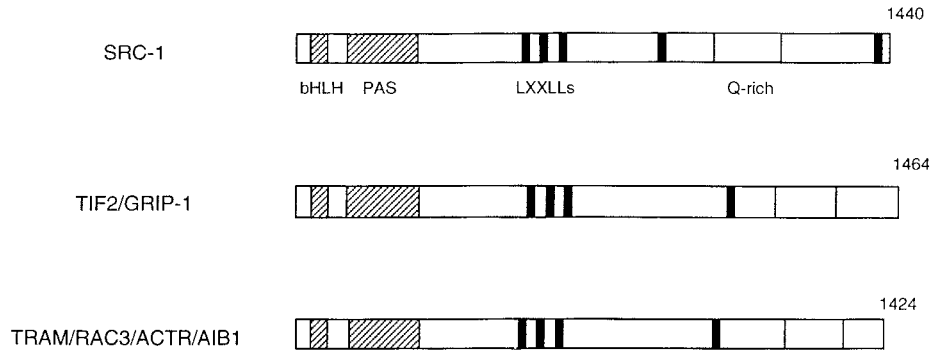

Fig. 6. Comparison of the organization and structure of putative nuclear hormone receptor coactivators.

terone receptor and enhanced ligand-dependent transcriptional activation. This protein also associated with TRs and several other members of the nuclear hormone receptor superfamily in a ligand-dependent manner, and enhanced their ligand-dependent transcription. Subsequent work has shown that the original cDNA clone was only partial and that the full-length clone encoded a 160-kDa protein *(108–110)* that may be one of the proteins described by Halachmi and colleagues *(105)*. Additionally, there may be alternative splicing of SRC-1 mRNA, leading to multiple SRC-1 isoforms *(110,111)*. Currently, the functional significance of the SRC-1 isoforms is not known although some splice variants may augment TR-mediated transcriptional activation more than others *(111)*. Another 160-kDa protein, TIF2/GRIP1, which interacts with liganded nuclear hormone receptors including TRs, has sequence homology with SRC-1, suggesting that there may be a family of coactivators related to SRC-1 *(112,113)*. Indeed, several other related 160-kDa proteins have been identified that interact with TRs or nuclear hormone receptors in a ligand-dependent manner and can augment ligand-dependent transcription (TRAM-1/ACTR/RAC3/AIB1) *(112–117)*. As seen in Fig. 6 there are several common features among these putative coactivators. First, there are multiple putative nuclear hormone receptor interaction sites that seem to bear a signature LXXLL sequence motif *(117,118)*. Second, several coactivators have a polyglutamine region, similar to androgen receptors. Third, in the aminoterminal region, there is a basic helix-loop-helix (bHLH) motif, suggesting that these coactivators may bind to DNA. Also located in this region is the so-called Per-Arnt-Sim (PAS) domain, which, interestingly, is also seen in several transcription factors that regulate circadian rhythm and in the heterodimer partner of the dioxin receptor *(119)*. Thus, the bHLH-PAS region may serve as a dimerization interface and potentially allow cross talk among other coactivators or transcription factors.

Moore and coworkers *(87,120)* recently identified several other proteins that interact with TRβ-1 LBD. These proteins, called TRIPs (TR-interacting proteins), are diverse; one of them is the human homolog of a yeast transcription factor, Sug 1, another is a new member of a class of nonhistone chromosomal proteins, and yet another contains a conserved domain associated with ubiquination of specific target proteins *(87,120)*. Cotransfection of plasmids encoding some of these proteins can augment TR-mediated transcriptional activation in yeast. Monden et al. *(121)* recently described a 120-kDa protein that has homology with skeletal muscle abundant protein, interacted with ligan-

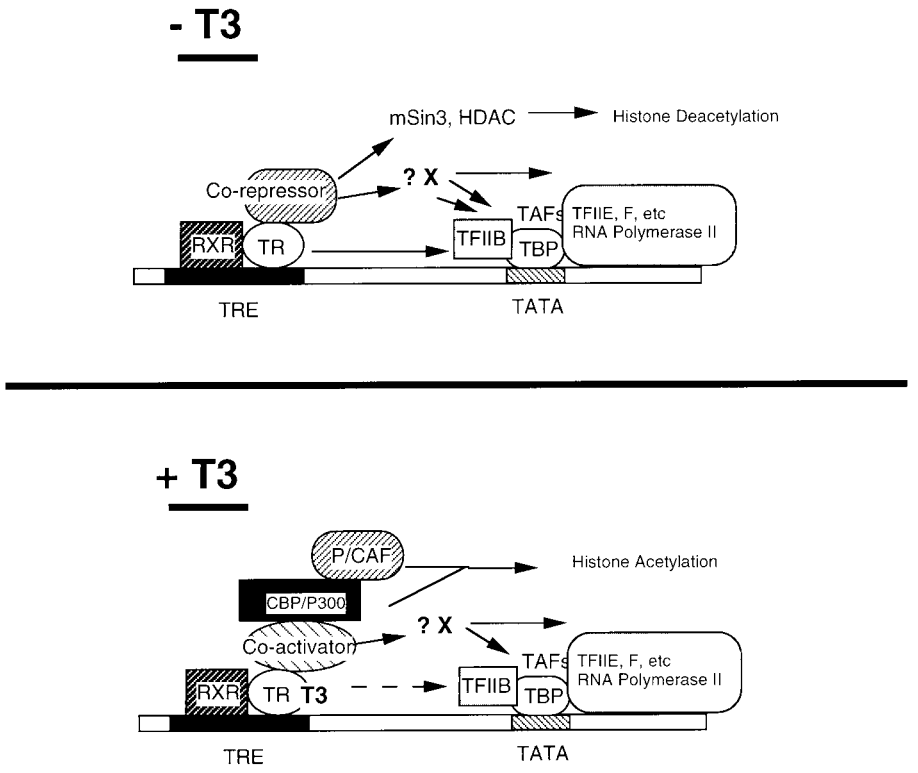

Fig. 7. Molecular model for **(A)** basal repression in the absence of T_3 and **(B)** transcriptional activation in the presence of T_3. X refers to possible additional cofactors that remain to be identified. See text for details.

ded TR, and enhanced ligand-dependent transcriptional activation. Finally, a number of other nuclear proteins have been identified by two-hybrid screening that interact with steroid hormone receptors and potentially may interact with TRs *(122)*. These findings suggest that there may be different families of adaptor proteins in addition to the SRC-1/TIF2 family. However, the functional significance of these proteins on TR-mediated transcription remains to be further defined.

The mechanism by which SRC-1 or other coactivators bridge the liganded TR to the basal transcription machinery is not known. Thus far there have not been any reports of their direct interaction with general transcription factors or TATA binding protein–associated factors. Recently, several groups showed that SRC-1 can interact with the cAMP-responsive element-binding protein (CBP), the putative coactivator for cAMP-stimulated transcription as well as the related protein, p300, which interacts with the viral coactivator E1A *(110,123,124)*. It is possible that this protein might serve as an integrator molecule for different signaling inputs such as protein kinase A- and protein kinase C-pathway-mediated transcription, as well as bridge-liganded TRs, with other adaptor molecules and/or the basal transcriptional machinery (Fig. 7). Additionally, recent studies have suggested that interaction of coactivators with the AF-2 domain of TR may be a critical step for release of corepressor from TR and derepression of basal repression *(96,125,126)*.

Finally, there is emerging evidence that liganded nuclear receptors may interact with chromatin structure. In vivo footprinting studies have suggested conformational changes in the chromatin structure near TREs and RAREs after ligand addition *(127–130).* Additionally, liganded receptor binding to the hormone response element allowed other enhancer elements in the promoter to be footprinted *(127).* Recent studies have supported the notion that liganded nuclear hormone receptors can remodel local chromatin structure by forming complexes with coactivators, CBP, and the histone acetylase, p300/CBP activating factor (P/CAF). Indeed, CBP and P/CAF as well as SRC-1 *(115,130,133)* have been shown to have intrinsic histone acetyltransferase, although the histone substrates are different for these proteins. This differential pattern of histone acetylation introduces yet another potential layer for regulation and specificity, since modulation of histone acetylation via liganded receptor and associated proteins and modulation of CBP activity via other pathways may act in concert to affect the local chromatin structure near the hormone response element. However, the precise nature of these interactions and the proteins involved in the rearrangement of chromatin structure remain to be elucidated.

SUMMARY

From all these recent data, we can attempt to construct a model for the mechanism of basal repression and transcriptional activation (Fig. 7). In the absence of ligand, TR homodimers or TR/RXR heterodimers are bound to the TRE and complexed with corepressor, which, in turn, interacts with sin3 or a related protein, and histone deacetylase. This complex may keep surrounding histones deacetylated and maintain chromatin near the TRE in a transcriptionally repressed state. In the presence of ligand, the TR/corepressor complex dissociates and is replaced by a coactivator complex that likely contains CBP and the histone acetylase P/CAF. These changes may result in remodeling of chromatin structure and nucleosome positioning, and lead to transcriptional activation. Recent studies using in vitro transcription systems, in vivo footprinting, and reconstituted nucleosomes have confirmed changes in chromatin structure near the TRE after addition of ligand *(128,130,134).* Although this model is intellectually satisfying, it is probably an oversimplification because there may be many other proteins that form the coactivator complex *(83,86,100,101)* that may interact directly with the basal transcriptional machinery or other transcription factors. Presumably some of these proteins may be recruited secondary to changes in the chromatin structure.

The molecular details of TH action have been accumulating at an accelerating pace, and have shed much light on both nuclear hormone action and general mechanisms of transcriptional regulation. As our understanding of the molecular details has improved, so has our understanding of the molecular basis of human diseases that involve TH receptors and coactivators. As discussed in Chapter 7, mutations of TRβ-1 have been associated with RTH, an autosomal disorder in which patients have elevated serum concentrations of TH and inappropriately normal thyrotropin levels. In general, these patients have mutations in the TRβ-1 LBD that decrease TH binding affinity but still allow the TR to bind to DNA *(135).* These receptors have dominant negative activity on wild-type receptors. Recent studies have shown that these mutant receptors may have defects in corepressor release and interactions with coactivators *(136,137).* Mutations in CBP have recently been associated with Rubenstein-Taybes syndrome, a congenital neurological disorder *(138).* Amplification and overexpression of the coactivator AIB-1

has been associated with human breast cancer *(114)*. Additionally, understanding the fine structure and mechanisms of transcription of TRs may lead to the development of TH antagonists or TR isoform-specific agonists that may be useful for treatment of obesity, hypercholesterolemia, or other diseases. In the next decade, it is likely that we will find novel actions of and applications for this familiar and important hormone.

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7 Models of Resistance to Thyroid Hormone

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CONTENTS

INTRODUCTION

Thyroid hormone plays an essential role in the regulation of development, growth, and metabolism. The nuclear actions of thyroid hormone are mediated by high-affinity nuclear receptors coded on two genes termed TRα and TRβ *(1–3)* (Fig. 1). Each gene has multiple alternative mRNA splice products coding for isoforms with differential expression in development and in adult tissues. The syndrome of resistance to thyroid hormone (RTH) is the result of a deletion or mutation in the thyroid hormone receptor β (TRβ) gene *(4)*. Affected individuals have goiter, elevated serum thyroid hormone levels, and a high incidence of attention deficit disorder and abnormal growth *(5)*. The RTH-associated mutant TRβ exhibits a dominant negative effect over the wild-type TRs. In recent years, investigators have used various genetic approaches to model RTH at the cellular and whole-animal levels *(2)*. These models provide a greater understanding of RTH, but are also used to identify TR-isoform-specific actions in development and in the adult animal.

DEVELOPMENTAL AND TISSUE-SPECIFIC DISTRIBUTION OF TR ISOFORMS

Tissue Distribution of TR Isoforms

Based on initial analysis of TR mRNA distribution among tissues by Northern analysis, TRα1, TRα2, and TRβ1 transcripts were found in a wide range of tissues

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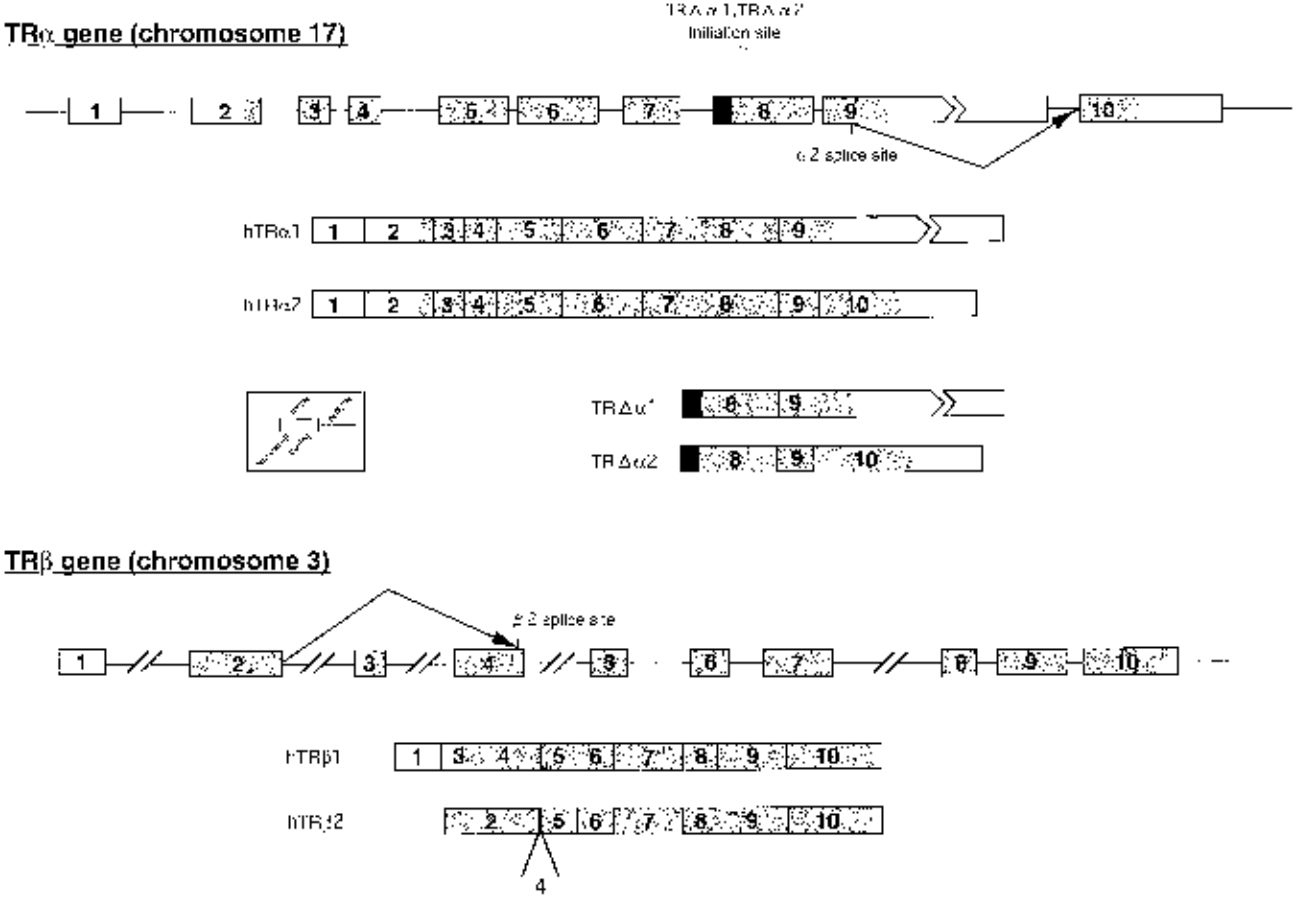

Fig. 1. TRα and TRβ genomic structures. A schematic of the intron and exon structure is shown as a synthesis of studies from human, murine, and rat. The TRβ gene is approx 250 kb and the TRα gene is approx 30 kb. Presumed splice sites are shown for various TR isoforms. (Reproduced with permission from ref. *2*.)

(6–8), whereas TRβ2 transcripts were limited to the pituitary *(9)*. By comparing the absolute concentration of the mRNAs of TRα1 and TRβ1 to the T_3 nuclear binding capacity measured by in vitro saturation analysis in several rat tissues, it was found that the T_3 binding capacity in any tissue is not simply a function of the level of the mRNA *(10)*. The report that during the maturation of embryonic chick erythroblasts, the concentration of TRα mRNA fell 10- to 20-fold whereas the TRα protein levels rose 3- to 5-fold is another example of the dissociation between the level of TR mRNAs and the amount of receptor proteins *(11)*. Direct measurement of the TRα1 and TRβ1 receptor proteins in nuclear extracts from rat fetal liver and brain and adult liver, kidney, brain, and heart has shown differential expression of TR isoforms *(12,13)* (Fig. 2A). In fetal brain and liver, TRα is the dominant TR isoform with only a trace of TRβ. The adult liver is 80% TRβ1 and 20% TRα1, closely corresponding to relative levels of mRNAs. In heart and kidney, there are almost equal amounts of TRβ1 and TRα1. The adult brain is predominantly TRα (72%). Marked variations in specific protein/mRNA ratios were noted among these tissues *(14)*. The protein/mRNA ratio varied by 18-fold for TRα1 and 17-fold for TRβ1 among the tissues analyzed. The T_3-binding capacity was greatest in the liver, with the next highest in the brain and heart *(12)*. Estimation of the protein content of TRα1 and TRβ1 in rat tissues by Western blotting showed that the relative concentrations of TR proteins among tissues paralleled TR levels measured by T_3-binding assay *(15)*.

TR Isoform Expression in Brain Development

Several groups have examined the expression of the TRα and TRβ genes in brain development *(16–18)*. Utilizing *in situ* hybridization histochemistry, it has been shown that in rat, on embryonic d 14 (E14), TRα1 mRNA is already widely expressed at a low level in the developing brain. Expression of TRα1 mRNA peaks during the first postnatal week in the cerebral cortex, amygadala, hippocampus, and cerebellum. The level of TRβ-mRNA is very low or absent in the central nervous system (CNS) before birth, but the TRβ transcript is clearly detectable in some nonneural tissue, such as liver. At birth a dramatic increase in expression of TRβ1 is seen, with high expression in the mitral cell layer of the olfactory bulb, nucleus accumbens, caudate, and hippocampal CA1 field *(18)*. In general, the levels of TRα1 mRNA are higher than those of TRβ1 during fetal and early postnatal development. In the adult, the TRβ1 transcript is relatively abundant compared to TRα1 in several areas of the brain *(18)*. TRα2 mRNA is widely expressed in a pattern similar to that of TRα1 *(18)*. The TRβ2 isoform, which was originally described as pituitary specific, is also detected in the developing hippocampus and striatum *(17)*. The highest level of expression of TRα is in the fetal neocortical plate, the site of cortical neuronal differentiation, and prominent expression of TRβ1 is in zones of neuroblast proliferation such as the germinal trigone and the cortical ventricular layer. TRβ may contribute to regulation of neuroblast proliferation, whereas TRα may play a predominant role in neuronal differentiation *(17)*.

In chick brain development, TRα mRNA is present from the early stages (d E4) and its levels increase two-fold through development *(19)*. TRβ is sharply induced after d E19, coinciding with the hormone-sensitive period. There is no detectable TRα2 in chicken. Surprisingly, both TRα and TRβ genes were expressed in early cerebellar outgrowth at d E9, a stage when thyroid hormone has no effect on development of this area. Expression of TRβ mRNA is restricted to the ventricular epithelium of the

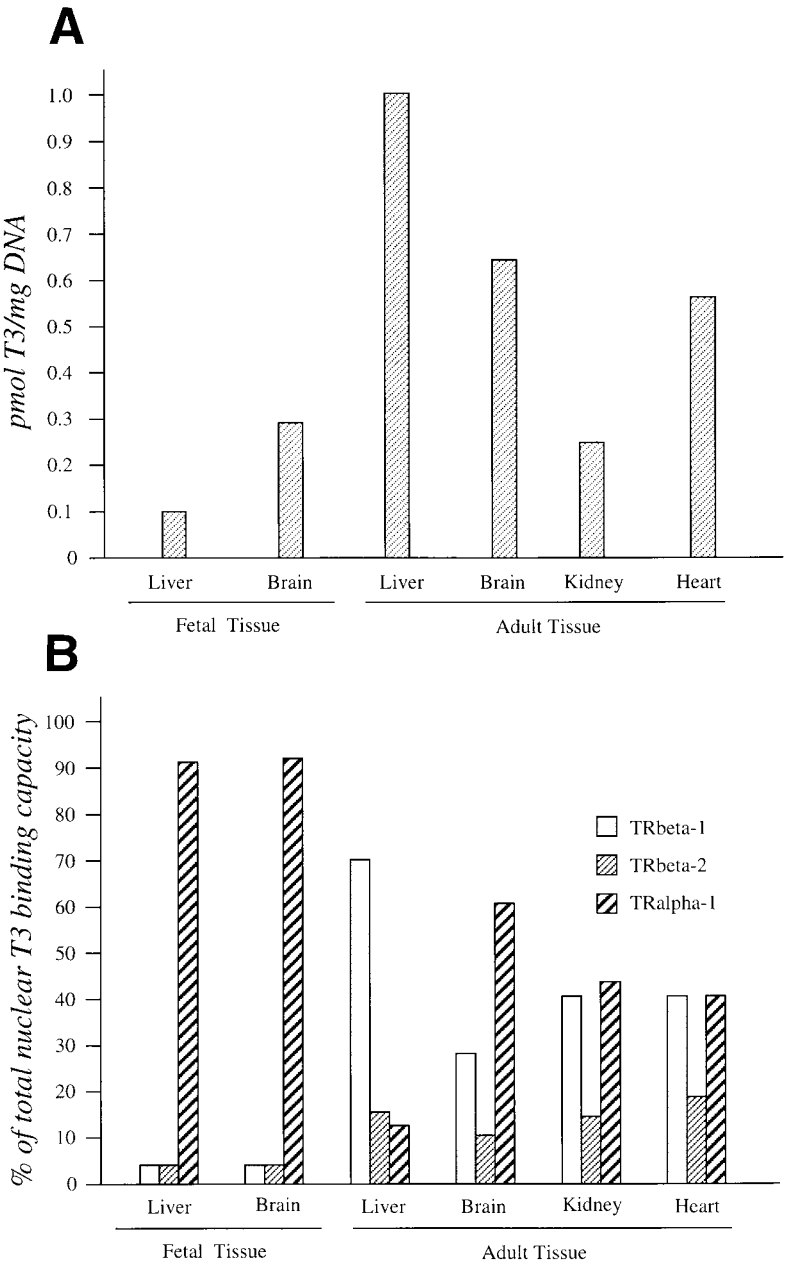

Fig. 2. Nuclear T_3-binding capacity **(A)** and TR isoform distribution **(B)** in fetal and adult rat tissue. Data are based on determination of nuclear T_3 binding capacity and TR-isoform-specific antibodies. (Reproduced with permission from ref. *2*, based on data from refs. *12* and *13*.)

metencephalon and expression of TRα to migrating cells and the early granular layer *(16)*. The widespread early expression of TRα mRNA suggests that TRα could serve as a T_3-independent repressor in immature neural cells *(16)*. This notion is consistent with the earlier observation made by Koenig et al. *(20)* that the rat TRα2-cDNA product could antagonize the effects of cotransfected TRα1 and TRβ1, suggesting that the high level of TRα2 could account for the unresponsiveness to T_3 in rat brain.

Examination of neonatal rats revealed that mRNA levels from three genes expressed in cerebellar Purkinje cells (myoinositol-1,4,5-triphosphate receptor, calbindin, and PCP-2) increase from neonatal d 1 to d 15. This increase comes after the surge of T_3 and TRβ1 in brain. Such regulation appears to have phases of both T_3-dependent and T_3-independent expression *(21)*. A 40-fold increase in rat brain β1 mRNA occurs in the transition between the 19-d gestational fetus and the 10-d-old neonate. By contrast, the levels of TRα1 and TRα2 are already high in the prenatal state. These two mRNAs increase only transiently around the period of birth (Fig. 2B) *(10)*. The surge of TRβ1 synchronizes with the period during which the T_3 content rises in brain and during which T_3 is known to influence CNS development. Therefore, TRβ1 may play a primary role in mediating T_3 effects in developing and adult animals *(10)*.

It has been shown that in the rat, TRβ is prominently expressed very early in inner-ear development *(22)*. As early as d E-12.5, both TRβ1 and TRβ2 mRNA expression are restricted to the portion of the embryonic inner ear that gives rise to the cochlea. The cochlea converts sound into a neural impulse. The TRα gene is also prominent in the developing cochlea, but is expressed throughout the inner ear. TRα1 and TRα2 transcripts are also found in inner-ear structures responsible for balance *(22)*. Mice with inactivation of TRβ have a hearing loss *(23)*, suggesting that cochlear function is a TRβ-isoform-specific action.

Recently deletion of the TRβ1 receptor in GH3 cells has been achieved through a stable transfected antisense construct. It was demonstrated that TRβ1 is not required for T_3 induction of the GH gene in GH3 cells and that TRβ1 and TRβ2 are not equivalent in their effects on basal repression of the GH promoter *(24)*. This illustrates the potential for isoform-specific dissociation of ligand-independent and ligand-dependent activation *(24)*. RTH TR mutations associated with specific resistance at pituitary levels show the ability to antagonize thyroid-stimulating hormone (TSH) regulation in the TRβ2 but not TRβ1 background *(25)*.

TR Expression in Amphibian Metamorphosis

Thyroid hormone stimulates amphibian metamorphosis, and prolactin (PRL) delays or arrests it *(26)*. Almost every cell type in the tadpole responds to thyroid hormone and activates a diverse set of developmental programs. These programs include remodeling of the CNS, limb formation, and regression of the tail and gills.

Amphibians, unlike mammals, have two genes each for TRα and TRβ *(27)*. The TRα gene structure of both genes, however, is quite similar to that of their mammalian counterparts. *Xenopus* TRβ mRNA exhibited a highly complex pattern of alternative splicing within the 5′ untranslated region. The TRβ mRNA has multiple transcriptional start sites. A minimum of two amino termini for each of the two TRβ proteins was identified. The significance of the multiple *Xenopus* TRβ isoforms is not known, although they may be important for regulation of metamorphosis.

Normally the *Xenopus* tadpole thyroid gland begins to secrete thyroid hormone after developmental stage 54 (2 to 3 mo after fertilization) and metamorphosis is initiated. However, as early as the first week after fertilization (stage 44), the tadpole responds to exogenous thyroid hormone stimulation *(28–30)*. This indicates that TR is expressed much earlier than the target cells would normally be exposed to endogenous thyroid hormones.

TRα transcripts can be detected by stage 44 and TRβ at subsequent stages *(31)*. The transcripts accumulate rapidly during this phase of development and reach the maximum level by metamorphic climax (stages 58–62). On completion of metamorphosis, the transcripts are almost undetectable *(32)*. This pattern of expression fits well with two observations: (1) the rapidly increasing sensitivity of tadpole tissue to thyroid hormone associated with metamorphosis, and (2) the absence of response to thyroid hormone in adult amphibia *(28)*.

In situ hybridization analysis also confirms the presence of TR mRNAs in early developmental stages of *Xenopus* tadpoles and provides a framework for spatial as well as temporal regulation of development *(32)*. TR mRNAs were found in the tadpole brain, spinal cord, intestinal epithelium, tail, and liver as early as 1 wk after fertilization (stage 44). Strong hybridization signals were also recorded in the hind limb buds, which undergo *de novo* morphogenesis at midmetamorphosis.

Autoregulation of Thyroid Hormone Gene Expression

In situ hybridization analysis revealed high levels of accumulation of TR transcripts in stage 1 and 2 oocytes of the developing froglet ovary *(32)*. Although these transcripts did not increase further with the growth of the oocytes, they were quite stable. This raised the possibility that the trace level of TR mRNAs found in early embryos was of maternal origin and that the response to thyroid hormone of tadpoles at early stages *(42–44)* may be owing to the TR synthesized from mRNAs of maternal origin.

The phenomenon of autoinduction of TR genes by thyroid hormone has been described *(31,32)*. Exposure of tadpoles at premetamorphic stages *(48–52)* to exogenous T_3 increased the accumulation of TR mRNA substantially. This could explain the rapid increase in sensitivity of tadpoles to thyroid hormones at the onset of metamorphosis. The autoinduction of TR mRNA is rapid (within 4 h of thyroid hormone treatment) and is more substantial for TRβ than TRα.

Substantial amounts of PRL are detected in the pituitary and blood in many amphibia during early tadpole development. PRL acts as a juvenilizing hormone in amphibian metamorphosis. Around the onset of metamorphosis, circulating PRL levels fall very sharply. The kinetics of its disappearance and the rapidly increasing appearance of thyroid hormone in blood show a close reciprocal relationship.

The 20- to 50-fold increase of TRβ gene transcripts by T_3 in vivo has also been reproduced in *Xenopus* cell lines *(33,34)*. A direct interaction between the TRβ-retinoid X receptor (RXR) heterodimer and thyroid hormone response element (TRE) in the promoter of the TRβ gene could explain the upregulation of TR by T_3 *(35,36)*. Recently it was shown that the dominant-negative TRs block the T_3-stimulated transcription from *Xenopus* TRβ gene in cultured *Xenopus* tail. This finding further supports the intimate relationship between TR gene expression and amphibian metamorphic response *(37)*.

TR AND RTH SYNDROME

Mutations Associated with RTH in Two Regions of the TRβ Gene

The RTH syndrome is characterized by diffuse goiter, varying manifestations of hypothyroidism, elevated serum concentrations of T_3 and T_4, and inappropriately "normal" (or elevated) serum thyrotropin concentrations *(4)*. RTH is associated with abnormalities in the TRβ gene on chromosome 3. A large number of mutations have been identified in patients with RTH. Interestingly, the distribution of mutations in the TRβ gene is centered at the carboxy terminal of the gene encoding the ligand-binding domain (LBD) of the receptor. The mutations are clustered in two regions of the LBD. One is found between amino acids 310 and 349, whereas the second region is located between amino acids 429 and 460, at the end of the LBD. No mutations have been found within the putative dimerization domain that lies between these two "hot areas" of mutation. It has been reported that 69% of mutations occur in GC-rich areas, particularly in the CpG dinucleotide hot spot (47%) *(38)*. Attempts have been made to introduce artificial mutations in the CpG dinucleotide or in the LBD outside the two hot areas of the TRβ gene *(39)*. The resultant TRβ mutants showed either normal thyroid hormone–binding affinity or very mild impairment. Such TRβ mutations therefore may not be detected because they are unlikely to produce the clinical phenotype of RTH *(39)*. In most kindreds, RTH is inherited as a dominant trait.

Models for Dominant Negative Mechanism of Hormone Resistance

The RTH mutations in the LBD of the TR produce receptors that are not only inactive but also exert a dominant negative influence over the wild-type receptor. Three different models for the dominant negative action have been proposed: formation of inactive heterodimers that leads to depletion of wild-type TR or RXR partners, mutant receptors that compete with wild-type receptor for receptor binding sites, or titration of limiting transacting factors *(40)*. To test these possibilities, mutations were introduced into the DNA-binding domain (DBD) of receptors that contained an RTH-associated mutation *(41)*. Such receptors lost their dominant negative activities, indicating that DNA binding is necessary for this action. When a dimerization mutant was introduced into a receptor that already contained an RTH mutation, the resultant receptor also lost dominant negative action *(42)*. This result indicates that heterodimerization was necessary for dominant negative activity. It is likely that receptor dimerization is necessary for high-affinity interactions of the receptor with DNA. A model in which an RTH mutant receptor must retain the ability to form heterodimers and to interact with DNA in order to exert dominant negative activity is consistent with these results *(40)*.

TRα2 cannot form homodimers; however, TRα2-RXR heterodimers inhibit wild-type receptor function mediated by a DR4 TRE *(43)*. Deletion of the DBD of TRα2 abolished the dominant negative effect of TRα2, which indicates that DNA binding is required. Thyroid hormone–responsive genes that have a DR4 type of response element may be strongly inhibited by the TRα2 isoform, whereas other target genes would escape α2 isoform inhibition *(43)*. The RTH mutants, as discussed for TRα2, selectively inhibit target gene expression. Preferential inhibition of receptor isoform by the RTH mutant receptors has also been reported. Both TRE structure and the isoform of endogenously active receptor could determine the degree of inhibition of a specific gene in individuals with RTH *(44)*.

Interactions with Transcriptional Coactivators and Corepressors

Steroid receptor coactivator-1 (SRC-1) is a recently isolated steroid receptor coactivator *(45)*. It functions as a positive regulator of the TR-mediated transactivation pathway. Deletion of six amino acids (451–456) in the extreme COOH-terminal region of TRβ results in a receptor that retains the ability to bind T_3 but fails to be stimulated by SRC-1 *(46)*. This suggests that the AF-2 domain of TRβ is critical for interaction with SRC-1. A natural mutation in codon 454 (L454V) of the TRβ with interesting properties has been reported recently *(47)*. It retains DNA binding, hormone binding, and dimerization function, but this mutant receptor transactivates target genes poorly and is a powerful dominant negative inhibitor of wild-type receptor action *(47)*. The interaction of this natural mutant with receptor-interacting protein 140 *(48)* and SRC-1 is markedly reduced. Therefore, this RTH mutant receptor has impaired interaction with putative transcriptional coactivators. This adds a critical factor to be considered in the building of a model that could explain the dominant negative effect of RTH mutant receptor. It has been noted that mutations of key acidic (Glu452, Glu455, Asp456) and hydrophobic (Phe454) amino acids, which impair the ligand-induced transactivation by TR, also abolish the ability of the peptide to interfere with SRC-1 binding to TR *(46)*. Interestingly, there are natural RTH mutants at two of these four amino acid positions.

Amino acids in the N-terminal region of the LBD of TR that are important for the corepressor and the silencing function of TR have been identified *(49)*. Although few RTH patients with mutations in the hinge region of TRβ have been reported *(38),* these few amino acids that have changes in RTH patients are not among those identified to be important for the silencing function. It was also shown that RTH-associated mutant TRs interact aberrantly with a newly recognized family of transcriptional corepressors including nuclear receptor corepressor (N-CoR), RXR interacting protein-13 (RIP-13), silencing mediator for retinoic and thyroid hormone receptor (SMRT), and thyroid hormone receptor–associating cofactor (TRAC) *(50)*. All RTH-associated mutant TRs examined exhibited an impaired ability to dissociate from corepressors in the presence of thyroid hormone. The phenotypes of the RTH mutants examined include normal SMRT association but requiring higher than normal levels of T_3 for dissociation, normal SMRT association but with little or no T_3-mediated dissociation, and unusually strong interaction with SMRT under all T_3 conditions tested. Artificial mutants that abolish corepressor binding also abrogate the dominant negative activity of RTH mutants. It has been reported that orphan receptor COUP-TFI and TRβ share a common corepressor for their silencing activity *(51)*. Orphan receptors such as COUP–TFI and RevErbA can function as a repressor in vivo by utilizing corepressors that are common for members of the TR and RAR subfamily. These data suggest that it is likely that the RTH mutant receptors could act in a similar way to exert the dominant negative activity.

Molecular Basis of the Phenotypic Heterogeneity in RTH

The variable manifestation of RTH within and between kindreds suggests that the specific amino acid mutations in TR alone are not responsible for the resulting abnormal phenotype. There is an inverse correlation in a subset of RTH individuals between mutant TR-binding affinity and serum thyroid hormone levels *(52)*. This suggests a compensation in some patients for reduced T_3 binding to TR. Studies in a large family

with the R320H TRβ mutation suggest that variability in factors that contribute to the action of thyroid hormone may modulate the phenotype of RTH *(38)*.

ANIMAL AND CELLULAR MODELS OF RTH

Somatic Gene Transfer of a Mutant TR

The first animal model of RTH utilized a replication-defective adenovirus vector for somatic gene transfer of a mutant TRβ to a mouse *(53)*. Replication-defective recombinant adenoviruses were constructed that express the human wild-type TRβ; a human mutant TRβ, G345R, identified in a family with RTH *(54);* and a reporter under the control of a TRE *(53)*. This TRβ mutant has no detectable T_3 binding and has a strong dominant effect over the wild-type TRβ *(39,55,56)*. Hypothyroid mice were infected with the mutant TRβ-expressing virus together with the reporter virus. Most of the virus was incorporated into the liver under these conditions. Immunocytochemical analysis of liver section revealed that more than 90% of the hepatocytes of the animals infected with the reporter gene expressed the reporter and TRβ proteins. The introduction of the wild-type TRβ augmented the T_3 response by 66-fold. By contrast, the induction of reporter activity by T_3 was abolished in mice infected with the mutant TRβ gene, showing the strong dominant action of the TRβ mutant on the endogenous TR. Type I 5′-iodothyronine deiodinase and spot 14 are well-characterized T_3-responsive genes whose transcriptional regulation has been extensively studied in rat liver *(57,58)*. T_3 treatment of wild-type TRβ-infected mice showed increased expression of these two genes, whereas T_3 treatment of mutant TRβ-infected mice showed no changes in the expression of these two genes. Compared with mice infected with reporter alone, T_3 treatment of mutant TRβ-infected mice showed no change in liver weight either. This finding is in agreement with clinical observations that the mutant TRβ blocks the catabolic effect of T_3. Serum cholesterol levels in mice expressing the TRβ mutant were significantly higher than those in the control group after T_3 treatment, indicating that T_3 action on endogenous cholesterol was inhibited by the mutant TRβ.

Ubiquitous Expression of a Mutant TR

The classic approach to create transgenic mice is by direct injection of a vector containing the transgene into the male pronucleus of a fertilized mouse egg. Injected eggs are then transferred to the oviduct of the foster female mouse. The transgenic mice delivered by the foster female mice harbor one to several hundred copies of injected DNA. Transgenic mice harboring a dominant negative human TRβ1 mutant were developed *(59)*. The mutation, called PV, has a C-insertion at codon 448, which results in a frame shift mutation in the last 16 amino acids at the carboxyl end. The mutant TRβ protein lacks the domain for T_3 binding and transcriptional activation. The expression of this mutant was directed by a constitutive β-actin promoter. The mutant PV mRNA was detected in all tissues of transgenic mice. However, the levels of mRNA varied with tissues and different founder lines.

Immunohistochemical localization with anti-PV antibody showed the presence of PV protein in the nuclei of liver and brain of the transgenic mice. The transgenic mice had a 1.5-fold higher serum total thyroxine level than that of wild-type mice. TSH levels were not significantly different from those of wild-type mice, despite an elevated

thyroxine concentration. The transgenic mice had reduced body weights and a behavioral phenotype characterized by hyperactivity.

TRβ Gene Inactivation

To inactivate the mouse TRβ gene, a 3.0-kb deletion was introduced into the exon encoding the first zinc finger of the DBD (exon 3). These cells were then used to generate chimeric mice that transmitted the mutation through the germline *(60)*. TRβ–/– mice were viable, displayed normal growth rates and weight gain, and were fertile. The thyroid gland was enlarged in TRβ–/– mice and T_4 levels were elevated about 2.5-fold. Levels of TSHα and TSHβ mRNAs were elevated 2.5- and 3.3-fold, respectively, compared with TRβ+/+ mice, suggesting a specific role for TRβ in mediating the negative regulation of TSH subunit genes. The effects of hypothyroidism and subsequent treatment with incremental doses of T_3 on TSH levels was examined. Upregulation of TSH in hypothyroidism in TRβ–/– was normal whereas the ability of TH to downregulate TSH was impaired. TRβ appears to be essential for the thyroid hormone–mediated suppression of TSH *(61)*. Surprisingly, given the critical role of T_3 in brain development, TRβ–/– mice displayed no overt abnormality in neuroanatomy or behavior. These data suggest that TRβ plays a more subtle role in brain development than suspected, or may be owing to compensation from TRα. The endocrine disorder of the TRβ–/– mouse closely resembles the characteristic findings in human RTH syndrome. Thus, the TRβ–/– mouse is a recessive model for this disease *(60)*.

TRβ1 has been implicated in playing a primary role in mediating T_3 effects in developing and adult animals *(10)*. TRβ1-specific regulation of Pcp-2 gene during cerebellar development is an example of the special role played by TRβ1 *(21)*. However, recently it has been shown that in the late fetal rat, neither increased nor reduced levels of T_3 alter expression of myelin basic protein, Pcp-2, or calmodulin kinase IV genes *(62)*. At the late fetal stage in the rat, the developing brain appears to be unresponsive to thyroid hormone despite the presence of TRs. Recent studies with the TRβ-null mouse revealed no difference in ontogeny of expression of myelin basic protein or Pcp-2 mRNAs when compared with wild-type mice *(63)*.

TRβ–/– mice have also been found to exhibit a permanent deficit in auditory function across a wide range of frequencies, although they show no other overt neurological defects *(23)*. Cochlear structure, however, is normal. These findings suggest that TRβ controls the maturation of auditory function but not the morphogenesis of the cochlea. Deafness is a feature of the RTH kindred homozygous for the TRβ deletion *(4)*.

TRα Gene Inactivation

TRα1 knockout mice *(64)* and TRα1 and TRα2 gene knockout mice *(65)* have been developed. In the TRα1 knockout mice, the functional TRα1 gene was deleted, but the splice variant, TRα2, and the related orphan receptor, rev-erb Aα, were still expressed. In the TRα1 and TRα2 gene knockout mice, both TRα1 and TRα2 were inactivated *(65)*.

To inactivate the TRα gene, Fraichard et al. *(65)* introduced a recombination cassette in the first coding exon (exon 2), immediately downstream of the TRα initiation codon. This prevents transcription of both TRα1 and TRα2 mRNAs. The expression of two truncated mRNAs from an internal promoter located in intron 7 of the TRα gene (Fig. 1), however, was not affected. In vitro, these truncated products antagonize

T_3 action. The homozygous mice became progressively hypothyroid and exhibited growth arrest. From the fourth week on, homozygous mutants lost 30–50% of their weight and died. In TRα–/– mice the overall structure of the small intestine was properly developed but maturation was significantly retarded. Bone development was also delayed in these mice. Some of the 3-wk-old homozygous mutants were rescued by 1 wk of T_3 injections. These data indicate that the products of TRα gene are required for thyroid hormone production and necessary for normal postnatal development *(65)*.

A targeting vector that replaced the TRα1-specific coding sequence with that of TRα2 was designed to delete TRα1 but retain expression of TRα2 and rev-erbAα *(64)*. TRα1–/– homozygous animals were viable and survived to at least 18 mo of age. Both female and male animals were fertile and no overt abnormalities were detected at autopsy. Data showed that expression of TRα2 was not affected by the TRα1 knockout. No compensatory increase of TRβ gene expression in TRα–/– mice was observed. The TRα–/– male mice had lower levels of free T_4 than wild-type animals; however, their T_3 levels were normal. No abnormalities in thyroid glands were found. The mice had an average heart rate 20% lower than that of control animals. The deficiency in TRα1 expression resulted in a lower intrinsic heart rate regardless of thyroid hormone status. The mice had a body temperature 0.5°C lower than normal. This study showed the important role of TRα1 in regulation of cardiac pacemaking, ventricular repolarization in myocardium, and body temperature *(64)*.

The abnormal phenotype seen in TRα knockout animals is distinct from that seen in TRβ knockout animals and does not resemble the clinical syndrome of RTH.

Differentiation of Embryonic Stem Cells with TRα Gene Inactivation

TRα and the non-T_3-binding variant TRα2 are expressed early in development, during a period of embryogenesis that is largely retinoic acid (RA) sensitive. Physiological coregulation by RA and T_3 has been demonstrated with the rat growth hormone gene *(66)*. The role of TRα as a T_3-independent repressor of gene expression in early development has been proposed *(16,67–69)*. To determine the role of unliganded TRα in early development and on RA-stimulated neural development, we used homologous recombination techniques to inactivate both TRα gene alleles in mouse embryonic stem (ES) cells *(70)*. Loss of both TRα alleles resulted in an increase in basal and RA-induced expression of the endogenous RA-responsive genes. Cotransfection experiments demonstrated that inhibition of the RA response could be mediated by TRα1. The addition of TRα1, but not the TRα variant c-*erb*Aα2, to TRα-null ES cells restored the inhibitory effect of RA-induced gene expression. A modified protocol was used to induce the differentiation of ES cells to neural tissue under the influence of RA. RA-stimulated neural differentiation was seen in the wild-type but not in TRα-null ES cells, which is consistent with reports of abnormal neural development as a consequence of premature RA stimulation. The results showed that TRα specifically inhibits the RA response and that the modulation of the RA responses and RA-stimulated neural differentiation may represent a critical role of early TRα expression during the RA-sensitive period. Initial analysis of TRα knockout animals, however, has not demonstrated a significant abnormality in neural development or function correlated to these in vitro studies. Further studies of the TRα knockout animals may show more subtle neural defects, or mechanisms for compensation in the whole animal.

Table 1
Animal Models of RTH and TR Isoform Function

Model	*Thyroid status*	*T_3 Action*	*Special features*	*Reference*
Somatic gene transfer	Euthyroid	Reduced T_3-stimulated spot 14 and type I deiodinase	Elevated serum cholesterol in TR mutant animals	*53*
Ubiquitous expression of mutant TR	Elevated serum T_4, inappropriately normal TSH	Impairment of TSH regulation by T_3	Reduced body weight, demonstrated hyperactivity in behavioral studies	*59*
TRβ gene knockout	Goiter, elevated serum T_4	Impairment of TSH regulation by T_3	Functional cochlear defect	*23,60,61*
TRα1 gene knockout	Normal thyroid, low serum T_4	Reduced serum TSH	Reduced heart rate, prolonged QT, reduced body temperature	*64*
TRα1/α2 gene knockout	Hypoplastic thyroid, low serum T_4	Demineralized bone, low serum TSH	Arrested maturation of small intestine, neonatal lethal at 4–6 wk	*65*

SUMMARY

The complexity of the role of thyroid hormone in development and in the adult is being investigated using a variety of molecular models. The quite different abnormalities in phenotypes that have emerged from TRα and TRβ knockout studies suggest important TR-isoform-specific actions and are summarized in Table 1.

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8 Thyroid Hormone Receptor Family Members

Homodimers, Heterodimers, and Mechanisms of Transcriptional Repression

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CONTENTS

INTRODUCTION

The thyroid hormone receptor (TR) is a member of the nuclear hormone receptor (NHR) superfamily. These receptors are hormone-dependent transcription factors that regulate gene transcription by binding to regulatory regions of DNA termed *hormone response elements* (HREs) *(1)*. TR binds to DNA in the presence or absence of its ligand, triiodothyronine (T_3), and is capable of upregulating or downregulating gene transcription depending on the nature of the underlying response element. Genes that are stimulated by T_3 are regulated by positive thyroid hormone response elements (pTREs); genes that are repressed by T_3 are regulated by negative thyroid hormone response elements (nTREs). nTREs are particularly important in feedback inhibition in the hypothalamic-pituitary-thyroid axis, and are present in the promoter regions of the TRH, thyroid stimulating hormone β (TSHβ), and common pituitary glycoprotein α-subunit genes. TR binds to TREs as monomer, homodimer, or heterodimer with thyroid hormone receptor accessory proteins (TRAPs). In addition, TR is capable of binding to other nuclear proteins, including corepressors and coactivators, which enable it to modulate the transcription of T_3-dependent genes. Thus, the presence of associated

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nuclear factors, as well as the nature of the underlying DNA response element, enables TR to regulate gene expression differentially.

TR AND ITS ISOFORMS

Thyroid hormone exerts its effects by binding to nuclear receptors. In 1986 two groups identified c-erbA gene products as TRs, but the cDNAs cloned differed significantly in a number of regions, particularly the amino termini *(2,3)*. These two distinct TR isoforms were subsequently termed *TR*α and *TR*β. TRα is the isoform that was originally cloned from a chicken embryo library *(3);* its gene is located on human chromosome 17. By contrast, TRβ was cloned from a human placental library *(2)* and is located on human chromosome 3.

The structure of both TRs is homologous to other members of the NHR family *(1),* and includes a central DNA-binding domain (DBD) and a C-terminal ligand-binding domain (LBD) (*see* Chapter 6). The N-terminal region, or so-called A/B domain, of many NHRs contains an activation function (ligand-independent), or AF-1 domain. The DBD, by contrast, imparts specificity of DNA binding. Its structure includes two zinc finger motifs that form α-helices *(4)*. The crystal structure of the glucocorticoid receptor (another NHR family member) when complexed to DNA has revealed that the DBD dimerizes on DNA binding *(5)*. This places the two dimer subunits at an appropriate distance to contract the corresponding HRE. There are two short sequences within the DBD, termed the *P-box* and *D-box* that specify binding of the NHR to its response element *(6)*. In fact, a substitution of just three amino acids in the estrogen receptor (ER) forms a chimeric receptor that binds to the glucocorticoid response element instead of the estrogen response element *(7)*. The P box of the TR is identical to the corresponding region of the retinoic acid receptor (RAR), retinoid X receptor (RXR), vitamin D receptor (VDR), and peroxisome proliferator-activated receptor (PPAR) *(8),* and thus defines a family of NHRs. Because of the conservation of amino acid sequence of the P-boxes, these receptors bind to similar DNA sequences (that are arranged with distinct spacing).

The "hinge" region separates the DBD from the LBD and contains the so-called CoR box; this region binds corepressors *(9–11)*. Other regions of the TR, in particular the ninth heptad of the LBD, may also be involved in corepressor binding *(12)*. The LBD itself imparts specificity of ligand binding. The LBD of the TR enables it to interact specifically with T_3. The LBD also contains important dimerization and transactivation (AF-2) domains *(8)*. Proteins that mediate ligand-dependent activation (termed *coactivators*) bind to the AF-2 region of the LBD.

The crystal structure of the rat TRα1 LBD bound to ligand reveals that the LBD is composed of 12 α-helices and four short β-strands *(13)*. T_3 appears to be buried within a hydrophobic core. The size of this cavity is about the same size as a molecule of T_3. T_4 binds TR less well because its binding is impaired by specific amino acids in the binding pocket, such as Met 256 and His 381 *(13)*. The presence of ligand results in a conformational change in the receptor, enabling it to interact with other nuclear proteins, such as coactivators *(13)*.

Whereas two separate genes encode for TRα and TRβ, additional isoforms of these receptors are generated by alternative splicing. In particular, two distinct TRβ receptor

types, termed *TRβ1* and *TRβ2,* are generated using alternative 5′ exons and possibly separate promoters *(14,15).* The two TRβ isoforms differ only in their distinct N-terminal A/B domains. The TRβ1 and TRβ2 A/B regions contain distinct transactivation domains *(16–19).* In addition, both regions bind TFIIB, although TRβ1 may interact more strongly *(17).*

The TRβ isoforms exhibit tissue-specific expression. In particular, TRβ1 is ubiquitously expressed, but TRβ2 is located primarily in the pituitary and hypothalamus *(15,20),* which are important sites of negative regulation by thyroid hormone. Therefore, it has been proposed that TRβ2 may play an important role in this process *(19).* Using anti-TRβ2-specific antisera, Schwartz et al. *(21)* have also detected TRβ2 protein in a variety of peripheral tissues, including liver, kidney, and heart.

Multiple isoforms of TRα are also generated by alternative splicing *(8).* TRα1 is an ubiquitously expressed receptor that binds T_3. By contrast, TRα2 includes a distinct C-terminal region, is deficient in T_3 binding, and lacks the TRα AF-2 transactivation domain. It does not form homodimers, but generates heterodimers on certain TREs *(22).* Since it does not bind T_3 but binds to TREs, it may function as an endogenous inhibitor of TR action. Dephosphorylation of the unique TRα2 C-terminus may enhance its dominant negative activity *(23).* TRα3 is another TRα isoform and is similar to TRα2, but lacks 39 amino acids that form a portion of the TRα2 C-terminus *(8,24).* Finally, short isoforms of TRα appear to be formed from an internal promoter in intron 7, termed *TRΔα1* and *TRΔα2,* which appear to inhibit TRα1 and/or RAR action *(25).* The exact physiological significance of these additional isoforms, though, remains unclear.

Although not a true TR isoform, RevErb is a member of the NHR family that is encoded on the antisense strand of the TRα gene *(26,27).* It is an orphan receptor, with no known ligand, and it lacks an AF-2 domain. RevErb is a transcriptional repressor on certain DR+2 elements *(28)* and binds corepressor *(29).* Its physiological significance is not yet known.

TR isoforms have distinct functional capabilities. In transient transfection experiments, TRα1 is most potent in ligand-independent repression and ligand-dependent activation (on positive TREs); this activity depends on its distinct amino terminus *(30).* Furthermore, the TRα1 amino terminus interacts directly with TFIIB and influences dimerization and transcriptional activation *(31).* By contrast, TRβ2 is most potent in achieving ligand-independent activation on negative TREs *(19).* TRβ2 also appears to functionally interact less well with corepressors than does TRβ1 *(32).* TRβ2 contains a unique N-terminal activation domain not found in TRβ1 *(18,19).*

Gene knockout experiments have further defined the roles of the TR isoforms. Knockout of the TRα gene generates mice lacking both TRα1 and TRα2. Homozygous mice die by 5 wk of age and are severely hypothyroid *(33).* Interestingly, mice specifically lacking the TRα1 isoforms are viable, but exhibit slower heart rates and lower body temperatures than do control animals; they are mildly hypothyroid *(34).* These animals have decreased TSH levels (including lower TSHα mRNA levels, but slightly higher TSHβ mRNA). By contrast, TRβ knockout mice exhibit high TSH and T_4 levels, suggesting that TRβ regulates transcription of TSHβ and α-subunit genes *(35).* The mice have goiters, but their pituitaries are histologically normal. When rendered hypothyroid, knockout and transgenic mice have similar elevations in TSH levels, but in response to exogenous T_3, knockout animals do not suppress TSH normally *(36).* However, this

study *(36)* did not directly evaluate TSH subunit mRNA levels, nor were animals completely hypothyroid, suggesting that the specific role of TR in the absence of ligand remains to be clarified. However, these data do suggest that TRβ isoforms may be important in negative regulation by thyroid hormone of the hypothalamic-pituitary-thyroid axis.

HOMODIMERS AND HETERODIMERS

TRs, RARs, VDRs, and PPARs recognize a 6-bp consensus sequence of DNA (AGGTCA), termed a *half-site,* in the regulatory regions of genes *(37–39)*. The DBDs of these receptors contain identical P-boxes (*see* "TR and Its Isoforms") that mediate binding to this core motif *(6,7)*. Actual response elements in vivo often deviate from this consensus sequence, but still permit protein-DNA interactions. Recently, it has been suggested, that the optimal TR binding site may actually consist of an 8-bp sequence (TAAGGTCA) *(40,41)*. The spacing between half-sites contributes to receptor specificity. Direct repeats with three, four, and five spaces represent binding sites for the VDR, TR, and RAR, respectively *(38);* these are called DR+3, DR+4, and DR+5 elements. These receptors bind DNA as heterodimers with RXR, another class of NHRs *(42–44)*. RXR is usually positioned as the upstream or 5′ partner in these heterodimers *(45)*. TR additionally can bind DNA as a homodimer and monomer.

One well-characterized DR+4 element is in the promoter region of the malic enzyme gene *(46,47)*. In addition to a DR+4 element, TR is capable of binding half-sites arranged as palindromic or everted palindromic sequences. Interestingly, the rat growth hormone (GH) gene has a sequence of three half-sites, in which the first two form a direct-repeat element and the latter two form a palindromic element *(39)*. The TREs of genes negatively regulated by thyroid hormone have not been as well characterized but appear to bind TR as monomers, homodimers, or heterodimers *(48,49)*.

RXRs represent an important class of transcription factors that homodimerize with each other and can heterodimerize with a variety of other NHRs, as well as certain orphan nuclear receptors such as LXR *(50)* and NGFIB *(51)*. There are three RXR isoforms: RXRα, RXRβ, and RXRγ *(52)*. The RXR isoforms are all members of the NHR superfamily and bind 9-*cis* retinoic acid as ligand *(53)*.

There are two classes of RXR-NHR heterodimers. "Permissive" RXR heterodimers permit ligand-dependent signaling by 9-*cis* RA. By contrast, when RXR heterodimerizes with a "nonpermissive" partner (such as TR or RAR), 9-*cis* RA signaling is blocked. This action depends on the ability of TR or RAR to bind corepressors in the absence of their own cognate ligands *(54)*. In addition, nonpermissive heterodimerization impairs the ability of RXR to bind its own ligand, 9-*cis* RA *(51,55)*. Lala et al. *(52)* identified a novel synthetic ligand of RXR, LG100754, which is capable of activating a nonpermissive RAR-RXR heterodimer *(57);* interestingly, this RXR-specific ligand activates via the RAR AF-2 domain, suggesting that interactions involving one heterodimeric partner can be transmitted to the other *(57)*.

The ability of TR to dimerize with itself and/or RXR depends on a region in the LBD termed the *ninth heptad (4)*. Mutations in the ninth heptad can selectively impair homo- or heterodimerization, as well as interactions with corepressors *(12)*. A second dimerization domain also exists, present in the DBD, that promotes binding of dimers to direct-repeat response elements *(58,59)*. RXR-containing heterodimers can induce

DNA bending, which may be important for transcriptional regulation *(60)*. One model proposes that TR and RXR dimerize in solution in a "head-to-tail configuration" that promotes asymmetric binding to a DR+4 element with RXR as the upstream partner; by contrast, the DBDs of TR homodimers are situated in such a way to bind preferentially to inverted palindromic elements *(4)*. Certain other NHRs (and orphan receptors) may also heterodimerize with TR, including COUP-TF *(61)*, ER *(62)*, and PPAR *(63)*. However, the RXR isoforms appear to be the most important heterodimerization partners of TR.

The ability of TR to bind DNA as a monomer, homodimer, or heterodimer (with RXR) depends on the TR isoform, the underlying TRE, other nuclear proteins, the presence or absence of ligand, and the activity of cellular signaling pathways. For example, interactions between TR and RXR depend on the presence or absence of T_3. Yen et al. *(64)* showed that T_3 decreases TR homodimer formation on DNA, but not TR-RXR heterodimers. Collingwood et al. *(65)* showed that T_3 is actually capable of enhancing the interactions between TRβ and RXR *(65)*. This effect does not depend on an intact TR AF-2 domain and does not involve interactions with coactivators or corepressors. Kakizawa et al. *(66)* similarly showed that the interactions between TRα1 and RXRα are ligand dependent.

In addition, the presence of other nuclear cofactors may be able to modulate the ability of TR complexes to bind DNA. As discussed subsequently, corepressors bind TR homodimer and TR-RXR heterodimer *(12,67)*. Corepressors, though, are also able to stabilize the TR homodimer *(67)*. Cellular signaling pathways are known to affect corepressor interactions with NHRs *(68,69)*. In addition, phosphorylation of the TR itself regulates which TR complex binds DNA. For example, protein kinase A phosphorylation of TRα1 preserves the ability of the receptor to dimerize, but inhibits TRα1 monomer binding to DNA *(70)*. Thus, a variety of nuclear signals affect TR complex formation on DNA.

RECEPTOR-MODULATING PROTEINS

Coactivators

Ligand-dependent and ligand-independent activities of TR are mediated by nuclear proteins, termed *coactivators* and *corepressors (71)*. On TREs, gene transcription is silenced in the absence of T_3 (ligand-independent repression) and stimulated in its presence (ligand-dependent activation). On TREs, by contrast, transcription is enhanced in the absence (ligand-independent activation) and actively repressed in its presence (ligand-dependent repression) of T_3. The function of cofactors on pTREs has been more fully defined than that of nTREs, but both functions appear to involve additional nuclear proteins.

The binding of T_3 to the LBD enables the TR AF-2 domain to bind coactivators. These coactivators include steroid receptor coactivator-1 (SRC-1)/NcoA-1 *(72,73)*, TIF2/GRIP-1/NCoA-2 *(74)*, CBP/p300 *(75,76)*, RIP140 *(77)*, p/CAF *(78–80)*, AIB1/p/CIP/ACTR/TRAM *(81,82)*, and p120 *(83)*. A variety of in vitro data supports the notion that these coactivators are important in ligand-dependent activation. For example, overexpression of coactivators in transient transfections systems enhance ligand-dependent activation of appropriate receptors. In addition, these constructs retain a transactivation function when transferred to a heterologous element. CBP and p300 interact with

a variety of other coactivators as well as NHRs, which suggests that they may play an integrator role *(76)*.

Gene knockout experiments suggest that coactivators play an important in vivo role in ligand-dependent signaling. Knockout of the SRC-1 gene in transgenic mice yields only a mild phenotype. However, there is partial hormone resistance *(84)*. Homozygote animals are fertile and have normal growth. Decidual response to mechanical stimulation of the uterus (a progesterone receptor[PR]–mediated response) is impaired in the female knockout animals; mammary duct branching is also reduced. Males have small prostates, urethras, and testes. TIF2 (but not p/CIP) mRNA expression is increased in knockout mice, suggesting that overexpression of TIF2 might be able to counteract the effects of SRC-1 deficiency *(84)*.

Coactivators contain one or more LXXLL motifs, which enables them to interact with NHRs in the presence of ligand *(85)*. Feng et al. *(86)* performed mutagenesis of the TR LBD and found that the amino acids that define the AF-2 domain contain charged and hydrophobic residues at their periphery, and hydrophobic residues in the center; the LXXLL motif presumably interacts with the hydrophobic cleft that is formed on hormone binding *(86)*. However, not all proteins that contain LXXLL motifs function as true coactivators. In particular, RIP140 is a nuclear cofactor that includes LXXLL regions and interacts with the AF-2 domains of NHRs. It appears, though, that RIP140 competes with other coactivators for NHRs and, in this way, negatively regulates ligand-dependent activation *(87)*.

Several coactivators, including CBP *(88,89)*, p/CAF *(78)*, and SRC-1 *(90)*, contain intrinsic histone acetylase activity, which destabilizes chromatin structure, placing it into a more transcriptionally active state. p/CAF was cloned based on its ability to interact with p300/CBP *(78)*. E1A, a viral transforming protein, was noted to impair p/CAF-p300 binding; p/CAF inhibits the E1A mitogenic effect *(78)*. In addition, p/CAF is able to interact with proteins identical to TBP-associated factors, subunits of TIFIID *(91)*.

Although multiple coactivators exist, the specific functions of each have not yet been defined. Northern analysis of SRC-1 and p300 mRNA transcripts from a variety of tissues reveal that, although both are ubiquitously expressed, there are tissue-specific differences in expression *(92)*. For example, relatively high levels of SRC-1 expression are present in the brain and pituitary. In addition, female rat pituitaries contain less SRC-1 than do male rat pituitaries. By contrast, no such gender-related difference in p300 expression was noted *(92)*.

Corepressors

Relief of repression may be as important as activation in T_3-dependent signaling on pTREs. In particular, TR and RAR silence gene expression in the absence of their cognate ligands. This is an active process mediated by protein-protein interactions. When ligand is not present, TR and RAR bind corepressors, including the nuclear receptor corepressor protein (N-CoR/RIP13) *(9,93)* and the silencing mediator for retinoic and thyroid hormone receptors (SMRT) *(10,11)*. N-CoR and SMRT are ubiquitously expressed *(92)*. They not only bind TR and RAR, but also interact with ERs and PRs in the presence of their antagonists *(94,95)*. Corepressors bind to a portion of the hinge region of the NHRs, called the CoR box *(9)*. Mutations in the ninth heptad of the TR inhibit corepressor binding as well *(12)*. N-CoR also interacts with certain

orphan receptors, such as RevErb; however, the portion of RevErb that binds N-CoR is not homologous to the CoR box *(29)*.

N-CoR and SMRT are modular proteins and contain N-terminal repressing domains, and two C-terminal interacting domains *(10)*. Both interacting domains of N-CoR bind TR, and each is capable of functionally interacting with TR on DNA *(67)*. The binding of TR to corepressors depends not only on the absence or presence of ligand, but also the presence of other factors. The formation of TRβ-RXR heterodimers decreases TRβ-N-CoR interactions *(67)*. However, N-CoR is able to bind TR-RXR heterodimers, and the CoR box also plays a role in the binding of TR to RXR in the absence of T_3 *(12)*. Various signaling pathways influence NHR-corepressor interactions. The growth factor/tyrosine kinase signal transduction pathway decreases TR-corepressor interactions and impairs TR-mediated repression *(96)*. The cAMP pathway has been shown to interfere with interactions between corepressors and PR *(68)*. The Ras pathway allows PPAR to function as a repressor and also interact with corepressors *(69)*. Therefore, a variety of cellular signaling pathways and nuclear proteins mediate the interactions between NHRs and N-CoR/SMRT.

The polarity of the NHR-RXR heterodimer also influences corepressor interactions *(97)*. RAR heterodimerizes with RXR on DR+1 and DR+5 elements, but with opposite polarities. On the DR+5 element, RXR occupies the upstream position. On this element, RAR mediates ligand-independent repression and ligand-dependent activation. On the DR+1 element, however, RXR occupies the downstream half-site. On this element, RAR is a constitutive repressor, and corepressors are unable to dissociate even in the presence of ligand *(97)*. It is not known whether similar TREs exist.

The underlying DNA response element limits NHR-corepressor interactions. For example, corepressors do not bind TR monomer well on DNA, even though binding occurs in solution *(98)*. RevErb interacts with N-CoR but not SMRT on DNA, although it interacts with both corepressors in solution *(29)*. TRβ interacts with N-CoR and SMRT equally well in solution and is capable of interacting with each on DNA *(98)*. However, interactions are stronger between TRβ and N-CoR on DNA response elements than with SMRT *(67)*.

Since TRβ prefers to interact with N-CoR over SMRT on thyroid hormone response elements, N-CoR may be the more important cofactor for TR-mediated ligand-independent repression. Nuclear microinjection experiments support this hypothesis. Antibodies to N-CoR reverse TR-mediated ligand-independent repression, whereas antibodies to SMRT are less effective *(69)*. Similar data have been obtained using transfected inhibitors of N-CoR and SMRT function *(67)*. Therefore, N-CoR may be the more physiologically important corepressor for TR. In the future, corepressor knockout experiments will further delineate the distinct roles of N-CoR and SMRT.

The ability of TR to interact with corepressors is isoform-specific. For example, the TRβ2 isoform does not appear to interact functionally with N-CoR as well as TRβ1 (although structural binding is observed) *(32)*. Furthermore, a TRβ construct lacking the N-terminal A/B domain functions similarly to TRβ1, suggesting that the TRβ2 amino terminus specifically impairs TR-N-CoR interactions *(32)*.

While corepressors are central to the process of ligand-independent repression, the mechanisms underlying this process have not yet been fully characterized. Certain of the repressing domains appear to interact with mSin3 and the histone deacetylase RPD3 (HDAC) *(99–101)*. Thus, the degree of histone acetylation as dictated by corepressors

and coactivators appears to play a key role in transcriptional regulation. Another nuclear protein, SunCor, that contains no significant homology to N-CoR or SMRT binds TR, N-CoR, and SMRT and potentiates TR-mediated repression, suggesting that it is another component of the repression complex *(102)*. Finally, antirepressors, short N-CoR or SMRT isoforms that interfere with corepressor function, may also exist *(11,32,103)*, adding yet another layer of complexity.

NEGATIVE REGULATION BY TH

A variety of genes are negatively regulated by thyroid hormone; this process is termed *ligand-dependent repression*. These include genes involved in feedback inhibition of the hypothalamic-pituitary-thyroid axis, including thyrotropin-releasing hormone (TRH), TSHβ, and the common α-subunit. In addition, a number of genes in the periphery are also negatively regulated by T_3, including those for keratin *(104)* and the β myosin heavy chain (*MHC*β) gene *(105,106)*. In the absence of T_3, transcription of these genes is enhanced (ligand-independent activation). For example, in hypothyroid patients, TSHβ and TSHα (and TRH) gene expression is increased, leading to the elevated levels of TSH (and TRH) seen in this state.

The mechanisms underlying TR effects on nTREs have not been as well characterized as those on pTREs. TR half-sites exist in the promoter regions of genes negatively regulated by T_3. For example, the promoter for the TRH gene contains three half-sites and binds TR monomer, homodimer, and TR-RXR heterodimer *(48)*. There is an additional element downstream of the transcription start site that may also be important for negative regulation of the TRH gene (though it does not bind TR) *(107)*. In addition, a region in exon 1 of the TRH gene, from +6 to +84, was found to increase promoter activity in transgenic mice *(108)*.

Naar et al. *(109)* generated DNA sequences of direct repeats of the consensus sequence TCAGGTCA, and found that a direct repeat of this sequence with no spacing generated an nTRE; this was similar to an nTRE in the mouse TSHβ gene *(109)*. However, this sequence in the TSHβ is not conserved across all species. Further data suggested that exon 1 of the TSHβ gene appears to contain an nTRE *(49,110–112)* consisting of two domains. One domain appears to bind TR homodimer, and the other binds TR monomer. Transfer of DNA sequence from +3 to +37 to a heterologous element resulted in negative regulation by T_3 *(49)*. Other data suggest that TR monomer or TR/TRAP heterodimer can bind the nTRE in the TSHβ gene *(113)*.

On the common α-subunit promoter, Chatterjee et al. *(114)* localized a TR binding site to positions −22 to −7, which was just downstream from the TATA box. These investigators suggested that TR might interfere with binding of factor(s) to the TATA box to mediate repression. Datta et al. *(115)* also suggested that TR may block access of other nuclear proteins to the α promoter, since when TR was added after the formation of a preinitiation complex (in a cell-free system), repression did not occur. However, Burnside et al. *(116)* found an additional nTRE in the area from −74 to −38 of the 5′ flanking region of the rat α gene. Furthermore, Brent et al. *(117)* placed pTREs and nTREs (including a fragment of the α promoter) at a variety of positions, either in the GH promoter, or downstream of the transcriptional start site. These investigators found that nTREs resulted in T_3-mediated repression of DNA transcription *(117)*. The position of the TRE influenced the strength of this interaction, but the overall effect was dependent

on the nature of the TRE itself. Therefore, negative regulation appears to be an active process and is not merely the inhibition of positive transcriptional activity.

The location of nTREs may be more varied than that of pTREs. Zhang et al. *(118)* located an nTRE of the GH gene that was present in the 3′ untranslated region of the gene. Similarly, Bigler and Eisenman *(119)* found a sequence corresponding to an nTRE in a 3′ untranslated sequence. Taken together, these data suggest that nTREs may be present in the promoter regions of genes, in the vicinity of the TATA box, the coding regions of negatively regulated genes, or the 3′ untranslated regions of these genes. Thus, there is great heterogeneity in the structure of nTREs.

The mechanisms underlying negative regulation by thyroid hormone remain to be defined. However, interactions with other nuclear proteins will likely play an important role. A few studies have already suggested that corepressors may modulate ligand-independent activity on nTREs. For example, an inhibitor of N-CoR, N-CoRI, enhances ligand-dependent activation on a TRH reporter element *(32)*. By contrast, Tagami et al. *(120)* reported that full-length N-CoR itself also activates, rather than represses, transcription on negative elements. These investigators suggested that this action might occur via protein-protein interactions off of DNA *(120)*. However, N-CoRI appears to be a more potent activator than does N-CoR, suggesting that the activation function maps to the C-terminal region of N-CoR (which is present in N-CoRI) *(67)*. Although these studies are not in agreement on the nature of the effects of corepressors on nTREs, they all suggest that corepressors play an important role in ligand-independent activation.

The actions of coactivators on nTREs is less clear. However, coactivator mRNA expression has been detected in the pituitary and hypothalamus, sites of important T_3-mediated negative regulation *(92)*.

IMPLICATIONS FOR RESISTANCE TO TH

Resistance to thyroid hormone (RTH) is a clinical syndrome manifested by variable degrees of organ resistance to the action of triiodothyronine (T_3) *(121)*. It is characterized by elevated thyroxine (T_4) and T_3 levels, and an "inappropriately" nonsuppressed TSH, in the absence of a thyroid hormone binding abnormality or TSH-secreting pituitary tumor. RTH is generally caused by mutations in the TRβ gene *(121)*. These mutations cluster in three "hot spots" of the TR LBD, and usually interfere with T_3 binding. Two of these regions occur in the LBD and account for the majority of TR mutations *(122);* recently, certain mutations in the hinge region of TRβ have been shown to cause RTH, defining a third hot spot *(123–125)*. Finally, some patients may have mutations in other genes *(126),* but such mutations have not yet been identified.

Most cases of RTH are autosomal dominant, suggesting that the mutant TRs interfere with wild-type TR function. This effect has been termed *dominant negative inhibition*. Mutant TRs dimerize with wild-type TRs *(127)*. Mutations in TRβ that impair dimerization *(128)* or DNA binding *(129)* impair dominant negative activity, suggesting that mutant receptors compete with wild-type TRs for TRAPs, other nuclear factors, and TREs to mediate their dominant negative effect.

Clinically, most patients with RTH have so-called generalized RTH (GRTH), in which resistance is manifested throughout the body. As such, there is an overall compensated euthyroid state, since the elevated circulating thyroxine generated from high TSH levels (from pituitary resistance) compensates for peripheral thyroid hormone resistance. In

fact, such patients often have a combination of tissue-dependent hypo- and hyperthyroid signs and symptoms, including goiter, hyperactivity, growth failure, delayed bone maturation, sensorineural deafness, and tachycardia *(121)*. Tachycardia likely results from T_3-mediated effects on TRα receptors, which remain wild-type *(34)*. No mutations of TRα have been identified to date in patients with RTH.

Mutant TRs that have decreased T_3 binding are expected to exhibit impaired corepressor dissociation and impaired coactivator recruitment. In fact, most mutant TRs that cause RTH probably behave in this respect. In the extreme, mutant TRs that do not bind T_3 do not release corepressor *(130)*. However, mutations in TRβ have also been identified that result in altered corepressor interactions independent of T_3 binding *(131)*. Certain mutant TRs (including P453A and P453H) do not dissociate from corepressors even though they bind T_3; other mutant TRs (Δ430M and Δ432G) exhibit enhanced corepressor binding in the absence of ligand *(131)*. The introduction of a P214H mutation, which decreases TR interactions with corepressors, impairs dominant negative activity of mutant TRs found in syndromes of RTH *(130)*. These data suggest that interactions with corepressors are important in the pathogenesis of RTH. Alterations in coactivator function may also play a role. An L454V mutation from a patient with RTH was found to have preserved T_3 binding, but impaired interactions with the coactivators SRC-1 and RIP140 *(132)*. As a caveat, it is important to note that T_3 binding by mutant TRs in solution may not correlate with T_3 binding on DNA *(133)*. Finally, it is likely that still other mechanisms lead to impaired TR-mediated activation, and thus TH resistance, including alterations in corepressors or coactivators themselves. However, such mutations causing RTH have not yet been identified *(126)*.

In contrast to individuals with GRTH, some RTH patients exhibit predominantly thyrotoxic symptoms. These individuals appear to have resistance in the hypothalamus and pituitary, with relatively preserved T_3 action in the periphery *(121)*. Therefore, elevated T_4 and T_3 levels cause peripheral tissue hyperthyroidism. This subtype of RTH has been called pituitary resistance to thyroid hormone (PRTH). It has been suggested that such patients represent merely a mild form of RTH along a clinical spectrum of disease *(134)*. Alternatively, these patients may have mutations that selectively produce resistance at the level of the pituitary and/or the hypothalamus. In fact, targeted pituitary expression of a mutant TR in transgenic mice has suggested that the elevated thyroid hormone levels seen in RTH result from resistance at the level of both the pituitary and the hypothalamus *(135)*. Thus, PRTH might be more accurately called "central" RTH.

Certain TRβ mutations do appear to selectively cause PRTH rather than GRTH. One of the first of these to be well characterized involves a mutation that converts an arginine to a glutamine at position 429 of TRβ (R429Q) *(136)*. Another such mutation, R383H, has also recently been described *(137)*. In fact, the arginine in position 383 has been predicted to interact with the arginine in position 429 of TRβ, based on the TRα crystal structure *(137)*. Thus, these mutations may define a novel domain of TRβ important in negative regulation.

Since the TRβ2 isoform is predominantly expressed in the hypothalamus and pituitary, it has been considered a potential mediator of negative regulation of the TRH and TSH subunit genes *(138)*. The specific effects of RTH mutations on TRβ2 function have been assessed by transient transfection. Safer et al. *(138)* found that mutations that cause predominantly PRTH specifically exhibit dominant negative activity as TRβ2 isoforms on nTREs. By contrast, these mutations do not have dominant negative activity

when expressed as TRβ1 mutations. Such mutations, therefore, selectively cause central (hypothalamic and pituitary) resistance. Since T_3 remains active on pTREs in the periphery, patients with these mutations are thyrotoxic.

SUMMARY

Mutant TRs found in patients with resistance to thyroid hormone have highlighted the importance of corepressors and coactivators in mediating thyroid hormone action. These cofactors enable TR to modulate gene expression in the absence and presence of ligand. Although the exact mechanism of action of corepressors and coactivators has not yet been defined, these proteins mediate their effects, in part, by influencing the degree of histone acetylation. In addition, the presence of multiple TR isoforms, as well as the existence of a variety of coactivators and corepressors (and potentially antirepressors), allows for a complex network to regulate expression of T_3-responsive genes. A further understanding of the regulation of gene transcription will enable us to understand more fully the mechanisms of thyroid hormone action.

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9 SF-1 and DAX-1

A Dynamic Duo in Endocrine Development

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Contents

INTRODUCTION

The cloning of the first steroid/hormone nuclear receptor a decade ago has led to the identification of a multigene family with well over 60 members *(1)*. Nuclear receptors are known to affect a wide array of important physiological effects in growth, development, and homeostasis and are key regulators of complex endocrine pathways. The field of reproductive endocrinology has been given a molecular boost by the discovery of two important members of the nuclear receptor gene family: steroidogenic factor-1 (SF-1) and DAX-1 (dosage-sensitive sex-reversal adrenal hypoplasia congenita critical region of the X chromosome 1). The striking phenotypes displayed in the loss-of-function (LOF) *SF-1* and *DAX-1* mutants, either in mice or in men, respectively, have brought both of these gene products to the forefront of developmental endocrinology. We now appreciate that both SF-1 and DAX-1 are critical for the development of the hypothalamic-pituitary-gonadal axis and the adrenal gland. Here we explore the individual and coordinate roles of SF-1 and Dax-1 in reproductive endocrine organ development delineated from expression and genetic analyses. Additionally, we present the current understanding of the physical and functional interactions between these two nuclear receptors. Finally, we highlight the major unresolved questions that have

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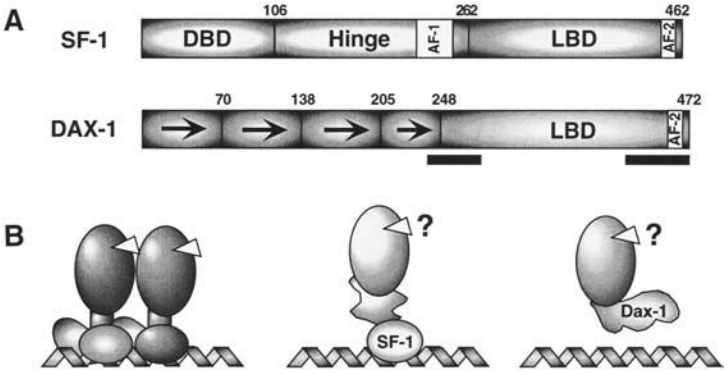

Fig. 1. SF-1 and Dax-1: divergent members of a supergene family. **(A)** Schematic representation of mouse SF-1 and mDax-1, with corresponding amino acids is shown. The classic DBD consisting of two Cys_2-Cys_2 zinc finger motifs, a hinge region, and a putative LBD of SF-1 is shown. Two regions important for transactivation function are also shown, AF-1 and AF-2. Arrows indicate the three and half cysteine-alanine-glycine-rich repeats and solid black bars indicate the approximate location of the silencing domains. Putative LBD regions for both SF-1 and Dax-1 are shown based on sequence identity with other steroid/hormone nuclear receptors. Based on high-resolution structural analyses of other nuclear receptors, the LBD of SF-1 might begin at residue 221. **(B)** Classic nuclear receptors bind discrete DNA response elements in a homo- or heterodimeric fashion and can affect transcription in a ligand- (triangle) dependent manner. SF-1 binds DNA as a monomer to stimulate transcription in the absence of ligand; however, an unknown ligand may modulate SF-1 activity (?). Dax-1 is unable to bind to classic nuclear hormone receptor response elements, but has been shown to bind DNA hairpin loops *(31)*. Similar to SF-1, a ligand for Dax-1 has not been identified.

emerged for those wishing to understand the molecular and genetic nature of the SF-1 and Dax-1 partnership.

SF-1 and Dax-1: Unique Cousins in the Nuclear Receptor Superfamily

Unlike most members of the nuclear receptor superfamily, SF-1 and Dax-1 are highly divergent in their domain topography, DNA-binding properties and requirement for a ligand; Figure 1A & B illustrates these differences. SF-1, but not Dax-1, contains a classic Cys_2-Cys_2 zinc finger DNA-binding motif that enables SF-1 to bind as a monomer to the T/CCAAGGTCA response element with high affinity and without addition of ligand. The single common feature shared among SF-1, Dax-1, and other nuclear receptors is a conserved ligand-binding domain (LBD); this domain normally renders many nuclear receptors active when bound by their cognate ligand. To date, bona fide ligands have not been identified for SF-1 and Dax-1, leading some to speculate that these "orphans" are early ancestors in this large superfamily. As such, these orphans could remain ligandless possibly inferring that SF-1 and Dax-1 will be regulated by other paradigms in which availability of cofactors, cellular localization, and posttranslational modifications refine transcriptional activity.

The ability to regulate nuclear receptors by small molecules has greatly aided the structure-function analyses of this transcription factor superfamily. Thus, the availability of a ligand for either SF-1 or Dax-1 would expand greatly our current structural knowledge of these two nuclear receptors. Indeed, oxysterols have been proposed to activate SF-1 in a ligand-dependent manner, but the nature of this activation is still

elusive. In the monkey kidney CV1 cell line, 25-hydroxycholesterol elevated SF-1-mediated transcription by 2- to 16-fold, depending on which promoter construct was used (*[2]* J. Strauss, personal communication). Others have provided convincing evidence that oxysterols were not effective in modulating SF-1-mediated transcription in steroidogenic, Chinese hamster ovary, or the CV1-related COS cells (*[3]* J. Strauss, personal communication). Nonetheless, the ability to activate SF-1 by a small molecule in the correct cellular context could portend a ligand for SF-1. Future studies aimed at obtaining high-resolution structures for either SF-1 or Dax-1 LBD protein are likely to provide important evidence to support or refute the ongoing debate about their requirement for ligands. For example, structural information concerning the presence of a ligand-binding pocket or the conformation of helix 12, known to change on ligand binding, might provide some clear insights into this issue. Obtaining high-resolution diffractable crystals may prove challenging for the unliganded LBDs of SF-1 and Dax-1 given that almost all LBD high-resolution structures are those bound by ligand *(4)*.

STEROIDOGENIC FACTOR-1

Because a comprehensive review of SF-1 and its role in steroidogenic tissues has been published recently *(5)*, herein we confine our discussion to SF-1's role in nonsteroidogenic cell types within the reproductive system. While SF-1 was identified initially as a transcription factor that contributes to the tissue-specific regulation of the cytochrome P450 steroid hydroxylases, subsequent studies revealed that SF-1 was expressed in nonsteroidogenic tissues and cell types, including cells in the gonads, pituitary, hypothalamus and placenta (Fig. 2, Table 1). Cell transfection assays have now identified multiple putative SF-1 target genes that are expressed in nonsteroidogenic cell types; these are discussed in section "In Vitro Analyses of DAX-1 and SF-1." Much of the excitement surrounding SF-1 has been fueled by the initial observation of Parker and colleagues *(6)* showing that SF-1 was critical for endocrine organogenesis in mice. Their work and that of others demonstrated that SF-1 is a key regulator in endocrine development; and perhaps this embryonic role supersedes its postulated role in the regulation of endocrine-specific adult genes. To date, the phenotype exhibited by *SF1–/–* is one of the most dramatic observed for all targeted disruptions of nuclear receptor genes in mice *(7)*.

LOF SF-1 Mutations in Mice

Targeted ablation of the *Ftz-f1* gene (encoding SF-1) in mice demonstrated that SF-1 plays a much broader role than first suspected in the development of endocrine organs. Mice null for *SF-1* exhibit adrenal and gonadal agenesis, loss of pituitary gonadotropes, and abnormal development of ventromedial hypothalamic nuclei (*[6,8–10]; see* Table 1). These results show that, at least in mice, SF-1 is critical for the development and maintenance of reproductive and steroidogenic tissues at all levels of the endocrine axis. Note that the actual developmental target genes of SF-1 in the adrenal and gonadal primordia have not yet been identified; this remains an important and difficult task for those working on SF-1 biology. Interestingly, SF-1 is expressed in the placenta; however, SF-1 null mice have normal placental architecture and maintain expression of P450c17 and P450scc transcripts, suggesting that SF-1 is not an obligate regulatory protein for placental steroid production *(9)*. Surprisingly, no human SF-1 mutations have been

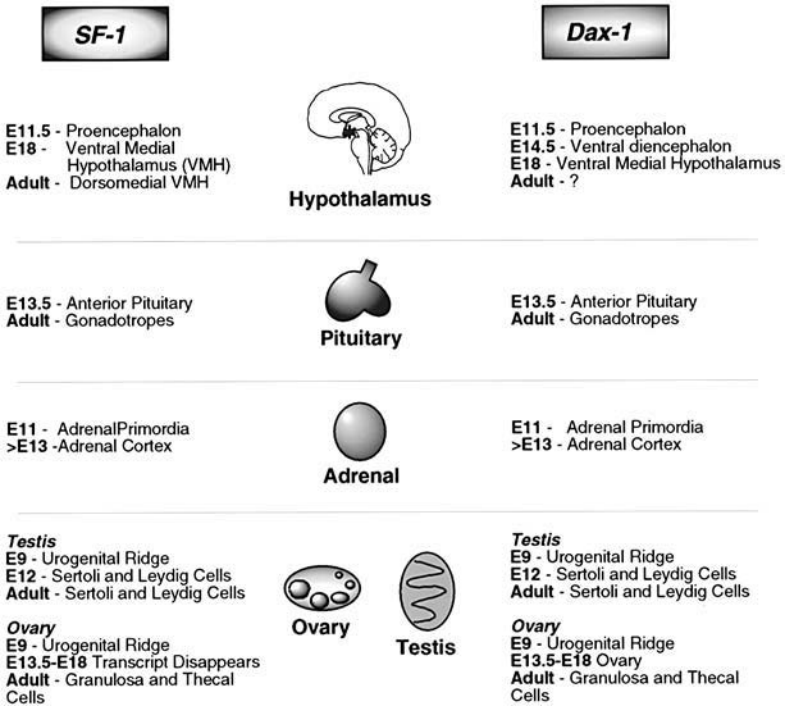

Fig. 2. Developmental expression of SF-1 and Dax-1 in endocrine tissues. The overlapping pattern of SF-1 and *Dax-1* expression is listed for all endocrine tissues. Stage-specific expression profiles are indicated for embryonic and adult mice: embryonic day (E) with plug date as d 0. A sexually dimorphic expression profile for SF-1 is found in the gonads, where it is initially in both males and females; however, it is repressed in females at the point of sexual differentiation. SF-1 expression is activated postnatally in the ovary. In the adult testis, *Dax-1* transcripts are localized to Sertoli and Leydig cells *(29)*. Cell lines reported to express both of these nuclear receptors include the pituitary gonadotrope cell line α-T3; mouse Leydig tumor cell line, MA10; and the human adrenalcortical carcinoma cell line, NCI-H295 *(19,22)*. Mouse adrenocortical Y1 cells do not express Dax-1, nor have rat R2C Leydig cells been reported to express Dax-1.

identified to date, leading to speculation that naturally occurring SF-1 mutant alleles are either weak or incompatible with life *in utero*.

DAX-1

Human Dax-1 Mutants

Human *DAX-1* resides on the short arm of the X chromosome (Xp21), and deletion or point mutations were mapped to the locus associated with familial X-linked adrenal hypoplasia congenita locus (*AHC*) *(11,12)*. Affected AHC patients fail to develop the adult permanent zone of the adrenal cortex. Coincident with the adrenal phenotype, these male patients also develop hypogonadotropic hypogonadism (HHG) postulated to arise from a primary hypothalamic or pituitary defect (*see* Table 1 for a summary of the AHC phenotype). Thus far, all missense mutations in the DAX-1 coding region associated with the AHC syndrome are restricted to the putative LBD region, suggesting that mutations outside the LBD do not lead to AHC (reviewed in ref. *13*). Moreover,

Table 1
Summary of LOF and GOF SF-1 and Dax-1 Mutants in Mice and Men

Tissue	*SF-1 LOF in mice*	*Dax-1 in humans*	
		GOF	*LOF*
Adrenal	Complete agenesis at E13	Normal adrenal function	Absence of permanent zone of cortex
Testis	Complete agenesis at E13; primordial germ cells in genital ridge; express Dax-1	XY sex reversal with variable penetrance	Normal testis development, but onset of HHG at puberty
Ovary	Complete agenesis at E13; primordial germ cells in genital ridge; express Dax-1 transcripts	Normal ovarian development and function	Normal ovarian development and function in heterozygote carriers
Putuitary	Loss of GnRH; gonadotropin expression; perhaps some persistence of a gonadotrope precursor cell type	ND	HHG
Hypothalamus	Aplasia or hypoplasia of VMH; precursors that disappear at E18-P1; persistence of some VMH neurons with abnormalities in ventrolateral VMH and dorsomedial hypothalamic neurons Normal presence of GnRH neurons	ND	HHG
Placenta	Normal development; normal expression of P450scc and P450c17		

[a]ND, not determined.

no mutations are found in the putative ligand-binding pocket of Dax-1. A mouse model of *Dax-1* deletions, AHC mutants, and other *Dax-1* mutants may help delineate how Dax-1 functions in adrenal, pituitary, and hypothalamic development.

Dosage-sensitive sex-reversal syndrome (DSS) manifests as a male-to-female sex conversion and is genetically characterized by a tandem duplication of the short arm of the X chromosome (Xp21) that overlaps with AHC *(14)*. Genetic mapping delimited the *DSS* locus to a 160-kb region on X, and, to date, all duplications in these DSS patients contain the *DAX-1* gene. When duplicated, this 160-kb region is sufficient to cause the DSS phenotype, but when deleted does not lead to abnormal testicular development. An extra dosage of the *DAX-1* gene is postulated to antagonize the male program in these DSS XY patients, who display a wide range of gonadal abnormalities ranging from immature testes to streak gonads (*see* Table 1; *[14]*). Furthermore, it is

assumed that the *DSS* locus undergoes X inactivation because of its chromosomal location on X. This assumption is further supported by the dominant male program in Klinefelter's syndrome (47,XXY), in which the additional copy of *DSS* would otherwise phenocopy sex reversal, as observed in DSS patients.

Do Gain-of-Function (GOF) Dax-1 Mutations Account for DSS?

Although the best candidate gene for DSS is *DAX-1,* the hypothesis that overexpression of DAX-1 causes XY sex reversal, in either mice or men, has not been proved. Disappointingly, transgenic XY mice carrying multiple copies of *Dax-1* were not feminized despite producing an excess of both Dax-1 mRNA and protein in the testes *(15).* However, an antagonistic effect of Dax-1 on male development was unmasked in a mouse strain (*Mus domesticus poschiavinus* × *C57/B16*) exhibiting a high frequency of spontaneous sex reversal. When crossed to Dax-1 transgenic mice, the F1 progeny displayed a higher incidence of XY females compared with the parental strain. Going one step further to prove that excess Dax-1 accounts for DSS, the investigators took advantage of a previously defined sex-converted mouse model in which XX trangenic *Sry* mice initiate testes development. If these same XX *Sry* transgenic mice also carried multiple copies of the *Dax-1* transgene, they would be unable to initiate the male program, suggesting that Dax-1 can antagonize testes development.

Collectively, these data are provocative and suggestive, but fail to provide definitive data supporting *Dax-1* as the *DSS* gene. The underlying factors that account for the lack of sex reversal in a normal mouse *Sry* genetic background are intriguing and may suggest that the mechanism of sex reversal in DSS patients is more complex than a simple dosage effect of Dax-1. Specifically, the site of integration in these transgenic mice might not recapitulate a tandem duplication in DSS patients. Perhaps surrounding chromosomal information or other architectural features in this 160-kb region also contribute to the DSS phenotype. Finally, it is formally possible that another gene in this region is responsible for DSS.

Evolution and Dax-1

Interestingly, both *Sry* and *Dax-1* are rapidly evolving genes, and the high divergence between human and mouse *Dax-1* genes is just as puzzling as that observed for *Sry (16).* Why are these two genes evolving so rapidly and could this divergence contribute to speciation? The answers remain unknown. However, in contrast to *Sry,* which is Y linked in all mammals, *Dax-1* is autosomal in marsupials, thereby excluding a role for Dax-1 in X-linked dosage sex determination *(17).* Furthermore, marsupials maintain *Dax-1* and its X-linked neighbors on chromosome 5, implying that this entire cluster was translocated to X in placental mammals. Dax-1 must have acquired its role in sex determination within the last 80–130 million yr, after divergence of the marsupial and eutherian (placental) mammalian lineages.

DAX-1 AND SF-1 IN ENDOCRINE ORGAN DEVELOPMENT

Colocalization of SF-1 and Dax-1 in multiple endocrine tissues suggested strongly that these two nuclear receptors are functionally linked (Fig. 2; *[18,19]*). Expression of SF-1 and Dax-1 commences prior to, or at organogenesis of the adrenal, gonads, pituitary, and hypothalamus. Genetic analyses of mice and humans have confirmed

their importance in endocrine development. Although there is a plethora of SF-1 endocrine-specific target genes present in the adrenal, gonad, and pituitary, virtually nothing is known about the relevant hypothalamic targets. Immunohistochemical analyses of SF-1 and Dax-1 could establish which cell types within the ventromedial hypothalamus (VMH) express these two factors. Currently, all prior analyses have relied on radioactive *in situ* hybridization, making it difficult to establish colocalization of SF-1 and Dax-1 definitively. Human HHG patients harboring *DAX-1* mutations suggest that this nuclear receptor is important for normal hypothalamic function, at least at puberty. Candidate targets such as gonadotropin-releasing hormone (GnRH) can be excluded given that neither SF-1 nor *Dax-1* is expressed in GnRH neurons and in the immortalized GT-1 cell line *(10,20)* (H. A. Ingraham, unpublished results). Finding downstream candidate genes for both SF-1 and Dax-1 in the VMH promises to be both challenging and exciting. Here, the ability to create a conditional SF-1 knockout in the hypothalamus via a Cre-loxP targeted disruption would circumvent the problems of postnatal lethality attributed to adrenal agenesis observed in the SF-1 null mice. This technology may provide insight into SF-1's function in the hypothalamus, but successful execution awaits the identification of a VMH-specific promoter.

Adrenal Development

Clinical data from human DAX-1 mutants underscore the role of DAX-1 in adrenal development. In the absence of a single functional copy of the *DAX-1* allele, development of the adrenal cortex is prematurely arrested *(11,12).* This phenotype contrasts the complete agenesis of the adrenal primordia observed in SF-1-null mice. The less severe adrenal phenotype displayed by human DAX-1 mutants vs the SF-1 null mice implies that *Dax-1* is epistatic and downstream of *SF-1*. Establishing the precise relationship between SF-1 and Dax-1 has proven elusive. Persistence of *Dax-1* transcripts is observed in selective tissues of the SF-1–/– mice *(19),* suggesting that SF-1 does not regulate *Dax-1* gene expression. However, the presence of an SF-1 binding site within the *DAX-1* human promoter has prompted the examination of this putative SF-1 binding site. Although one analysis in mouse MA-10 cells reported this site to be unimportant *(19),* two other studies showed that Dax-1 reporter activity was largely dependent on a single SF-1 binding site *(21–23).* Moreover, transfection of an SF-1 expression vector leads to significant activation of Dax-1 promoter constructs in cells that either do or do not express endogenous *Dax-1 (22,24).* Further studies may help resolve this unsettled issue. Nonetheless, genetic data imply that SF-1 and Dax-1 act sequentially or coordinately to regulate target genes required for adrenal cortex development.

Gonadal Development

Testis and ovary development is completely absent in SF-1 null mice, implying that SF-1 is essential for establishing the gonadal primordia *(6,9).* Just after the onset of sexual differentiation, SF-1 expression rises dramatically in testes and is repressed in the ovary *(25–27).* The sexually dimorphic expression pattern exhibited by SF-1 and the role of SF-1 in regulating the Müllerian inhibiting substance gene (MIS) and testosterone synthesis provide strong evidence that this orphan nuclear receptor mediates male-specific gene expression. Because *DAX-1* male patients present with normal gonadal function and the HHG observed in these patients is attributed to a hypothalamic or pituitary deficiency, Dax-1 appears nonessential for testis development *(11).* How-

ever, prominent *Dax-1* expression is observed in the late embryonic testis *(19,28),* despite one report that showed *Dax-1* to be downregulated at later stages of testicular development *(18).* This testicular expression pattern is difficult to reconcile with the notion that Dax-1, when present in a single gene copy, acts solely as a repressor or "antitestis factor." Instead, these data suggest that Dax-1 contributes to embryonic and adult testis function in both Sertoli *(29)* and Leydig cells. As in the adrenal, it seems likely that Dax-1 is acting in concert with SF-1, but how remains unclear.

The excitement following the discovery of the *DAX-1* gene arose, in part, because of its putative role as the long-awaited factor that might dictate ovarian development. Unfortunately, little can be said about the role of DAX-1 in ovarian development, since homozygous *DAX-1* mutant female patients have not been identified and AHC heterozygous female carriers have normal fertility. Thus a single copy of the *DAX-1* allele appears sufficient for normal ovarian development. The observation that female AHC patients are undetected suggested that these AHC female carriers always accurately inactivate the mutant *DAX-1* allele. Alternatively, carriers may develop mosaic adrenal glands, expressing either wild-type or mutant DAX-1 protein, but do not present with an obvious AHC phenotype. A mouse model for *Dax-1* null mutants, not present in the human population, should help answer two important questions: Is *Dax-1* required for ovarian development? and Is *Dax-1* subject to X inactivation?

IN VITRO ANALYSES OF DAX-1 AND SF-1

The concordance of *Dax-1* and *SF-1* expression originally suggested that the product of these two genes would interact to modify gene expression. Recent in vitro studies have now proved that a direct interaction between these two molecules occurs. Unexpectedly, the first molecular dissection of SF-1 and Dax-1 by Jameson and colleagues *(30)* showed that these two factors interact in an antagonistic manner; their findings have now been repeated on many, but not all, SF-1-responsive promoters *(28–32).* Although this observation is consistent with their postulated antagonistic roles in testis determination, it is paradoxical to the adrenal, pituitary, and hypothalamus phenotypes displayed by human Dax-1 LOF AHC patients, where SF-1 and Dax-1 appear to function similarly.

Transcriptional Targets of SF-1: An Expanding Family

Since the cloning of SF-1, many genes have been found to be upregulated by this orphan receptor. In addition to genes required for steroidogenesis, nonsteroidogenic targets are also activated by SF-1 *(5,33).* These nonsteroidogenic SF-1 target genes include *MIS (26),* luteinizing hormone β (*LH*β) *(34,35),* the α-glycoprotein subunit (α*GSU*) *(36),* oxytocin *(37),* and the receptors for GnRH *(38),* adrenocorticotropic hormone *(39),* and prolactin *(40).* Nominally, these genes have been identified because their promoters contain one or more SF-1 binding sites and all can be activated with SF-1 in standard cellular transfection experiments. Thus far, the requirement of the SF-1 binding site for gene expression has been confirmed for both *LH*β and *MIS* promoters in transgenic mouse studies *(35,41).*

SF-1: An Activator

In contrast to classic steroid/hormone nuclear receptors that repress basal transcription when unliganded, SF-1 activates reporter constructs in the apparent absence of ligand.

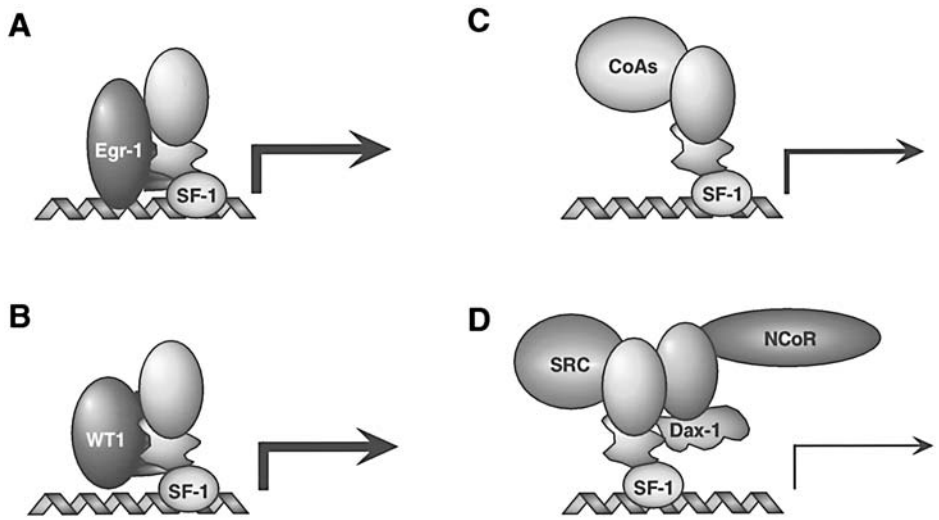

Fig. 3. SF-1-binding partners. Four models illustrating distinct examples of how SF-1-mediated transcription is modulated. For each scenario, relative levels of transcriptional activity are denoted by the thickness of arrow. **(A)** SF-1 and the zinc finger protein Egr-1 interact as well as bind discrete elements, resulting in synergistic activation of the target promoter. **(B)** Synergistic activiation of SF-1 target genes also occurs following WT1 interaction with SF-1; however, WT1 has not been shown to contact DNA directly. **(C)** Binding of ubiquitous coactivators, such as SRC, to the AF-2 region within the LBD of SF-1 (large ball) results in a more modest increase in transcription. **(D)** On several SF-1 target genes, Dax-1 interacts with SF-1 to repress transcription, possibly by recruiting corepresesors such as N-CoR, via its AF-2 domain.

Constitutive activation by SF-1 is still not fully understood, and even more perplexing is SF-1's ability to regulate multiple target genes in several cell types. Because SF-1 is essential for the development of multiple endocrine tissues, mechanisms must exist to restrict expression of SF-1 target genes, to the appropriate cell types. As with other developmental programs, the underlying molecular basis resulting in cell-specific SF-1-mediated transcription appears to rely on a combinatorial code of protein-protein interaction. Currently two cofactors have been proposed to interact with SF-1 leading to synergistic transcriptional activity: early growth response protein 1 (Egr-1) and Wilms' tumor 1 (WT1) (*see* Fig. 3).

In pituitary gonadotropes, in vitro and in vivo data demonstrate that SF-1 and Egr-1 synergistically activate the *LH*β promoter *(30,42)*. These two factors bind independently to closely spaced sites and are able to stimulate transcription ~20-fold over the level induced by SF-1 or Egr-1 alone (Fig. 3A). SF-1 and Egr-1 interact in vitro, based on GST pull-down experiments, but heteromeric complexes are not readily observed in gel-shift analyses *(42)*. Similarly, we have demonstrated a synergistic interaction between SF-1 and WT1, a transcription factor related to Egr1, on the *MIS* promoter (Fig. 3B; **[28]**). Unlike Egr1, WT1 is unable to bind or activate the *MIS* promoter, despite the presence of a related Egr1 consensus site adjacent to the SF-1 high-affinity-binding site.

Although SF-1 and WT1 are expressed in several tissues, they colocalize exclusively in Sertoli and granulosa cells and are essential for establishing the bipotential gonad

in both sexes *(6,43)*. Furthermore, known human WT1 mutations are associated with persistent Müllerian duct structures or *MIS* dysregulation. Our results suggest that SF-1 directs cell-specific gene expression by recruiting a cofactor (WT1) unrelated to the nuclear receptor superfamily. A similar paradigm involving other cofactors present in the hypothalamus, pituitary, and adrenal might account for restricted expression of SF-1 target genes in these endocrine organs.

Consistent with *DAX-1* as the *DSS* gene, we might predict that overexpressing *DAX-1* silences male-specific genes, such as *MIS*. Indeed Dax-1 repressed the synergistic activity of SF-1 and WT1 in our in vitro system (similar to Fig. 3C). Our molecular evidence suggested that gene dosage of *Dax-1,* as well as *WT1,* is important for male sexual development and that Dax-1 and WT1 oppose each other to affect SF-1-mediated transactivation of male-specific genes.

SF-1 Has an AF-2 and a Unique AF-1 Domain

Activation by SF-1 has been attributed to two domains: a C-terminal activation function-2 (AF-2) domain that is highly conserved among all nuclear receptors, and an AF-1 domain located in the distal hinge region (*see* Fig. 1A) *(44,45)*. Recently the C-terminal AF-2 hexamer was found to interact with one of the many previously identified nuclear receptor coactivators, steroid receptor coactivator (SRC) (*see* Fig. 3C) *(44,46)*. It follows that interaction with SRC confers the activation properties of this AF-2 domain; however, unlike other nuclear receptors, this interaction between SF-1 and SRC is ligand independent. Could this data be telling us that SF-1 is really ligandless? Given that 25-hydroxycholesterol is a proposed ligand for SF-1, it would be informative to know whether addition of this compound alters the affinity or nature of the SF-1/SRC interaction.

In addition to the classic AF-2 domain, an independent activation domain (AF-1) has been mapped to the large divergent hinge region of SF-1 that bifurcates the DNA-binding domain (DBD) from the LBD (*see* Fig. 1A) *(44,45)*. This region is proline rich and hydrophilic, and would be predicted to be unstructured. We might hypothesize that this region participates in coactivator binding or is modified posttranslationally by phosphorylation, especially since there are multiple proline-directed kinase consensus sites sprinkled throughout this domain. SF-1 is phosphorylated by cAMP protein dependent kinase in vitro *(47,48)* and is a phosphoprotein in vivo *(45,47)*. Where and how phosphorylation affects SF-1-mediated transcription is still unknown; however, note that forskolin stimulation promoted activation of an SF-1 target gene, aromatase, providing indirect evidence that SF-1 activity may be modulated by phosphorylation *(47)*.

Day 1: Potential Mechanisms of SF-1 Repression

Based on in vivo data showing that SF-1 and *DAX-1* LOF mutations result in similar phenotypes, one might have predicted that DAX-1 would activate SF-1 target genes or interact with SF-1 to positively affect gene expression in the adrenal. Unexpectedly, when the activation potential of these molecules was characterized using GAL4 fusion proteins, Dax-1 failed to activate and, instead, repressed basal levels of transcription *(30,32,49)*. Both the activation and repression by SF-1 and Dax-1, respectively, in the GAL4 system are ligand independent. In vivo data generated from yeast and mammalian two-hybrid assays, as well as in vitro GST pull-down experiments have demonstrated that, indeed, DAX-1 physically interacts with SF-1, most likely through discrete regions

within the LBD of SF-1 *(28,32)*. Interestingly, all known AHC mutations map within the LBD of DAX-1 and, accordingly, may interfere with its ability to bind to other factors. Structure-function analyses of Dax-1 repression showed that the novel repeats in the N-terminus are largely dispensable for repressor function *(28,30,49)*. Despite the low identity shared between human and mouse Dax-1 (<65%), the N-terminal repeats remain conserved and may constitute an unorthodox zinc finger motif, potentially binding to DNA. That Dax-1 functions as a classic DNA-binding protein appears unlikely; however, data by Sassone-Corsi and colleagues *(31)* demonstrated that DAX-1 binds single-stranded hairpin structures. Thus, binding to these unusual DNA structures by Dax-1 may represent a novel mechanism whereby Dax-1 inhibits transcription for some genes.

Structure-function analyses of Dax-1 have mapped repressor activity to two domains within the LBD that work cooperatively *(30,49)*. One of these domains maps to the C-terminal conserved AF-2 motif in Dax-1 (Fig. 1A). The nuclear receptor corepressor (N-CoR) interacts with the AF-2 region of Dax-1 and is proposed to mediate repression of SF-1 via this interaction (Fig. 3D) *(32)*. Recreation of human DAX-1 mutations associated with AHC/HHG abrogate interaction with N-CoR in vitro and fail to repress transcription *(30,32,49)*. Classic nuclear receptors require ligand to relieve repression by corepressors such as silencing mediator for retinoic acid receptor and thyroid hormone receptor and N-CoR *(50)*. By analogy, one might hypothesize that the binding of a "mystery" ligand to Dax-1 converts Dax-1 from a repressor to an activator. This general mechanism may also alter association of Dax-1 with other ubiquitous coactivators or corepressors. Perhaps this ligand is continuously present in tissues such as the adrenal cortex; if so, Dax-1 would not function as a repressor as observed in most in vitro settings, but would be active and potentially synergize with SF-1.

Other DAX-1-Like Receptors

Another member of the nuclear receptor superfamily, short heterodimeric partner (SHP) was found to resemble Dax-1 and shares a high degree of identity (41%) with the LBD of Dax-1, but is divergent in its N-terminal region *(51–53)*. Similar to Dax-1, SHP represses heterodimer retinoid X receptor β-retinoid acid receptor α (RXRβ-RARα)-mediated transcription. Repression may occur by SHP interacting with the heterodimer or by attenuating DNA binding *(52)* since increasing concentrations of recombinant SHP protein were able to displace a TRβ-RXR heterodimer complex from a classic RARβ element; by itself, SHP is not reported to bind DNA (D. D. Moore, personal communication). Similar attempts to abrogate SF-1 binding following addition of Dax-1 protein have failed (D. Enyeart-VanHouten and H. A. Ingraham, unpublished data *[30]*). Coexpression of SHP and the SF-1-related nuclear receptor, fetoprotein transcription factor, in the liver may be analogous to the dynamic dual partnership of SF-1 and Dax-1 in the endocrine system, and future comparisons between SHP and Dax-1 may help delineate further the relationship between SF-1 and Dax-1.

SUMMARY

Over the last 5 years SF-1 and Dax-1 have obtained prominence in the area of developmental endocrinology. As we have discussed, SF-1 and Dax-1 are integrally linked in their endocrine expression and their essential roles in endocrine organ develop-

ment. Now several major issues remain to be resolved in this orphan receptor partnership. A burning question in this field is, Do SF-1 and Dax-1 have a bona fide ligand as predicted by the presence of a conserved LBD? Thus far, there is scant evidence to predict that endogenous ligands activate these two orphan receptors. Rather, much more of the data we summarized predicts that these receptors function in a ligand-independent manner. Nonetheless, discovery of natural or synthetic ligands for SF-1 and Dax-1 has tremendous therapeutic implications in reproductive and adrenal physiology.

In addition, we would like to gain a more precise molecular understanding of the SF-1 and Dax-1 interaction. For instance: Does Dax-1 always oppose SF-1? What other specific cofactors interact with the SF-1/Dax-1 complex? How does DNA contribute to the SF-1/Dax-1 interaction? and How do ubiquitous coactivators or corepressors contribute to SF-1/Dax-1 function? Such molecular information may also tell us how SF-1 and Dax-1 govern sex differentiation. Finally, a significant challenge to the field will be identifying genes that are regulated by SF-1 and Dax-1 in early embryogenesis. Identification of these targets in early embryonic adrenal, hypothalamic, and gonadal development could tell us much about the molecular blueprint of endocrine organogenesis. The next 5 years should bring answers to many of these exciting questions.

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10 Gene Knockout Models to Study the Hypothalamic-Pituitary-Gonadal Axis

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Contents

INTRODUCTION

Reproduction is a complex physiological process that involves a network of interactions between diverse factors secreted from the hypothalamic-pituitary-gonadal (or the reproductive) axis. The reproductive axis is evolutionarily conserved both structurally and functionally in all the vertebrates. Proper reproductive function is fundamental to a species' existence. Therefore, the reproductive axis is regulated in a highly coordinated manner integrating a diverse array of molecular signals *(1,2)*. These signals often exert their functions in both positive and negative loops. In humans, alterations within this network of interactions may lead to aberrant forms of reproduction including infertility and pituitary and gonadal cancers *(1,2)*. A thorough understanding of the molecular mechanisms and the genetic basis of reproduction is essential to manipulate effectively the reproductive ability of both humans and farm animals.

Unfortunately, there are only a limited number of naturally occurring mutant strains of laboratory mice in which the reproductive axis is affected (Table 1), thus precluding

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Table 1.
Naturally Occurring Mouse Mutants with Well-Characterized Reproductive Defects[a]

Mutation	*Chromosome*	*Locus affected*	*Reproductive defects*
Atrichosis (at)	10	?	Recessive; male and female sterility; hypogonadal; very few germ cells
Abnormal spermatozoon head shape (azh)	?	?	Recessive; abnormal sperm with ladle shape; 40% of sperm lack flagella; reduced fertility
Hypogonadal (hpg)	14	GnRH	Recessive; male and female sterility; reduced testis and ovaries; suppressed serum FSH and LH
Juvenile spermatogonial depletion (jsd)	1	?	Recessive; small testes; normal serum testosterone; elevated FSH levels; azoospermia; testicular germ cell depletion by 8–10 wk
Osteopetrosis ($csfm^{op}$)	3	Colony-stimulating factor (CSF)-1	Recessive; reduced male fertility; low testosterone levels; decreased sperm number; viability; depletion of macrophages in reproductive tracts; reduced fertility in females; lower pregnancy rate owing to implantation defects
Oligotriche (olt)	?	?	Only male sterility; normal spermatogenesis until the spermatid stage; no mature spermatozoa in the seminiferous tubules or the epididymis
Postaxial hemimelia (px)	6	Wnt 7a	Recessive; limb defects; both male and female sterility owing to anomalies of the Müllerian ducts in females such as partly or wholly double vagina and uncoiled oviducts; persistent Müllerian ducts in the male
Steel (Sl)	10	Steel factor or kit ligand (KL)	Semidominant; anemic homozygotes; die *in utero* by 15 to 16 d; primordial germ cells absent
Testicular feminization (tfm)	X	Androgen receptor	Androgen receptor insensitivity; spermatogenesis block at meiotic prophase; hemizygous males phenotypically resemble females
Dominant spotting (W)	5	c-*kit*	Multiple alleles; pigmentation defects; severe deficiency of primordial germ cells; migration defects in ovarian tumors owing to overproduction of pituitary gonadotropins in W/W strain of mice

[a]Adapted from *Genetic Variants and Strains of the Laboratory Mouse,* eds. M. F. Lyon and A. G. Searle, Oxford University Press, New York, 1990.

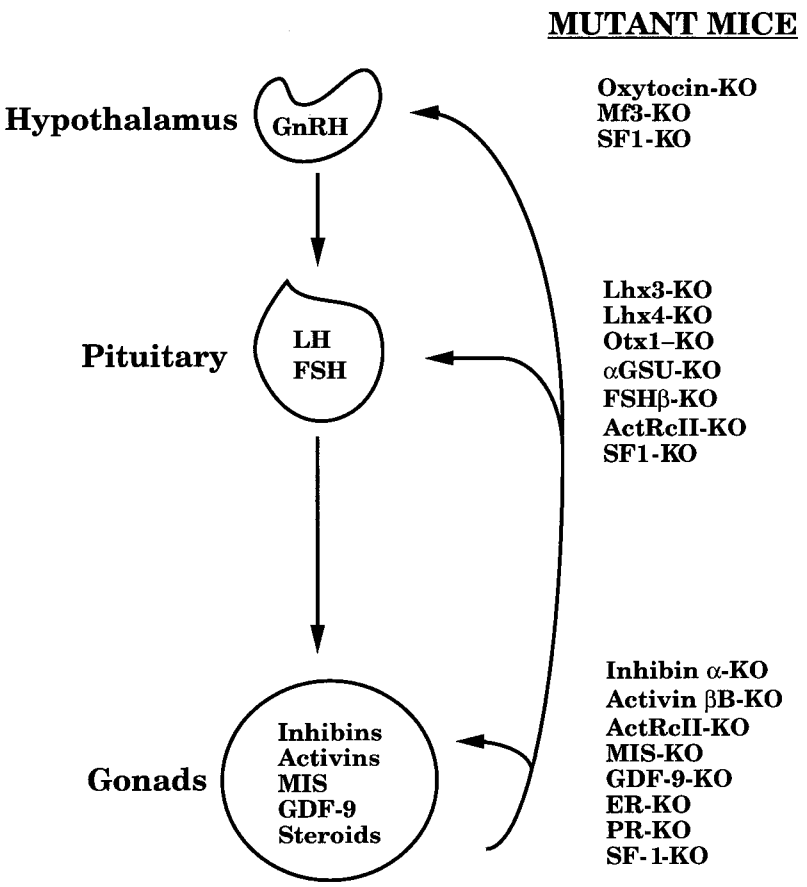

Fig. 1. The reproductive axis and important mouse KO models.

a comprehensive analysis of the regulation of the axis. With the recent advent of tools to genetically manipulate the mouse genome by introducing site-specific mutations into defined loci, it is now feasible to generate a number of defined mutant mouse models to study reproduction in a systematic way (Fig. 1) *(3–5)*. The mouse is an ideal animal model for reproduction research. Mice are relatively inexpensive and easy to breed compared to most of the laboratory animals *(6)*. In addition, a wealth of genetic mapping data is already available for mice. Both the proteins and the genes that encode most of the factors involved in reproduction are structurally and functionally highly conserved between humans and mice. Methods of manipulating the mouse oocyte and embryo have been practiced extensively and perfected in the past two to three decades.

In this chapter, we first briefly describe the early events during the differentiation of the mouse reproductive axis, then outline the principles of targeted mutagenesis in mouse embryonic stem (ES) cells, and finally describe reproductive phenotypes of the mutant mice created by ES cell technology in which specific genes encoding either regulatory proteins, growth factors/receptors, steroid hormone receptors, enzymes, or structural proteins associated with hypothalamic-pituitary-gonadal function are "knocked out."

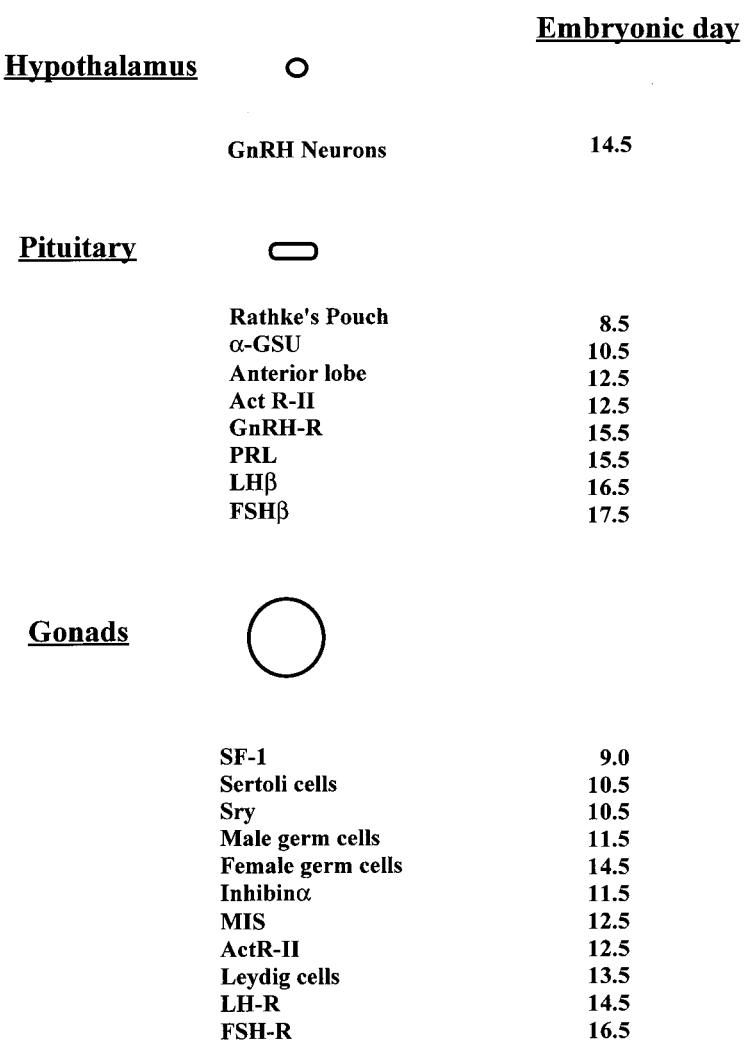

Fig. 2. Embryonic timetable of the mouse reproductive axis.

DIFFERENTIATION OF THE MOUSE REPRODUCTIVE AXIS

In the mouse, differentiation of the reproductive axis occurs in a highly coordinated fashion such that different anatomical structures are formed that consist of distinct cell types (Fig. 2). First, the hypothalamus and pituitary gland originate from distinct ectodermal primordia *(6)*. The hypothalamus arises from the third ventricular neuroepithelium ventral to the hypothalamic sulcus, with neurogenesis occurring between embryonic (E) days 8 and 16 *(7)*. Functionally related neurons are generated sequentially following an outside-in gradient, ultimately yielding stratified arrangements of mature neuronal phenotypes. The hypothalamic neurons that produce gonadotropin-releasing hormone (GnRH) are developmentally unusual. They originate from extraneuronal tissue in the olfactory-placode and migrate along the nervus terminalis to reach the preoptic area and hypothalamus *(8,9)*. The signals that guide migration of GnRH neuroblasts and the targeting of their axons caudally to the median eminence are unknown.

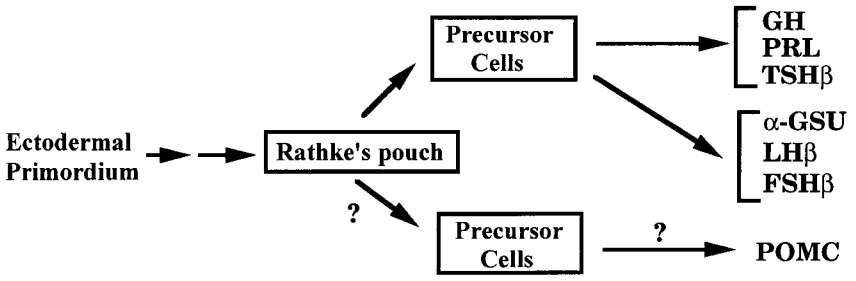

Fig. 3. Development of the anterior pituitary.

At E11.5 the anterior and intermediate lobes of the pituitary gland develop from an invagination of the stomodeal ectoderm known as Rathke's pouch. At the same time, the floor of the diencephalon eventually forms the posterior lobe and remains connected to the hypothalamus by the pituitary stalk or pars tuberalis *(10)*. By E12.5 Rathke's pouch gradually closes and becomes detached from the palate epithelium. The cells of this pituitary precursor proliferate in response to stimuli emanating from the surrounding structures, the mesenchyme, and diencephalon. Concomitant with these events, expression of the α-subunit of glycoprotein hormones (α-GSU), the first pituitary marker, is initially detected in the placode that becomes Rathke's pouch *(11)*. Subsequently, much later, five distinct cell types, each characterized by the expression of a unique hormone, appear in a highly spatial- and temporal-specific fashion, suggesting that distinct pathways regulate terminal differentiation of each cell type (Fig. 3). Several transcription factors, in particular those belonging to the homeodomain class, are expressed in a differentially restricted fashion early in pituitary development *(12)*. At E16.5–17.5 the expression of the gonadotropin subunits luteinizing hormone β (LHβ) and follicle-stimulating hormone β (FSHβ) is restricted to only gonadotropes, whereas αGSU is expressed in both gonadotropes and thyrotropes *(13)*. The noncovalently associated heterodimers of the pituitary gonadotropins LH and FSH are glycosylated and secreted into the blood in response to the hypothalamic GnRH *(14)*.

The initiation of gonadal development into either testis or ovary is the key step in mammalian sex determination. Molecular mechanisms that establish this cell/organ fate are not yet completely known. Genetic analysis of a number of human patients with sex chromosome anomalies and generation of mutant mouse strains has helped formulate a working model (Fig. 4) *(15)*. The genital ridge, under the influence perhaps of important transcription factors such as steroidogenic factor-1 (SF-1), Wilms' tumor 1 (WT1), and others, gives rise to a bipotential gonad very early during embryogenesis around E9.5. Depending on the expression of a Y chromosome–specific gene called *Sry* (all eutherian mammals have conserved this gene on the short arm of Y chromosome) around E10.5–E12, and a related gene called *Sox9,* the genital ridge differentiates into somatic cell precursors of the testis and forms testicular cords *(16,17)*. *Sry* and *SOX9* encode HMG-box-containing proteins that transcriptionally regulate yet-unidentified gene targets. The precursor cells form Sertoli cells and Leydig cells of the testis and eventually support the germ cells directly or indirectly. Sertoli cells express Müllerian-inhibiting substance (MIS) immediately after *Sry* gene expression is turned off. MIS promotes regression of the Müllerian duct that would normally give rise to the female

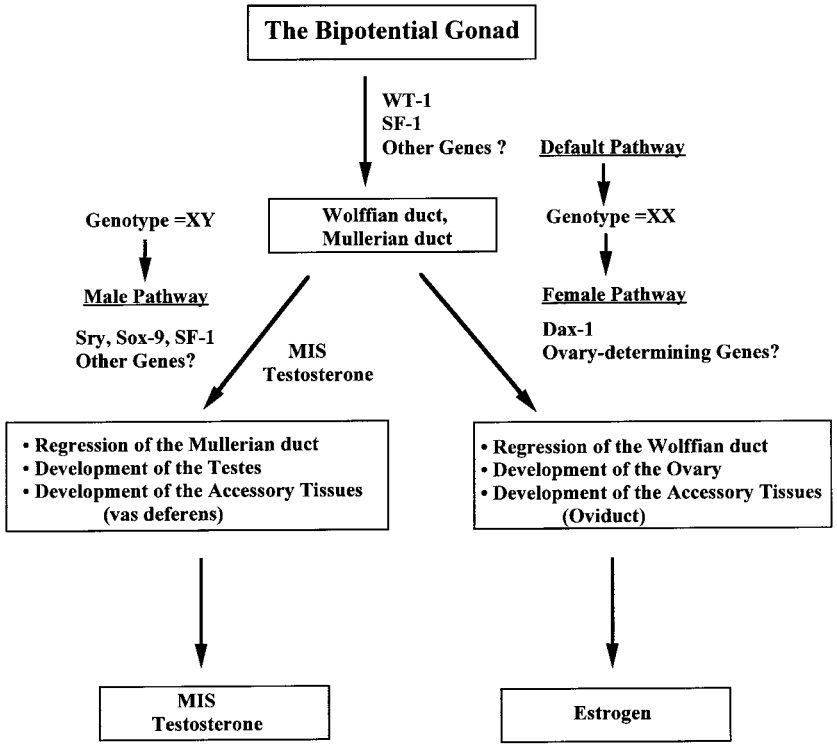

Fig. 4. Differentiation of the mouse gonads and reproductive tracts.

reproductive structures such as oviduct, uterine horns, and the upper part of the vagina *(18,19)*.

The steroidogenic cells of the testis—the Leydig cells—produce testosterone, the male sex steroid that influences the formation of male internal and external genitalia. Less completely understood is the specification of the somatic cells of the ovary (i.e., the granulosa and thecal cells). However, a gene product encoded by *Dax-1,* a member of the nuclear hormone receptor superfamily, antagonizes Sry action in mammalian sex determination when overexpressed and diverts the pathway toward ovary formation *(19)*. While the bifurcating pathways of the male and female gonad specification seem to operate through the somatic cell lineages, yet another complex pathway involves the migration, multiplication, and differentiation of the germ cells in the somatic cell milieu *(20)*. Once proper autocrine and paracrine interactions between the intragonadal and extragonadal factors are established within the testis or ovary, both steroidogenesis and gametogenesis are achieved. Finally, both positive and negative feedback loops are established and finely tune the overall process of reproduction *(1,2)*.

TARGETED MUTAGENESIS IN ES CELLS AND GENERATION OF KNOCKOUT MICE

The primary goal of targeted mutagenesis in ES cells is to engineer a mutation into a desired gene locus. These mutations are as subtle as a base pair change to large-scale megabase-range chromosomal deletions or rearrangements *(21)*. Thus, a "desired

Step 1:	Maintain ES cells (derived from an agouti coat color mouse) in an undifferentiated state on fibroblast feeder layers
Step 2:	Design and construct a targeting vector for, e.g., with isogenic homologous DNA sequences flanking the selection cassette gene sequences
Step 3:	Electroporate the targeting constuct into ES cells, apply drug selection, isolate, and expand the ES cell clones
Step 4:	Isolate ES cell DNA and identify the mutant ES cells by diagnostic Southern blot or polymerase chain reaction analysis using specific probes/primers
Step 5:	Inject mutant ES cells into blastocysts (obtained from a black or white coat color mouse)
Step 6:	Transfer the injected blastocysts into uteri of pseudopregnant females
Step 7:	Generate chimeras with high percentage agouti coat color
Step 8:	Breed chimeras to wild-type mice to confirm germline transmission of the mutant allele (heterozygous F1 progeny)
Step 9:	Breed F1 heterozygous male and female mice (if viable) to obtain F2 homozygous mice
Step 10:	Analyze the mutant phenotypes

Fig. 5. Important steps in production of knockout mice.

mouse can be designed and produced" by this powerful genetic manipulation technique (Fig. 5).

ES cells are pluripotent stem cells derived from the inner cell mass (ICM) of the blastocyst. When mixed with the host cells either by aggregation or by microinjection into blastocysts, the ES cells have the ability to contribute to all lineages including the germ cell lineage of the developing host *(22–24)*. The initial step of the ES cell technology is to maintain ES cell lines in vitro to retain their pluripotency under specified culture conditions for several passages. These cells are then transfected (usually by electroporation) with gene targeting vectors that contain target gene DNA homology sequences flanking an appropriate selection marker gene cassette. After appropriate drug selection conditions, those ES cells that have undergone the correct homologous recombination event at the desired locus are identified (by diagnostic restriction enzyme mapping) (Fig. 6), propagated, and finally combined with the host cells as mentioned above *(22–24)*. These blastocysts containing the modified ES cells are transferred into the uteri of pseudopregnant females. If the ES cell lines are originally derived from an agouti coat color mouse, and the host blastocysts are from a black or white coat color mouse, then the resulting offspring chimeras will exhibit mixed or patchy coat colors. These chimeras are later bred to propagate the mutation into the germline and to generate mice heterozygous for the introduced mutation *(22–24)*. The heterozygous mice are subsequently intercrossed to generate homozygous mutant offspring that should

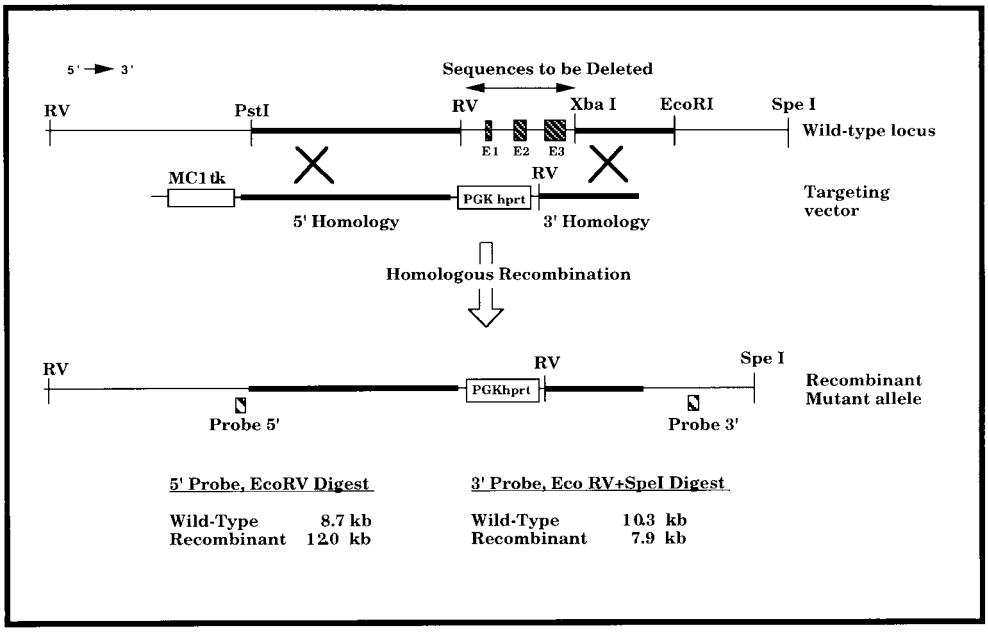

Fig. 6. Replacement strategy to delete a gene in mouse ES cells.

be produced at a Mendelian frequency of 25%, assuming the introduced mutation does not affect the embryonic survival (Fig. 5).

In recent years, several new strategies have been developed that circumvent the problems with the conventional gene-targeting approaches. Many genes are expressed in multiple tissues and at different times during development. In tissue-specific gene knockout experiments, the gene of interest is engineered in such a way that the sequences to be disrupted are flanked by a cre-recombinase enzyme recognition sequence called loxP and "floxed" mice are produced. Using tissue/cell-specific promoters driving cre-recombinase enzyme expression, transgenic mice are generated (by microinjection of embryos) and mated to the floxed mice. Because cre-recombinase recognizes only these phage loxP sites and deletes the DNA sequences between them, the gene of interest will be knocked out specifically in selected tissue/cell types *(25,26)*. Since in some instances, it is not always possible to achieve driving and expressing cre-recombinase in sufficiently large levels with different promoters to selected tissues, cre-recombinase is delivered through adenoviral expression systems to obtain maximum recombination efficiency *(27)*. Although spatial expression of a gene can be manipulated using "cre-lox" strategy, often it may be necessary to study gene expression in a temporal manner, e.g., only during embryogenesis or later during adulthood. This conditional gene-targeting approach is based on the principle that cre-recombinase is induced at selected times following the administration of a drug or an analog that normally does not affect any other gene(s) in mice *(28,29)*. In this strategy, the floxed mice are generated as mentioned before and mated to transgenic mice that harbor a tissue-specific promoter driving a mutant form of a steroid (estrogen or progesterone) receptor ligand-binding domain (LBD) fused to cre-recombinase gene sequences *(28,29)*. When these mice are supplemented at any given point in time with steroid analogs that do not bind endogenous

normal steroid receptors, cre-recombinase is expressed in high levels and results in a spatiotemporal gene deletion event. In recent years, techniques have also been developed to express cre-recombinase directly from an endogenous locus that is either inducible, e.g., to a hormone/growth factor stimulus and thus avoiding line-to-line variation with transgenic mice expressing cre-recombinase. Another important approach that has recently been developed is a knock-in approach *(30)*. This technique is useful particularly in instances in which different isoforms or closely related members of a superfamily can be substituted for one another and then examined for their abilities to function in a given pathway. For example, coding sequences of gene 1 will be replaced by those of its closely related member gene 2 (by gene targeting) but utilizing all the regulatory sequences of gene 1. Hence, gene 2 will be expressed in the same spatiotemporal expression pattern as gene 1 but in the absence of gene 1 *(31)*.

In the following sections, we describe some of the mutant mouse models that have been developed to study the hypothalamic-pituitary-gonadal axis.

KNOCKOUT MOUSE MODELS DEFICIENT IN HYPOTHALAMIC FACTORS INVOLVED IN REPRODUCTIVE FUNCTION

The hypothalamus consists of anatomically distinct regions that are the sites of synthesis of some of the important neuropeptides that directly or indirectly influence reproductive function. Multiple isoforms of neuropeptides are often generated by alternate splicing mechanisms, and these ligands bind different classes of receptors or different isoforms of the same receptor expressed differentially in many target cell types. This functional diversity among the neuropeptides and their cognate receptors is dependent on their discrete anatomical and cellular localization at which they are subject to multiple signals, some activating and others inactivating. Molecular mechanisms of this structural and functional diversity in the hypothalamus leading to reproductive function are relatively unknown. Expression of diverse classes of transcription factors in specific regions at precise time points and interactions among them are critical to the development and eventually to the function of the hypothalamic centers.

Steroidogenic Factor-1

Steroidogenic factor-1 (SF-1) was originally isolated as an obligatory regulator of the steroid hydroxylases. It is a nuclear receptor for a yet-unidentified ligand and is expressed in all three centers of the reproductive axis—the hypothalamus, the pituitary, and the gonads. SF-1 is expressed in the ventral diencephalon, which ultimately gives rise to the endocrine hypothalamus, around E11 during mouse embryogenesis *(32)*. This region of the hypothalamus is known to be important for female reproductive behavior, including the lordosis response, and contains high concentrations of sex steroid receptors. Mice deficient in SF-1 have been generated and characterized *(33)*.

In addition to the defects in the pituitary and gonads (described in later sections), both male and female mice deficient in SF-1 have structural abnormalities, in the ventromedial hypothalamus (VMH), with decreased cellularity and organization of the VMH nucleus *(34)*. The gonadotrope population in the anterior pituitary and the expression of three important markers (LH, FSH, and GnRH-R) are affected in SF-1-deficient mice despite normal GnRH neuronal migration, localization, and cell numbers in the hypothalamus. Injection of GnRH to the mutant mice restored the gonadotropin synthe-

sis, suggesting that normally VMH interacts directly or indirectly to determine the appropriate release of GnRH *(34)*. Thus, these studies have identified SF-1 as the first transcription factor that is localized to a single hypothalamic nucleus and regulates the VMH in the reproductive axis function.

Oxytocin

Oxytocin is the classic hypothalamic neuropeptide implicated in mammalian reproduction at multiple levels. Oxytocin, a nonapeptide, is synthesized as an oxytocin-neurophysin preprohormone. The genes encoding oxytocin and a structurally related member, vasopressin, are closely linked *(144)*. The predominant sites of oxytocin synthesis in the hypothalamus are the supraoptic nucleus and paraventricular nucleus. From these sites, oxytocin is transported to and stored in the posterior pituitary, where it can eventually be released into circulation. In addition, oxytocin is also synthesized peripherally in the corpus luteum of the ovary, uterus, and placenta in the female. Although the mouse testis is not known to be a source of oxytocin, experiments or gain of function in transgenic mice suggest that Leydig cell steroidogenesis may be influenced by oxytocin. Several physiological studies have suggested that oxytocin plays a role in milk ejection, initiation and maintenance of parturition, and regulation of LH pulses from the pituitary. Further, oxytocin has been implicated to play a major role in male and female mating behavior, male copulation and ejaculation, and female maternal behavior *(145)*. To complement the existing information on the complex roles of oxytocin in mammalian reproductive physiology and to test genetically many of these attributed functions, our group and others have produced oxytocin-deficient mice *(35,36)*. The mutation we generated includes the deletion of exon 1, which encodes the signal peptide, the oxytocin peptide, 3 amino acid endoprotease recognition sequence, and part of the neurophysin peptide. Both female and male mice deficient in oxytocin are viable, fertile, and do not display any defects in sexual behavior *(35)*. Oxytocin-deficient female mice have no obvious defects in gestation, parturition, and female maternal behavior. However, the mutant females display obvious nursing defects owing to a lack of the milk ejection in response to suckling. Postpartum injections of the oxytocin peptide to these oxytocin-deficient mothers restore milk ejection and rescue the otherwise dying offspring *(35,36)*. Thus, this gene knockout model clearly establishes the essential role of oxytocin only in milk ejection, at least in the mouse, despite the many postulated roles based on physiological studies. Because oxytocin-deficient mice have no obvious parturition defects (and it is well known that oxytocin injections induce labor in women), these mice may be an ideal source of identifying novel oxytocin-like material(s) that may be important for labor induction *(35)*.

Mf3

The mouse Mf3 gene belongs to an evolutionarily conserved winged helix family of transcription factors. Members of this family (e.g., HNF3β, HNF3α, Bf1, Bf2, and Mf2) are known to affect cell fate, proliferation, and tissue-specific gene expression and are expressed in the central nervous system (CNS) of the mouse embryo. At E9.5, Mf3 gene expression is detected in the developing diencephalon and midbrain region. Interestingly, late in gestation, the predominant region of its expression is the most caudal region of the hypothalamus within the mammillary bodies, where no functionally identified specific cell populations are apparent *(37)*. To understand the biological role

of Mf3 during mouse development, knockout mice have been generated in which the entire protein-coding region of the Mf3 gene has been deleted *(37)*. Homozygous mutant mice on an ×129 Black Swiss genetic background display variable phenotypes. Approximately 6% of the mutant embryos die *in utero* and demonstrate either an open neural tube in the diencephalon and midbrain region or a severe reduction of the posterior body axis. Mice that survive appear normal at birth; about one-third of these mice become growth retarded postnatally and die before weaning *(37)*. Serum levels of both growth hormone (GH) and thyroid-stimulating hormone (TSH), which normally influence body growth, are normal in the mutant mice compared with littermate controls, suggesting that these effects are not owing to altered pituitary function. Mice that survive past weaning are smaller than normal and fertile but show an abnormal clasping of the hind feet when suspended by the tail. Female homozygous mutant mice lack a milk-ejection reflex and can not nurse their offspring. This nursing defect can be corrected with injections of oxytocin *(37)*. Histologically, the hypothalamus in mutant mice appears normal. Oxytocin-producing cells are present; however, there is a 40% reduction in the number of cells synthesizing oxytocin in the supraoptic (SO) and paraventricular nuclei of the hypothalamus. It is therefore hypothesized that the oxytocin surge necessary to induce milk ejection either is not generated or is not of sufficient amplitude to be functional *(37)*. Although the functional interactions between different neurons within the mammillary bodies of the hypothalamus are unknown, elegant studies using Mf3 knockout mice have uncovered a link between a transcription factor expressed in this region of the hypothalamus and a growth regulatory and milk-ejection response.

More than 25 neuropeptides have been shown to be important for regulating the hypothalamic-pituitary-gonadal axis. Some are known to act directly on the pituitary at the gonadal level and some indirectly by altering the GnRH pulses from the hypothalamus. The best known example is β endorphin, an opioid peptide, synthesized from a polypeptide precursor, proopiomelanocortin, in all three centers of the reproductive axis. Several pharmacological and physiological studies have suggested that this peptide plays important roles in reproduction, but, surprisingly, β-endorphin-deficient mice are fertile without any reproductive defects *(38)*. Thus, ES technology, a powerful in vivo approach, has the potential to test genetically the physiological/pharmacologically known hypotheses and provide conclusive information on the specific actions of a number of neuropeptides implicated in regulating reproductive function.

KNOCKOUT MOUSE MODELS WITH DEFECTS IN THE PITUITARY

The specification of at least five distinct cell lineages from Rathke's pouch during pituitary organogenesis involves a series of developmental decisions controlled by multiple transcription regulators. These include global transcription factors that dictate the cell type identity enroute from a discrete cell lineage and cell-specific regulators that control the transcriptional regulation of the marker genes.

Lhx3 and Lhx4

Targeted mutations in two closely related LIM homeobox genes, *Lhx3* and *Lhx4,* were introduced and mutant mice were generated *(39,40)*. Subsequently, double mutants were obtained that lack both these transcription factors, and pituitary development was

analyzed in these mice *(40)*. Based on the phenotypes of these mice, it has been proposed that Rathke's pouch is formed in two steps: first as a rudiment and later as a pouch. Formation of the rudiment does not require the function of either of these transcription factors, whereas either Lhx3 or Lhx4 is required for the development of the rudiment into a definitive pouch. Precursor cell commitment in Rathke's pouch to pituitary organ fate is controlled by Lhx3 since in Lhx3-deficient mice this developmental transition step is blocked. Much later Lhx3 and Lhx4 regulate proliferation and differentiation of pituitary-specific cell lineages including the somatotropes, lactotropes, thyrotropes, and gonadotropes *(40)*.

The cell lineage–specific transcription factors involved in the regulation of GH, prolactin (PRL), TSH, and POMC gene expression have been characterized and well studied, both in vitro using specific cell lines or in vivo using transgenic or naturally occurring mutant mice. However, mechanisms of transcriptional regulation of the gonadotropin subunit genes are poorly understood owing to the lack of well-differentiated gonadotrope cell lines. Although no bonafide gonadotropin subunit-specific transcription factor gene has been cloned and well characterized, mutant mice with gene deletions in some transcription factors demonstrate defects in transcription of the gonadotropin subunit genes. We next briefly describe two such models.

NGFI-A

NGFI-A, also known as Egr-1, Krox-24, and Zif268, is a zinc finger transcription factor that binds GC-rich DNA sequences and can activate transcription of nearby genes *(41)*. It is a phosphoprotein, originally identified as an immediate-early serum response or nerve growth factor response gene product and was later shown to be rapidly induced by various stimuli. Even though NGFI-A is expressed widely during development, including in endothelial tissue, thymus, muscle, cartilage, bone, and part of the CNS and peripheral nervous system, none of these tissues are affected in NGFI-A knockout mice. Instead, the major defect in two independently generated mutant strains of NGFI-A knockout mice is female sterility owing primarily to reduced pituitary LHβ synthesis *(42)*. This observation led to the identification of a canonical NGFI-A binding site within the LHβ promoter that can synergize with SF-1 (whose binding site is also nearby within the LHβ promoter) to induce expression of reporter constructs driven by the LHβ promoter in αT3-1 cells (an immortalized gonadotrope cell line). The female sterility owing to this mutation in NGFI-A manifests in reduced uterine size, decreased serum progesterone levels, absence of corpora lutea, and an anestrous condition. Superovulation treatment leads to the rescue of these mutant female mice *(42)*. Male mice deficient in NGFI-A are fertile and have normal testicular morphology and function, normal accessory glands (seminal vesicles), and serum testosterone levels despite reduced LH levels. In both male and female mutant mice, gonadotrope number is unaffected *(42)*.

In contrast to these observations, the mutation in NGFI-A locus generated by a second group of investigators results in both male and female infertility *(43)*. This group found defects also in LH-receptor expression within the ovarian follicle cells, and superovulation treatment cannot restore the normal fertility *(43)*. Additionally, this group found proliferation defects in the somatotrope cell lineage in the pituitary. One explanation for these differences is that NGFI-A encodes a protein that has two functional domains and loss of the DNA-binding domain leads to defects in LHβ gene regulation

(mutation created by both groups), and loss of the N-terminal domain leads to somatotrope proliferation defects (present only in the second group's mutation) *(43)*. Irrespective of these differences, the important fact is that creation of NGFI-A mutant mice by ES cell technology led to the identification of a novel mechanism of selective regulation of only LHβ gene transcription by NGFI-A within the pituitary gonadotropes.

Otx1

Otx1 is a homeobox-containing gene that belongs to the Otx family. Unlike most of the other regulatory transcription factors controlling pituitary gene activation, *Otx1* is not expressed during embryonic pituitary development postnatally through the adult stage *(44)*. In cell culture experiments, Otx1 has been shown to transactivate α-GSU, LHβ, FSHβ, and GH promoters. Otx1-deficient mice have been generated, and analyses of these mice provided the most intriguing results *(44)*. About 75% of the mutant mice on a C57B16/DBA2 genetic background survive and by postnatal day 7, these mice exhibit an increasing dwarfism, with peak reduction in both size and body weight around dp 30. This growth retardation is transient and by 4 mo of age, the mice recover to normal size. Similarly, these mice exhibit a transient hypogonadism during the prepubescent stage and gradually recover and restore gonadal function by 4 mo of age. The serum profiles of GH, LH, FSH, and sex steroids show a parallel drop with a gradual increase by the recovery stage *(44)*. Likewise, the gonadal maturation is remarkably delayed but completely recovered. Expression of growth hormone-releasing hormone (GRH), gonadotropin-releasing hormone (GnRH), and their receptors in the anterior pituitary of the mutants do not show any changes suggesting that the ability to synthesize the trophic hormones is impaired *(44)*. Although the mechanism underlying the recovery from the transiently affected growth and reproductive phenotypes is not clear, it was suggested that this is a possible example of temporal-restricted competence in hormonal regulation of specific cell lineage by Otx1. Interestingly, the growth and reproductive phenotypes in these mice are reminiscent of the "catch-up growth" in children often referred to as CDGA, constitutional delay in growth and adolescence.

KNOCKOUT MOUSE MODELS DEMONSTRATING DEFECTS IN THE GONADS

Gonadal growth and differentiation are subject to multiple intra- and extragonadal signals. These signals include the pituitary gonadotropins, LH, and FSH, as well as several gonadal peptides and growth factors. Gonadal growth and differentiation ultimately is important in steroidogenesis and gametogenesis. Distinct steroid biosynthetic and modifying enzymes are compartmentalized in subsets of cells within the gonads and are regulated either by steroids themselves or by other peptides/growth factors. Several transcription factors, including steroid receptors and cell cycle regulators, act in a concerted fashion to regulate spermatogenesis and ovarian folliculogenesis. Both of these are cyclic processes and normal fertility is achieved as a result of successful production and fertilization of the gametes, implantation of the embryo, and proper delivery of the progeny.

In the following sections, we describe mouse models in which gonadal growth and differentiation, fertilization and postfertilization, implantation, and pregnancy are affected.

Peptides/Growth Factors and Receptors

Gonadotropins

FSH and LH are pituitary gonadotrope-derived glycoproteins. They are noncovalently linked heterodimers that share a common α-subunit (α-GSU, also expressed in thyrotropes), but differ in their hormone-specific β-subunits *(45)*. In the male, FSH receptors are localized to Sertoli cells whereas structurally related but distinct LH receptors are localized to Leydig cells of the testis. FSH is known to act as a mitogen to regulate Sertoli cell multiplication and differentiation, which eventually controls the spermatogenic potential of the male. LH binding to Leydig cell receptors stimulates androgen production, which promotes testicular and accessory gland differentiation *(45)*. In the female, FSH receptors are present on the granulosa cells and LH receptors are localized to thecal cells of the ovarian follicles. Subsequently, granulosa cells and corpora lutea also express LH receptors and acquire LH responsiveness *(45)*.

α-GSU is the earliest marker expressed during pituitary gland development prior to the onset of expression of TSHβ, LHβ, and FSHβ subunits. To study the functional role of α-GSU, mice deficient in this glycoprotein subunit and hence in TSH, LH, and FSH were generated *(46)*. The mutant mice exhibit profound hypothyroidism resulting in dwarfism. Thyroid development is arrested in late gestation, and pituitary thyrotropes exhibit hypertrophy and hyperplasia owing to a lack of thyroid hormone feedback *(46)*. Pituitary morphogenesis and GnRH neuron migration appear normal in the absence of α-GSU. The mutant mice are hypogonadal; however, sexual differentiation and genital development are unaffected in the absence of circulating gonadotropins. Homozygous male mice are infertile, have decreased testis size, and have undetectable serum testosterone levels. Epididymides and vas deferens are present, but the seminal vesicles are atrophied consistent with the absence of testosterone *(46)*. Histological analysis of the testis demonstrates normal fetal stage development, but at 8 wk of age, smaller seminiferous tubules are apparent, interstitial cells are rare, and there is a block in spermatogenesis at the first meiotic division. In female mutant mice, there is a failure of the vaginal orifice to open. These mice demonstrate small ovaries and thin uteri and have suppressed estradiol levels. Histologically, no antral follicles and corpora lutea are observed *(46)*.

Since the absence of α-GSU results in deficiency of both the gonadotropins in addition to TSH, and since thyroid status is important for reproductive development, α-GSU is not an ideal model to study the isolated effects of the absence of each of these hormones in reproductive development. To study the role of only FSH in gonadal growth and differentiation, our group generated mice deficient in FSHβ *(47)*. FSH-deficient male mice were fertile despite a decrease in testis size beginning at postnatal d 14. Histological analysis suggested a reduction in seminiferous tubule volume with no change in net number of Leydig cells per testis. All the stages of spermatogenesis appeared normal. Quantitation of sperm parameters indicated reduced sperm number and motility *(47)*. There were no changes in serum testosterone levels, and the male accessory sex glands appeared normal. In contrast to normal fertility in males, FSH-deficient female mice were infertile. They demonstrated small ovaries and variable uterine size, and had reduced progesterone, slightly elevated LH, and unaltered estradiol levels in serum. Ovarian histology indicated a preantral stage block in folliculogenesis with no corpora lutea. Primordial and multilayer preantral follicles appeared normal,

with apparently normal granulosa and thecal cells and oocytes. The infertility in females could be rescued by PMSG/hCG treatment *(47)*.

There has been a long-standing debate on the role of FSH in spermatogenesis, and species-specific differences have been observed by several groups in the past. FSH knockout mouse model phenocopies a human recessive FSH receptor mutation leading to ovarian dysgenesis in affected women *(48)*. However, their sibling brothers are fertile despite reduced testicular volume and sperm number *(146)*. Based on these mouse and human studies, it can be concluded that FSH signaling is dispensable for spermatogenesis but essential for ovarian folliculogenesis.

Prolactin and Prolactin Receptor

Prolactin (PRL) and its signaling through PRL-receptor (PRL-R), a transmembrane protein belonging to the cytokine receptor superfamily, have been extensively investigated for the past several decades *(49)*. In most mammals, PRL is synthesized and secreted from pituitary lactotropes. The presence of PRL isoforms or variants, PRL-like peptides, placental lactogens, and splice variants of PRL-R (long and short forms) in multiple tissues suggests a multitude of functions attributed to PRL *(49)*. This has made understanding the biology of PRL in mammary gland development and reproduction extremely complex. To study PRL-mediated signaling systematically, recently two groups generated independent mouse mutations, one deficient in PRL-R, and the other deficient in PRL-ligand *(50,51)*.

In heterozygous (PRL-R mutant/+) female mice, mammary gland development is greatly impaired, leading to lactational defects after their first, but not later pregnancies. Homozygous mutant female mice deficient in PRL-R are sterile and have multiple reproductive defects including irregular estrous cycles and impaired maternal behavior *(50)*. The ovarian histology indicates fewer primary follicles, and fewer eggs are released and fertilized when these mutant mice are mated. In addition, there is a complete arrest of preimplantation development of embryos in PRL-R knockout mice, however, a small proportion of embryos that progress to blastocyst stage fail to implant in the uteri. When the homozygous mutant female mice are mated with vasectomized males, they fail to exhibit pseudopregnancy 12 d after mating, indicated by the absence of an estrogen surge *(50)*. Approximately 50% of the homozygous mutant male mice are subfertile or exhibit delayed fertility. PRL is thought to be involved in regulating Leydig cell testosterone production via modulating LH levels or its receptor on Leydig cell. Although the reason for the male infertility is not yet known, because 50% of the homozygous mutant male mice are fertile and produce normal-sized litters, other factors may compensate for this process or perhaps it is dependent on the genetic background of the mice. Since the mutation introduced into the PRL-R locus is a premature stop codon, any readthrough transcript should only result in a form without any functional LBDs. Therefore, it is unclear which of the PRL-R isoforms (long or short) is important for the observed phenotypes *(50)*.

Similar to the phenotypes in the PRL-R-deficient mice, deficiency of the PRL ligand also leads to mammary gland developmental defects and infertility in female mice *(51)*. Heterozygous female mice are normal. The irregular estrous cycles and a failure to maintain pseudopregnancy in homozygous mutant female mice are similar between the ligand and receptor-deficient models. In sharp contrast to male mice deficient in PRL-R, PRL-deficient mice are normal and fertile *(51)*.

The ability to generate individual mouse mutations in the ligand and its cognate receptor by ES cell technology thus offers a useful approach to understanding the biology of complex mechanisms of signaling in vivo.

Transforming Growth Factor-β Superfamily Members

Members of the transforming growth factor-β (TGF-β) family are important signaling proteins with diverse cellular functions. These proteins are synthesized as prepropeptides and cleaved to form biologically active homo- or heterodimers that interact with at least two distinct classes of cell surface Ser/Thr kinase receptors containing transmembrane domains *(52)*. The type II receptors bind ligands and phosphorylate and recruit a type I receptor. This trimeric complex then transduces the signals downstream into the cytoplasm, where the recently discovered Smad proteins undergo phosphorylation and translocate to the nucleus to activate gene transcription either directly or indirectly *(53)*. Among the TGF-β family members, important proteins implicated to play a role in reproduction are inhibins and activins, MIS, bone morphogenetic proteins (BMP8a and 8b), and growth differentiation factor-9 (GDF-9).

Inhibins and activins were originally discovered as gonadal peptides based on their respective abilities to regulate negatively or positively FSH homeostasis in the pituitary. Inhibins and activins later were shown to be expressed in multiple tissues including pituitary during embryogenesis through adulthood *(54)*. Inhibins and activins are both dimeric proteins and consist of combinations of a single α- or one of two β-subunits. Activins are β-subunit-containing homodimers (βA:βA; βB:βB) or heterodimers (βA:βB), whereas inhibins are α:β heterodimers (α:βA = inhibin A; α:βB = inhibin B). In the testis, these peptides are synthesized primarily in the Sertoli cells and in the ovary, from granulosa cells *(54)*. A well-characterized activin receptor type II (ActRIIA) has been shown to be expressed in the reproductive axis. Expression of ActRIIA, activins, and inhibins in the reproductive axis suggests that they may have autocrine, paracrine, and endocrine interactions *(54)*. Functional analysis of these proteins has been undertaken through a genetic approach by creating mutant mouse models in which genes encoding these peptides have been deleted by ES cell technology. Mutant mice deficient in activin βA *(55),* or activin receptors subtypes ActRIB *(56)* and ActRIIB *(57),* all die embryonically or at birth and therefore reproductive phenotypes in these mice could not be examined. Inhibin-deficient mice (owing to deletion of the inhibin α-subunit) initially develop normally but ultimately develop hemorrhagic mixed gonadal sex cord-stromal tumors with 100% penetrance and have high levels of FSH *(58)*. These mice die because of a severe wasting syndrome accompanied by liver failure and loss of body weight. The gonadal tumors secrete large amounts of activins and estradiol into circulation. Ovarian transplantation experiments have suggested that loss of inhibin is the primary reason for the onset of these gonadal tumors and thus identified inhibin as a novel secreted type of tumor suppressor *(59)*. Activin βB subunit gene deletion results in viable mice and leads to eyelid closure defects. Whereas male mice deficient in activin βB are fertile, mutant female mice have reproductive defects *(60)*. These are manifested by increased gestation time and nursing defects of the mothers. The mammary glands develop properly and histologically appear normal. In the absence of the activin βB, activin βA is shown to be upregulated at least in ovaries. In contrast to the defects seen in mice lacking the activin βB subunit, deficiencies in activin βA lead to perinatal death owing to whisker and cleft palate defects *(55)*.

The majority of ActRIIA-deficient mice live up to adulthood and exhibit reproductive defects *(61)*. Consistent with activin expression in pituitary gonadotropes, ActRIIA-deficient mice have suppressed FSH levels in the pituitary and serum, but LH levels are unchanged. Mutant ActRIIA-deficient male mice exhibit a delay in fertility and have small testes, presumably owing to secondary reduced FSH levels *(61)*, or directly owing to the absence of activin signaling through ActRIIA locally in the testis. Female mutant mice are infertile and have thin uteri and small ovaries. Normal follicular development is obvious in the ovaries of these mutant mice; however, follicular atresia is often noticed with rare occurrence of corpora lutea *(61)*.

Gene-targeting approaches have confirmed that the well-established mesoderm-inducing activity attributed to activin in lower vertebrates is not present in mammals. However, mice deficient in ActRIB die embryonically owing to gastrulation defects *(56)*, and mice deficient in ActRIIB also die at birth owing to cardiac and craniofacial defects *(57)*. Thus, these approaches have uncovered novel role(s) of these important signaling proteins and receptors.

The simplest ligand-receptor signaling pathway established for a TGF-β superfamily member by gene-targeting experiments is that for MIS and its type II receptor *(62)*. Both of these mouse models phenocopy each other. Absence of MIS signaling (removal of either ligand or receptor) in males leads to normal testicular descent and production of functionally normal sperm. However, mutant male mice are infertile owing to Müllerian duct development and interference of these female reproductive organs with the sperm transfer in males. Leydig cell hyperplasia and an occasional tumor are noted in the mutant male testes. Generation of double mutant mice that lack both MIS and MIS-II receptor does not uncover any additional phenotypes compared to the single mutations *(62)*.

The BMPs constitute an important subfamily of the TGF-β superfamily and are important regulators of embryonic development, and cell-cycle control including apoptosis, cell fate specification, and differentiation *(63)*. At least 15 BMPs have been identified to date and some of their functions have been identified by analysis of mutant mice generated by ES cell technology. Unlike most of the other TGF-β family members, BMP ligands can bind and transduce through well-characterized type I receptors, although recently a BMP type II receptor was also cloned *(63)*. BMP-2 deficiency leads to cardiac defects and embryonic death in mice. BMP-4 and BMP-RI(ALK-2)-deficient mice die around gastrulation owing to defects in mesoderm formation and patterning, and BMP7-deficient mice die perinatally with major defects in eye, limb, and kidney development *(63)*.

Unlike inhibins, activins, and MIS, which are all gonadal somatic cell–derived products, two closely linked mouse genes on chromosome 4, BMP8a, and BMP8b, are expressed in the germ cells of the gonads. These two genes share high structural homology, and their expression patterns within the testis significantly overlap during specific stages of the mouse spermatogenesis cycle, particularly in stage VI–VIII round spermatids. To define the biological roles of these proteins, mice deficient in either BMP8a or BMP8b were generated *(64,65)*. Female mice deficient in either BMP8a or BMP8b are normal and no significant defects are observed in female fertility. Both mutations also do not result in any obvious defects in somatic cells of the testis and the Sertoli and Leydig cells. Consistent with a bimodal germ cell–specific expression of BMP8b, first during prepubertal/early germ cell proliferation and later restricted to

only spermatocytes during the adult stage, two distinct phenotypes are evident in the testes of BMP8b-deficient male mice. There is either a failure to proliferate or a reduction in proliferation and differentiation of germ cells during early puberty *(66)*. Additionally, in the testes of adult BMP8b-deficient mice, spermatocytes undergo increased apoptosis, leading to germ cell depletion and progressive sterility. In BMP8a-deficient male mice, testicular germ cell defects are not obvious during early stages of spermatogenesis. But approx 50% of adult BMP8a-deficient male mice eventually show degeneration of epididymal epithelium only in the caudal region with occasional formation of a granuloma *(63)*. Genetic analyses of these mutant mice have established definitive roles of these two closely linked genes in spermatogenesis. BMP8b is required for both initiation and maintenance *(65),* whereas BMP8a is required only for maintenance of spermatogenesis *(64)*. It is probably difficult to generate double mutant mice lacking both these genes via breeding, because genes are only 0.2 cM apart on mouse chromosome 4. With the advent of newly developed techniques of knock-in and conditional gene targeting, it will now be feasible (1) to test genetically in vivo whether functions of each of these proteins can be replaced by one another, and (2) to delete one or both genes at specific time points during spermatogenesis.

To date, at least 11 members of another TGF-β subfamily, called GDFs (growth differentiation factors), are known. GDF-9 is a unique member of this subfamily. It was originally cloned during a search for novel TGF-β superfamily members *(66)*. GDF-9 is expressed primarily only in the oocyte from the primary one-layer follicle stage until after ovulation. To study the function of this oocyte-restricted growth factor, we generated mice deficient in GDF-9 by deleting exon 2 of the *GDF-9* gene, which encodes the mature protein *(66)*. Mutant male mice were fertile without any gross defects. Homozygous mutant female mice were infertile with small ovaries and could not be rescued by superovulation treatments. Histological analysis revealed a folliculogenesis block at the primary one-layer follicle stage. Only primordial and primary (one-layer) follicles were apparent and follicles beyond this stage were not present *(66)*. In addition, ovaries from these GDF-9-deficient female mice demonstrated progressive oocyte degeneration with collapsed zona pellucida, and the presence of asymmetric, abnormal, and vacuolated granulosa cells in follicles that appeared luteinized. There was no distinct thecal cell layer apparent surrounding these follicles. In vitro analysis of oocytes from GDF-9-deficient mice suggested that normal transitions in chromatin organization were present in oocytes, but the acquisition of meiotic competence was significantly impaired. In addition, oocytes from the mutant ovaries cultured in vitro exhibited abnormal germinal vesicle breakdown owing to aggregation of chromatin and a failure to form proper meiotic spindles *(66)*. At a more advanced age of approx 4–6 mo, ovaries in the mutant females demonstrated fluid-filled follicular cysts, and because of a probable failure of gonadal steroid feedback on the pituitary, these mice had a hypergonadotropic (elevated LH and FSH) condition. Northern blot analysis revealed no changes in LH-R, FSH-R, and ActRIIA, but a reduction in aromatase mRNA levels. Thus, these gene-targeting approaches identified GDF-9 as the first oocyte-derived growth factor required for somatic cell function in vivo *(66)*. The observations that granulosa cells assume abnormal morphology leading presumably to altered function and that oocytes subsequently degenerate further emphasizes that reciprocal interactions between somatic cells and oocytes are critical for normal progression of ovarian folliculogenesis and oogenesis.

Cytokines/Growth Factors

Cytokines are hormones produced by an immune system cell that acts most often locally on either the same (autocrine) or another nearby cell (paracrine). This class of proteins typically does not include the traditional hormones that are endocrine organ derived and act at a distance from their source of origin. More than 50 distinct cytokines have been isolated and physiochemically characterized, and their receptors on target cells have been identified *(67)*. Diverse stimuli regulate the production of various cytokines from different cell types, although cells of the immune system are one major source of cytokines. Based on both structural and functional homology and chromosomal localization, most cytokines are categorized into families (e.g., interleukin [IL], colony-stimulating factor, insulin-like growth factor (IGF)). Biochemical studies and several in vitro cell culture experiments have identified both cell surface and soluble receptors that are unique or common to different cytokines *(67)*.

Interleukin-1 (IL-1) is a pleiotropic cytokine expressed in multiple tissues. The primary source of IL-1 production is tissue macrophages. At least 3 forms of IL-1 have been characterized. These are IL-1α, IL-1β, and IL-1 receptor antagonist protein. They act as either agonists or antagonists by binding to two receptors: the type I IL-1 receptor and the type II IL-1 receptor. In the reproductive tract, macrophage-derived IL-1 has been implicated as a regulator of gonadal steroidogenesis. The type I IL-1 receptor, which is the only receptor capable of signal transduction in response to IL-1, is localized to granulosa cells in the ovary and Leydig cells in the testis. Mice lacking a function type I IL-1 receptor have been generated *(68)*. Both male and female mutant mice are fertile and normal. These studies with IL-1 mutant mice suggest that IL-1 signal transduction via its type I receptor is redundant at least in the reproductive axis. In contrast to the normal fertility in IL-1 receptor null mice, female mice deficient in the IL-11 receptor signal transduction pathway are infertile *(69)*. Similar to IL-1, IL-11 is widely expressed in many tissues, and its actions or hematopoietic cells and cells of the gastrointestinal tract and nervous system have been well studied. Additionally, both IL-11 and IL-11 receptor are expressed in the pregnant uterus at the time of decidualization. The female infertility in IL-11 receptor–deficient mice is owing to a postimplantation defect resulting in the absence of a normal uterine decidual response required for successful pregnancy *(69)*.

Another example of a mutant strain of mice deficient in a cytokine, which shows normal development and hematopoiesis but implantation defects, is the leukemia inhibitory factor (LIF)–deficient model. LIF, in addition to its multitude of actions on several hematopoietic cell lineages, inhibits ES cell differentiation in vitro and is a known regulator of early implantation of the embryo. LIF is induced in mouse uterine endometrial glands on d 4 of pregnancy, coinciding with blastocyst implantation. LIF-deficient mice fail to become pregnant because of a preimplantation defect of the blastocysts in the uterus *(70)*. This defect can be rescued by injections of recombinant LIF into 3-d pregnant homozygous mutant female mice.

Unlike the female-specific defects, a null mutation in *IGF-1,* another important growth factor normally expressed in multiple reproductive organs, results in dwarfism and severe reproductive defects in both male and female mutant mice *(71)*. In mutant males, when compared to wild-type male mice, the testis size is decreased by 60%, and all of the accessory sex glands are severely hypoplastic with more than 80%

reduction in epididymis, vas deferens, seminal vesicle, and prostate. Sperm number in the testis and epididymis is reduced but the sperm motility is not affected. Leydig cell number and volume are also reduced in the mutant male testis. The basal and LH-induced testosterone production from mutant Leydig cells are downregulated in in vitro experiments. Although these defects are manifested in male infertility, capacitated sperm obtained from the mutant males function normally in vitro and fertilize ova that develop into two-cell embryos at comparable numbers to wild-type controls *(71)*.

Similar to the hypoplasia seen in Igf-1 mutant male reproductive organs, in female mutant mice, the ovaries and uteri are completely hypoplastic and their serum estradiol levels are reduced by 50% compared to those of controls. Ovarian histological analysis shows that antral follicles are present but the female mice fail to ovulate normally or even when analyzed by PMSG/hCG superovulation *(71)*. There is also a significant decrease in follicle size and oocyte diameter in the mutant ovaries. The uterus in mutant female mice, which does not exceed 13% of the normal weight, is thin and flaccid. Histologically, in these mutant females, the uterine endometrium is lined with well-differentiated columnar epithelial cells, but the secretory glandular elements are reduced. The most prominent feature in the absence of Igf-1 in the uterus is a severely hypoplastic myometrium with only a few layers of smooth muscle cells in the outer longitudinal layer. Thus, although the primary role attributed to Igf-1 is to mediate GH actions in the adult to controlling body size, Igf-1 knockout studies establish Igf-1 as an important local regulator of male and female reproductive function *(71)*.

Desert Hedgehog

In *Drosophila,* the segment polarity gene, hedgehog (*hh*) has been identified as a key regulator of embryonic and adult pattern formation. Three mammalian *hh* homologs have been identified: Desert hedgehog (*Dhh*), Indian hedgehog (*Ihh*), and Sonic hedgehog (*Shh*). All of these genes encode secreted signaling proteins. Dhh has been shown to be expressed sex-specifically only in Sertoli cells of the testis, shortly after the activation of *Sry*. This expression of Dhh in the testis persists into the adult *(72)*. To determine whether Dhh, a secreted signaling factor, plays a role in cell-cell interactions (which are well known) in the testis, Dhh-deficient mice have been generated. As expected, based on the male-restricted expression of Dhh, females deficient in Dhh are normal and fertile *(72)*. Dhh-deficient male mice are viable but infertile. The testis size is dramatically reduced (almost by 90% at 6 wk) in mutant male mice. Spermatogenesis in the mutant testis is blocked at different stages depending on the genetic background. In the *129/Sv* mouse inbred genetic background, pronounced apoptosis occurs in primary spermatocytes and no spermatids are apparent. By contrast, on a mixed *C57BL/6J-129/Sv* genetic background, some of the male mice have progression of germ cells through late stages of spermiogenesis despite a decrease in testis size. More precisely, step 16, a stage in which spermatids form spermatozoa as they approach the tubule lumen, is blocked *(72)*. Consequently, no mature spermatozoa are detected in either the testis or epididymis.

Further analyses indicated that the reduction in testis size is initiated during the embryonic stage and occurs after the first wave of germ cell proliferation, confirming that for normal germ cell proliferation, Dhh is not essential. Most important, Dhh deficiency causes loss of another sex-specific gene that encodes a transmembrane protein, called Patched (Ptc), in Leydig cells *(72)*. Both in *Drosophila* and mammals,

Ptc has been implicated in the Dhh signaling pathway. These results suggest that the effects of Dhh on spermatogenesis may be mediated indirectly through the Ptc protein that is expressed in Leydig cells. It will be of interest to examine whether Leydig cell steroidogenesis output (i.e., testosterone production) is affected in Dhh-deficient mice. These loss of function experiments establish that the Dhh signaling pathway is evolutionarily conserved across phylogeny in flies and mice.

Steroid Hormone Receptors and Transcription Factors

Estrogen Receptor α

In mammals, the gonad-derived sex steroids are estrogens and androgens. Estrogens can act directly or get enzymatically aromatized to androgens and thus may contribute indirectly to the proposed actions in males *(1,2)*. In addition, two types of estrogen receptors (ERs), ERα and ERβ, are expressed widely from embryonic through the adult stages in both sexes; however, the functional significance and mechanism of action in many of these tissues are relatively unknown. ER, similar to other well-known steroid receptors, belongs to a superfamily of transcription factors, is activated on ligand binding, and binds specific DNA sequences in target genes, commonly known as estrogen response elements (EREs). It is now a widely accepted notion that this binding recruits the newly identified accessory coactivators and more global transcription machinery components to bring about the overall process of gene transcription. Until recently, no known mutations had been identified in humans or mice that affect estrogen signaling and thus mammalian reproduction and development. To understand the consequences of the absence of ER signaling in vivo, ERα-deficient mice have been generated by an insertional mutagenesis strategy using ES cell technology *(73)*. In mice, ERα is expressed in the hypothalamus, pituitary, and gonads and all three of these organs are affected in the mutants. Both male and female ER-deficient mutant mice are infertile and mutant males exhibit impaired sexual behavior including decreased intromissions and ejaculations. At the level of the pituitary, estrogens are believed to be important for lactotrope development and function. However, lactotrope specification is not affected, but the number of PRL-expressing cells is reduced, with a concomitant reduction in PRL gene transcription in the pituitaries of ERα-deficient mice *(74)*.

The negative feedback effects owing to ER signaling within the gonadotropes have been well studied. Consistent with this, the gonadotropin subunit gene transcription is upregulated in ERα-deficient females, with the maximal induction in LHβ gene transcription compared to that of α-GSU and FSHβ genes. Consequently, the circulating levels of LH are elevated only in female ERα-deficient mice, thus reinforcing the idea of sex-specific differences in steroid-mediated transcriptional regulation of pituitary gonadotropin subunit gene expression. In the testes of ERα-deficient mice, there is a progressive atrophy of seminiferous tubules as early as 3 wk of age. By 12 wk, spermatogenesis is completely disrupted with reduced sperm counts, motility, and viability. The sperm are also incompetent in in vitro fertilization assays. One explanation attributed the failure of testicular function to defects in the intraluminal fluid dynamics and pressure caused by alterations in the blood testis barrier and Sertoli–Sertoli cell tight junctions.

More recently it was shown that estrogen regulates the reabsorption of luminal fluid in the head of the epididymis. Disruption of this function, as may be the situation in ERα-deficient male mice, would result in entry of "dilute" sperm into the epididymis,

leading to infertility *(75)*. The infertility in ERα-deficient female mice is associated with elevated ovarian estradiol and testosterone levels. Histologically, the ovaries are hemorrhagic and cystic and folliculogenesis is arrested at the secondary follicle stage, with many atretic follicles. Exogenous administration of gonadotropins to these mutant females does not cause superovulation, suggesting that the ovarian defects are perhaps owing to local effects of elevated androgens and estrogens *(73)*. The defects in accessory female reproductive structures include mammary agenesis with absent and alveolar development, very thin uteri that are unresponsive to estrogen stimulation, and enlarged androgen-sensitive preputial glands (which in wild-type female mice are normally inconspicuous).

Thus, ERα-deficient mice present multiple reproductive phenotypes and offer an excellent in vivo model to study estrogen signaling in mammals. Because the (elevated) steroid hormones can act locally and cause the observed defects directly, not all of the observed phenotypes can be accounted for by the altered hormonal milieu (elevated LH and decreased PRL). Interestingly, the absence of estrogen signaling does not result in defects in embryonic development although the fetal effects of estrogens are well known *(74)*. It will be interesting to generate ERβ-deficient mice in the future and compare the phenotypes of these two strains of mice to explore the possible redundancy and synergism between the two signaling pathways. In addition, tissue-specific and conditional gene-targeting approaches will further delineate the complex mechanisms of estrogen signaling in a more defined spatiotemporal pattern.

Whereas embryonic development proceeds normally in the absence of ERα, severe placental abnormalities are observed in embryos lacking an orphan nuclear receptor, the ER-related receptor β (ERRβ) *(76)*. This receptor is homologous to ERα and binds the ERE (estrogen response element in DNA) but is not activated by estrogens. The mutant embryos die *in utero* at d E10.5 with no detectable heartbeat and begin to undergo resorptions. Abnormal chorion development, often associated with an overabundance of trophoblast giant cells and deficiency of diploid trophoblasts, is seen in these embryos. E9.5 mutant embryos demonstrate growth failure and absence of chorioallantoic fusion, and contain multiple layers of giant cells with no labyrinthine trophoblasts or spongiotrophoblasts *(76)*. These mutant mice offer an excellent model to manipulate pharmacologically the activity of ERRβ to affect implantation and placentation processes, which may have implications for female contraception in humans.

Progesterone Receptor

Unlike ER, effects of progesterone receptor (PR) are more confined to the female reproductive axis. The primary source of progesterone is the ovarian corpus luteum. Regulated by pituitary or placental gonadotropins and PRL, progesterone is believed to be essential for uterine implantation of the embryo, as well as establishment and maintenance of pregnancy. PR is also expressed in the hypothalamus and pituitary and controls sexual behavior and gonadotropin surges. Later, during pregnancy and after parturition, the effects of progesterone on mammary gland development and lactation are also well characterized *(1,2)*. Two naturally occurring isoforms of PR, PR_A and PR_B, are encoded by the same gene and are known to bind progesterone, although the physiological significance of the two forms is unclear. In the majority of the tissues, PR is induced by estrogen. A well-characterized ERE is present in PR, implying that most of the reproductive functions attributed to progesterone may in fact be mediated

via estrogen signaling through estrogen receptor or owing to the combined effects of both estrogen and progesterone.

To distinguish clearly the biological roles of progesterone in vivo from those of estrogen and to understand signaling through PR further, PR knockout mice have been generated. The engineered mutation disrupts the transcription of both PR_A and PR_B isoforms *(77)*. PR-deficient mice are viable, similar to ERα-deficient mice. At least four distinct phenotypes are characterized as a result of null mutation in PR in mice. As expected, homozygous male mice are fertile, and homozygous females are infertile. The infertility in mutant female mice cannot be rescued with superovulation treatment, although histological analysis reveals the presence of an unusual number of mature preovulatory follicles but no corpora lutea. These anovulatory follicles, however, undergo cumulus expansion, considered as the last step prior to follicular rupture, and the granulosa cells do not show signs of luteinization *(77)*. The oocytes do not further divide and often an unexpected "necrosis" of the oocytes is observed. Therefore, these observations suggest that PR signaling is necessary for ovulation and luteinization within the ovary.

Estrogen-primed uterus normally responds to progesterone stimulation and exhibits marked morphological and histological changes. In addition, uterine response to decidual stimulation is well characterized in rodents. Female mice deficient in PR fail to demonstrate both these phenotypes, suggesting that the absence of PR signaling leads to uterine developmental and functional defects *(77)*. Interestingly, uterine hyperplasia accompanied by a strong local inflammatory response is observed in female mutant mice. Mammary gland development, both normal and neoplastic, is subject to multihormonal signaling and progesterone is known to play a crucial role in these processes. In PR-deficient mice, less-extensive ductal development and complete absence of lobular-alveolar structures, even after injecting high doses of estrogens and progesterone, are present. Finally PR-deficient mice fail to exhibit lordosis, the female receptivity to the mounting effects of sexually experienced wild-type male mice, even after estrogen priming *(77)*.

Most of the estrogen and progesterone effects in the female mice and the effects of androgens in males are clearly dependent on steroid receptor coactivator-1 (SRC-1), which is a histone acetyltransferase that binds and recruits components of the general transcription machinery in cells *(78)*. Mice deficient in SRC-1 are viable and fertile but exhibit partial hormone resistance to steroid hormone treatments and demonstrate only marginal increases in biological responses such as estrogen-primed uterine weight gain, decidual response, mammary gland branching, and androgen-induced prostate growth *(78)*. Because many clinical syndromes in humans are associated with partial hormone resistance, even though the corresponding receptors are intact, SRC-1 knockout mice may be a useful model in understanding the molecular basis of these disorders.

Retinoic Acid and Retinoic X Receptors

Pleiotropic effects mediated by only a limited number of ligands or heterodimeric partners of these ligands via multiple receptors or isoforms of receptors are best illustrated by retinoic acid (RA) signaling in the mouse. There are two major families of RA receptors (RARs); the RAR family (RARα, RARβ, and RARγ) members are activated by both all-*trans* and 9-*cis* RA, whereas the retinoid X receptor (RXR) family members are activated exclusively by the *cis*-isoform of the ligand. Further complexity

exists because each of these six different receptors also have isoforms, e.g., RARα1 and RARα2. RARs and RXRs belong to steroid receptor superfamily of transcriptional activators *(79)*. The receptors are widely expressed, and the signaling through these receptors in distinct pathways is known to be important for embryogenesis, homeostasis in skeletal morphogenesis, and vision. In addition, vitamin A and its derivatives are important and established regulators of male reproductive functions. Although the major aim of Chambon *(79)* in generating different mutant mouse strains that lack components of RA/RAR signaling pathway was to study in vivo the genetic redundancy between these members during embryogenesis and skeletal development, three of the mutant strains show interesting male reproductive phenotypes in addition to defects in other tissues.

One hundred percent of RARα-deficient (absence of all isoforms of RARα) male mice at more than 2 mo of age are sterile and do not sire any offspring *(80)*. By 4 to 5 mo of age, 50% of the mice exhibit severe degeneration of the germinal epithelium, with lesions in seminiferous tubules. These include atrophy and marked reduction of multiple stages of spermatogenic cells. In Sertoli cells, vacuolation is frequently observed with aberrant cytoplasmic projections into the lumen of the tubules. Very few sperm are present in the epididymis and the lumen of the many abnormally appearing tubules. The accessory glands (i.e., seminal vesicles and prostate) are normal in these mutant mice *(80)*.

In the male reproductive tract of mice, RXRβ is selectively expressed in Sertoli cells of the testis. The absence of RXRβ leads to embryonic or perinatal death in 50% of the mice, but in the other 50%, the males are sterile owing to abnormal spermatogenesis *(81)*. The defects appear to be more severe than in RXRα-deficient mice. There is germinal epithelium breakdown in RXRβ knockout mice also, and, more characteristically, these mice exhibit oligo-astheno-teratozoospermia, a condition in which there is a severe reduction in the number of spermatozoa, reduced motility, and a high percentage of abnormalities of acrosomes and tails of spermatozoa in the semen *(81)*. There is a progressive accumulation of unsaturated triglycerides within the Sertoli cells. Additionally, there is a failure in spermiogenesis leading to apoptosis of spermatids. The genital ducts, namely, the epididymis and vas deferens and accessory glands (i.e., seminal vesicles, prostate and bulbourethral glands), appear grossly and histologically normal *(81)*.

In contrast to the spermatogenesis defects in RARα- and RXRβ-deficient mice, null mutation in RARγ causes male reproductive defects primarily in accessory glands leading to infertility in mice that survive past 2 mo *(82)*. Both seminal vesicles and prostate glands exhibit squamous metaplasia and/or keratinization or glandular epithelia without any secretion product in their lumen. The mucosal folds and septa within the accessory glands are often absent, and histological demarcation of these two glands as distinct structures is indistinguishable. Hypertrophy of the prostate is also apparent in mutant male mice, probably owing to infection and focal destruction of the metaplastic epithelium composed of the keratinized cells *(82)*.

The most interesting observation with regard to the reproductive defects in these RA signaling pathway mutant mice is that only male fertility is affected. Females bearing these individual mutations are all normal and fertile. Further studies aimed at examining the effects on potential target genes and how they are affected will uncover the molecular basis of the observed male fertility defects in these mice.

Homeobox Family Members

Homeobox (Hox) genes encode transcriptional regulators that specify patterning of groups of cells into specified structures by activating cascades of genetic interactions. In mammals, Hox genes are clustered into a series of linked genes on distinct chromosomes (four clusters—A, B, C, D—are known), and their expression pattern is normally restricted to defined boundaries in segmented structures. There is also a colinearity between a given Hox gene and its expression pattern during development, with 5′ genes expression restricted to more caudal regions of the embryo. Because Hox genes are sequentially activated, targeted misexpression or misregulation of Hox genes within a given cluster can have profound consequences in skeletal, central nervous system (CNS), and limb morphogenesis. These are often known as homeotic transformations *(83)*.

Homeotic transformations of reproductive organs leading to infertility as a result of engineered null mutations in three distinct Hox genes have been analyzed. Although these mutations cause defects in other tissues as well, we describe here only those relevant to the reproductive axis. Hoxa-10 is expressed in the genital tubercle during embryogenesis. Later, by E15.5, the expression is also detected in the gubernaculum, the ligamentous cord, which connects the gonad to the labioscrotal swellings of the abdominal wall. Slightly preceding this expression, around E13.5–14.5, Hoxa-10 expression is also detectable in the developing abdominal wall and the future inguinal canal and scrotal sac. In females, the adult uterus persistently expresses Hoxa-10, consistent with the fact that the uterus undergoes cyclic changes of differentiation program during embryo implantation and pregnancy when it is under constant growth factor and cytokine stimuli. Male and female mice deficient in Hoxa-10 are infertile *(84)*. Mutant males have small testes owing to Sertoli cell vacuolation, germinal epithelium breakdown, and sloughing of germ cells into lumen of the tubules. These defects are secondary to prominent unilateral or bilateral cryptorchidism (i.e., failure of testicular descent). Further studies suggest that cryptorchidism in Hoxa-10-deficient mice is owing to an indirect effect of homeotic-anterior transformations of first and second lumbar spinal cord segments. These segments normally give rise to the genitofumeral nerve, which innervates the gubernaculum and the cremaster muscle, which are essential for proper testicular descent. There is also an apparent homeotic transformation at the junction of the genital ducts (i.e., epididymis and ductus deferens). Female mice deficient in Hoxa-10 have implantation defects leading to early resorptions of embryos. Parallel to the male genital duct transformation in mutant females, the proximal 25% of the uterus is homeotically transformed into oviduct. Implantation failure of wild-type embryos transferred into the mutant uterus distal to the homeotic transformation suggests that expression of Hoxa-10 in the uterus is critical for successful implantation proceeding to pregnancy *(84)*. Deficiency of Hoxa-10 in mutant females also results in decreased vascular permeability, which is normally elicited in response to implantation. There are defects in decidualization following artificial stimuli supplemental to the uterine horns in mutant female mice.

The fundamental principle of the specification of axial skeletal structures dictated by "Hox code" appears to operate universally when the male reproductive phenotypes in Hoxa-11-deficient mice are analyzed. Hoxa-11 is the next 5′ gene to Hoxa-10 in the Hoxa cluster. Male Hoxa-11 mutant mice show a partially overlapping homeotic transformation of the ductus deferens, when compared with that in the Hoxa-10 mutation

(85). The transformation as a result of Hoxa-11 deficiency, however, extends more distal to the region affected in Hoxa-10 mutant mice, with the vas deferens resembling the epididymis. The cryptorchidism observed in Hoxa-10-deficient male mice is also seen in Hoxa-11 mutant males. Hoxa-11 mutant female mice are infertile. Ovarian folliculogenesis and ovulation are normal, but there is an implantation failure of the embryos postfertilization. At early gestational stages (until d 8 of gestation), uterine stromal, decidual, and glandular cell development do not occur in these mutant female mice. In addition, the typical steroid-induced uterine cell proliferation, and oil-induced stromal decidualization as a consequence of pseudopregnancy are all absent in the Hoxa-11-deficient mice. The characteristic burst in LIF that is normally found at gestation d 4.5 is abolished in Hoxa-11-deficient uteri. There is also a partial transformation of the uterus to the more distal part of the oviduct *(85)*.

The defects observed in Hoxd-13-deficient male mice are in striking contrast to those in Hoxa-10- and Hoxa-11-deficient mice. These mice have accessory sex organ abnormalities including agenesis of bulbourethral gland, diminished mesenchymal folding in the seminal vesicles, and a decrease in size and diminished ductal branching in the prostate *(86)*. The expression of Hoxd-13 in these organs perfectly correlates to the time at which distinctive patterns of ductal branching is initiated during postnatal development.

The three Hox-mutant mouse models described here are invaluable in studying the homeotic transformations and Hox code in the reproductive axis. It will be interesting in the future to determine how the absence of these transcription factors brings about such remarkable tissue-specific phenotypes in the gonads and accessory sex organs in both sexes.

WT-1, SF-1, and EMX2

WT-1 and SF-1 are key transcription factors, and as mentioned in Chapters 9 and 12, play critical roles very early during urogenital and adrenal gland development *(16,17)*. Both are also expressed in multiple tissues outside the gonads. Mice deficient in WT-1 die embryonically around d E16.5. The defects related to the reproductive axis in these mice include a failure in gonadal development. At E11.5, in the mutant embryos, thickening of the coelomic surface of the urogenital ridge is reduced, the gonadal ridge develops into a small structure by E12.5, and by E14.5, no visible gonad remnant is seen in the remaining urogenital ridge *(87)*. Despite these quite early defects in gonadal morphogenesis, germ cell migration occurs normally and germ cell number remains almost the same compared to wild-type embryos at E12.5. Therefore, WT-1 is essential for the developmental transitions in gonad formation between E11.5 and E14.5 or even prior to E11.5, because the earliest embryonic expression of WT-1 is normally detected at E9.5 in mouse embryos *(87)*.

The earliest expression of SF-1 in mouse embryos, like WT-1, is also initiated around E9.5 in the urogenital ridge and, in addition, by E10.5 in the adrenal primordium. Based on the early expression analysis of SF-1, Sry, and MIS, and several in vitro cell transfection studies, SF-1 is thought to be important for switching the early "default" female sex determination pathway toward the male pathway. Later, in the adult ovary, SF-1 is expressed in the ovarian somatic cells, i.e., theca and granulosa cells, where its primary function is to support steroidogenesis. Mice deficient in SF-1 are growth retarded and die between p 3 and p 8 owing to adrenocortical insufficiency (i.e., dimin-

ished corticosteroid levels). The primary steroidogenic tissues are affected, and in the newborn mutant pups, the adrenal gland and gonads are completely absent *(88)*. Irrespective of the genetic sex, all the pups have female external genitalia owing to sex reversal. This is also apparent in internal sex organs; both male and female SF-1-deficient mice have normal oviducts, uterus, and vagina. Analysis of very early mutant embryos shows some degree of mesenchymal thickening in the region where the genital ridge normally develops, indicating that initiation of gonadal development does not require SF-1. SF-1 is also not required for primordial germ cell migration, because the germ cells are present in SF-1 mutant mice. Although gonadal initiation is not blocked, gonadal apoptosis is triggered in the absence of SF-1, leading to total regression of the gonads *(88)*.

Recently, mice deficient in *EMX2,* a homeobox gene (*Drosophila* homolog of empty spiracles) were generated. These mutant mice exhibit urogenital defects seen in WT-1 and SF-1 knockout mice. Interestingly, the mutant embryos show accelerated degeneration of Wolffian and mesonephric tubules without the formation of the Müllerian duct *(89)*. Adrenal glands and bladder develop normally, however.

Analysis of gonad development in WT-1 and SF-1 mutant mice and results from other studies provide a model whereby early events in sex determination/differentiation pathway can be formulated. Other components remain to be identified, particularly the potential down- and upstream gene targets for these transcription factors and how they are regulated to establish the phenotypic and functional sex characteristics.

Zfx

The genes encoding similar but nonidentical zinc-finger proteins, Zfx and Zfy, located on nonhomologous regions of human X and Y chromosomes are believed to be important for sex determination and gametogenesis *(90)*. The structural analysis of these proteins suggests that they may act as transcription activators. There are at least four homologs of this gene family in the mouse. These include Zfx on the X chromosome, Zfyl and Zfy2 on the Y chromosome; and a processed autosomal pseudogene on chromosome 10, Zfa. Zfx protein is evolutionarily conserved, and the gene is transcribed in all male and female tissues at all developmental stages compared to the restricted pattern of expression of Zfy and Zfa, and less similar conservation of protein sequence across phylogeny. Zfx-deficient mice were generated via gene targeting in ES cells *(90)*.

Mutant mice deficient in Zfx exhibit the normal sexual phenotypes corresponding to their genetic sex. Both male and female mice are growth retarded and less viable. Zfx-deficient male mice are fertile but their sperm counts are reduced by 50% *(90)*. By contrast, Zfx-deficient female mice have reduced fertility. Those mutant female mice that carry pregnancy and deliver pups have nursing defects. The mutant ovaries are hypoplastic and are depleted of primary and growing follicles, and there is a dramatic reduction in the number of oocytes much prior to the onset of puberty and reproductive cycling *(90)*. The reduced sperm counts and oocytes in the mutant male and female mice, respectively, is reflected in the reduction of primordial germ cells at d E11.5, prior to gonadal sex differentiation.

Zfx-deficient mice phenocopy clinical features exhibited by human females with the XO karyotype and women with premature ovarian failure owing to germ cell defects. Accordingly, this model will prove useful to address the molecular basis of these disorders.

Nhlh2

Neuronal helix-loop-helix-1 (Nhlh1) and Nhlh2 belong to the basic helix-loop-helix family of transcription factors, which are important regulators of growth and development. Nhlh1 and Nhlh2 share overlapping but distinct domains of expression during murine embryogenesis in the developing nervous system. More specifically, Nhlh2 is expressed in the ventral-medial and lateral hypothalamus, Rathke's pouch, and the adult anterior pituitary *(91)*.

To understand the role of Nhlh2 in vivo, mice deficient in Nhlh2 have been created *(91)*. The mutant mice exhibit progressive adult onset obesity owing to an excess of adipose tissue in the perirenal, perigonadal, and subcutaneous areas of the body. Male mice deficient in Nhlh2 are microphallic, hypogonadal with bilateral cryptorchidism, and infertile. In the mutant male mice, the preputial glands are either reduced or absent, and the seminal vesicles are severely atrophied *(91)*. Histologically, the adult testes shows small seminiferous tubules with rudimentary lumen and no apparent spermatids. The epididymis is completely devoid of sperm and greatly reduced in size. In the later stages of the adult testes, only remnants of the seminiferous tubules and very few Leydig cells are seen. The male mice have virtually undetectable levels of testosterone in serum, and they fail to copulate with female mice. Paradoxically, serum GH and LH in Nhlh2-deficient male mice are unchanged but FSH levels are decreased almost four-fold *(91)*.

Nhlh2-deficient female mice exhibit interesting variable phenotypes. When caged in isolation, these mutant females are infertile with very thin uteri and hypoplastic ovaries demonstrating an early antral-stage block in folliculogenesis. However, when the mutant female mice are raised in cages together with males, the fertility defects are partially restored, but with reduced litter sizes and delays between successive pregnancies *(91)*. This fertility restoration is presumably owing to male phenomone-induced hormonal surges that causes ovulation and changes in uterine morphology.

In the developing CNS of mouse embryos at E11.5, the highest expression of Nhlh2 is seen when proopiomelanocortin (POMC)- and GnRH-containing neurons are first detectable. Nhlh2-deficient mice do not display any defects in the central or peripheral systems *(91)*. There is a 25% reduction in cell number in the arcuate nucleus of the hypothalamus, but immunohistochemical staining of tyrosine hydroxylase–positive cells show no differences in the median eminence or arcuate nucleus. However, there is a marked reduction in staining intensities for POMC and GnRH in the arcuate nucleus, Nhlh2-deficient mice. Although Nhlh2 normally is expressed in the anterior pituitary, its deficiency in mice does not cause any changes in pituitary cell types, and all cell types are present in the mutant anterior pituitary.

Generation and characterization of Nhlh2-deficient mice offer a challenging model to study growth and developmental aspects of the reproductive axis.

In the previous sections, we described mouse models in which specific transcription factors are functionally deleted. Several mutations in other transcription factors have also been introduced and knockout mice have recently been generated. These include A-myb *(92)*, E2F-1 *(93)*, CREM *(94,95)*, FMR1 *(96,97)*, sperm-1 *(98)*, C/EBP-β *(99)*, and Stat5a and Stat5b *(100)* knockout mice. These mutant mice have either male, female, or both male and female reproductive defects. Tables 2–4 summarize these defects.

Table 2.
Knockout Models with Reproductive Defects Only in Females

Knockout mouse model	*Major reproductive findings*	*Reference*
c-*mos*	Decreased fertility in females only; ovarian cysts and teratomas	*112,103*
LIF	Infertility; implantation defect	*70*
Progesterone receptor	Infertility; defects in all reproductive tissues	*77*
Activin/inhibin βB	Large litters but delayed parturition; nursing defects	*60*
α-Lactalbumin	Normal fertility but inability to nurse offspring	*147*
GDF-9	Infertility; defect in folliculogenesis at one-layer follicle stage	*66*
Oxytocin	Nursing defect	*4,36*
Transcription factor NGFI-A	Infertility, luteinizing hormone suppression causing no corpora lutea (and/or ovulation)	*42,43*
Zona protein 3	Infertile; no zone pellucida	*134,135*
Steroid 5α-reductase type I	Reduced litter size; parturition defects (fetal death owing to excess estrogens)	*124,125*
Mf3	Nursing defect (also embryonic and postnatal defects)	*37*
Connexin 37	Infertile; defect in folliculogenesis at the Graafian follicle stage	*136*
Cox 2 (prostaglandin endoperoxide synthase-2)	Largely infertile; absence of corpora lutea owing to apparent ovulation defect	*129*
C/EBPβ	Infertile; ovulation and corpora lutea defects	*99*
IL-11 receptor α	Infertile; implantation defect	*69*
Prostaglandin F receptor	Infertile owing to lack of induction of oxytocin receptor	*148*
PRL	Infertile; irregular estrous cycles	*51*
Hmx3	Normal preimplantation development but implantation failure of embryos	*149*
SOD I	Reduced fertility; increase in embryonic lethality	*126,127*
Caspase-2	Excess number of germ cells in ovaries; oocytes resistant to cell death following exposure to chemotherapeutic drugs	*123*
Stat5a/Stat5b double mutants	Infertile; absence of corpora lutea, leading to implantation defect	*100*

Cell-Cycle Regulators and DNA Repair Enzymes

In response to a multitude of signals, eukaryotic somatic cells undergo a cascade of cyclic events, collectively known as the cell cycle, and ultimately differentiate. The cell cycle is divided into four major sequential phases: the gap 1 (G1), the DNA synthesis (S), the gap 2 (G2), and mitosis (M). Each of these transitions is controlled by several cyclin-dependent kinase inhibitors and phosphatases. In addition, tumor suppressors and oncogenes act as negative and positive regulators of the cell cycle, respectively *(101)*. Furthermore, in eukaryotic cells, an additional set of proteins, called

Table 3.
Knockout Models with Reproductive Defects Only in Males

Knockout mouse model	*Major reproductive findings*	*Reference*
MIS	Obstruction and secondary infertility in majority of mice caused by uteri in males	*150*
Inhibins/MIS double mutants	Granulosa/Sertoli cell tumors; Leydig cell neoplasia; large fluid-filled uteri; complete infertility	*151*
ACE	Reduced fertility owing to decreased ability of sperm to fertilize ova	*152*
Bclw	Infertile; spermatogenesis block during late spermatogenesis; eventual loss of all germ cells and Sertoli cells	*111*
HR6B ubiquitin-conjugating enzyme	Infertile; possible defect in histone polyubiquitination and degradation during spermatogenesis	*118*
PMS2 DNA mismatch-repair enzyme	Infertile; meiosis defects leading to abnormal spermatozoa	*115*
Bone morphogenetic protein 8A	Progressive infertility; germ cell degeneration, spermiogenesis defects, and epididymis degeneration	*64*
Bone morphogenetic protein 8B	Infertile; germ cell proliferation/depletion defects	*65*
MIS receptor	Partial fertility; presence of Müllerian duct causing physical blockage	*62*
Apolipoprotein B heterozygotes	Reduced fertility; spermatozoa fertilization defects	*131*
Fragile X mental retardation 1	Normal fertility; macroorchidism owing to increased embryonic Sertoli cell proliferation	*96,97*
Hsp70	Infertile; block at meiotic prophase and increased spermatocyte apoptosis	*105*
Desert hedgehog	Infertile; defects in germ cell development	*72*
Bax	Infertile; spermatogenesis block at premeiotic stage	*110*
CREM	Infertile; block at first stage of spermiogenesis	*94,95*
RARα	Male infertility secondary to seminiferous tubule degeneration	*80*
RARβ	Male infertility secondary to germ cell mutation defects and tubular degeneration	*81*
RARγ	Male sterility secondary to squamous metaplasia of the seminal vesicles and prostate	*82*
Sp4	Infertility owing to defects in male reproductive behavior	*153*
Sperm-1	Subfertile despite normal testicular morphology and sperm number	*98*
Hoxd-13	Defects in formation of the seminal vesicles, ventral and dorsal prostate, and bulbourethral gland	*86*
PC4	Infertile; impaired fertilizing ability of spermatozoa	*122*
Acrosin	Delayed fertility; normal binding and penetration of zona pellucida by sperm	*121*
Calmegin	Infertility owing to impairment of sperm binding to zona pellucida	*154*

Table 4.
Knockout Models with Reproductive Defects in Both Sexes

Knockout mouse model	*Major reproductive findings*	*Reference*
α-Inhibin	Infertility in females; secondary infertility in males; granulosa/Sertoli cell tumors; cachexia-like syndrome	*58,59*
ActRIIA	Infertility in females; delayed fertility in males; small gonads	*61*
ER	Uterine/ovarian defects in females; small testes, reduced number of spermatozoa in males	*73,74*
α-GSU	Infertile; hypogonadal and hypothyroid	*96*
Hoxa-10	Variable infertility in males and females owing to cryptorchidism and preimplantation embryonic loss, respectively	*84*
IGF-1	Hypogonadal and infertile; preantral block in folliculogenesis in females	*71*
Nhlh2	Males infertile; females fertile only in presence of males; hypothalamic defect	*91*
Zfx	Reduced germ cell number in both sexes owing to defective proliferation	*90*
FSHβ subunit	Female infertility; folliculogenesis block prior to antral follicle stage; males fertile but decreased testis size	*47*
$p27^{Kip1}$ CDK inhibitory protein	Female infertility; corpus luteum defects; males fertile and increased testis size	*107–109*
MLH1 DNA mismatch-repair enzyme	Male and female infertility; defective meiosis at pachytene stage (males) and failure to complete meiosis II (females)	*76,114*
Ataxia telangiectasia (Atm)	Male and female infertility; complete absence of germ cells	*112,113*
Cyclin D2	Female infertility secondary to block in folliculogenesis; males fertile but decreased testis size	*104*
PRL-R	Female infertility owing to multiple abnormalities including irregular estrous cycles and implantation defects; males infertile or subfertile of unknown origin	*50*
Dazla	Male and female infertility; loss of germ cells and complete absence of gamete production	*137*
β1,4-Galactosyltransferase	Male and female infertility owing to abnormal glycoprotein hormone glycosylation	*155*
A-myb	Male infertility; pachytene stage arrest of germ cells; nursing defects in females owing to underdevelopment of mammary glands	*92*
EMX2	Accelerated degeneration of Wolffian duct and mesonephric tubules without formation of Müllerian duct	*89*
Hoxa-11	Partial homeotic transformation of vas deferens to epididymis; failure of testicular descent; absence of uterine stromal, decidual, and glandular cells in females	*85*

continued

Table 4.
Continued

Knockout mouse model	*Major reproductive findings*	*Reference*
TIAR	Infertility; complete absence of primordial germ cells by d E13.5 leading to absence of spermatogonia and oogonia	*138*
DMC1	Arrest of spermatogenesis at zygotene stage in males; no oocytes in adult ovary	*116,117*
Telomerase	Progressive infertility in males and females; increased apoptosis in testicular germ cells and reduced testis size; decreased number of oocytes and uterine abnormalities	*130*

DNA repair enzymes, are present that act as cell-cycle "guardians." A variety of insults to the genome results in aberrant DNA replication, and these enzymes recognize such "damaged DNA spots," "repair" the DNA, and protect the cell from not accumulating various kinds of mutations. Another complex process that is critical during transition of cell proliferation into the differentiation state is the programmed cell death, now commonly referred to as apoptosis. This process appears to be crucial to the proper development/differentiation of almost every cell type within an organism, and occurs at defined time points during embryogenesis. *Escherichia coli* and both budding and fission species of yeast have provided excellent model systems to study cell-cycle regulation in general *(101)*.

Cell-cycle regulation within the reproductive axis is highly complex and relatively less explored to date. One well-studied pathway within the testis is spermatogenesis. In addition to mitosis, both male and female germ cells undergo meiosis during gametogenesis. The important somatic cells in the gonads, including testicular Sertoli and Leydig cells in the male and ovarian thecal and granulosa cells in the female, proliferate at discrete periods of time primarily under pituitary gonadotropin influence. Mutations that affect cell-cycle control in mice, including apoptosis and DNA repair pathways, have been created by a number of groups using ES cell technology. A few of these that have reproductive defects are described briefly next.

c-*mos*

The protein encoded by protooncogene c-*mos* is a 37- to 39-kDa cytoplasmic serine/threonine kinase. Earlier studies have suggested that it plays a role in the meiotic maturation events during male and female gametogenesis. c-*mos*-deficient male mice do not display any fertility defects and have normal spermatogenesis *(102,103)*. The mutation, however, affects females, which demonstrate reduced fertility. Normally, oocytes are released owing to a surge of LH. Consequently, the oocytes undergo germinal vesicle breakdown, and meiotic division is initiated leading to first polar body extrusion before they are fertilized by the sperm. All these aspects are apparently normal in c-*mos*-deficient mice, indicating that c-*mos* is not essential for oocyte maturation *(102,103)*. By a distinct parthenogenetic activation (i.e., without fertilization by sperm), c-*mos*-deficient oocytes fail to exhibit a meiotic arrest (which normally happens

in wild-type mice) and proceed developmentally to extrude the second polar body. However, some of the mutant eggs are fertilized shortly after maturation and before parthogenetic activation, and hence only reduced fertility is evident in female mice deficient in c-*mos*. Some of the older mice deficient in c-*mos* develop ovarian teratomas, many resembling benign cystic teratomas of the human ovary. Thus, these c-*mos*-deficient models may provide an animal model to investigate this type of human ovarian pathology *(102,103)*.

Cyclin D2

The three cyclin D members, D1, D2, and D3, are important G1 phase regulators in mammalian cells. They are ubiquitously expressed with overlapping pattern of expression in many proliferating cells during mouse embryogenesis *(104)*. Mice deficient in cyclin D2 have been generated and characterized. No abnormalities during embryonic development are obvious in these mice, indicating that there is functional redundancy in cyclin D family members. Cyclin D2 is predominantly expressed in the testicular Sertoli cells and spermatogonia and in the ovarian granulosa cells. In ovarian granulosa cells, cyclin D2 is induced by FSH stimulation via the protein kinase A pathway. Cyclin D2–deficient male mice are fertile despite a decrease in testis size and a corresponding reduction in sperm count. Their serum testosterone levels are normal and no additional histological abnormalities are found in the testis *(104)*.

Cyclin D2–deficient female mice are infertile and unresponsive to superovulation treatment. Histologically, the ovaries contain a similar number of ovarian follicles and oocytes compared with wild-type ovaries, but granulosa cells surrounding oocytes are reduced to only 3 to 4 layers surrounding small follicles, unlike up to 10 layers normally found in control female mice *(104)*. Both in vitro and in vivo, FSH treatment does not cause these mutant granulosa cells to proliferate. When FSH injections into mutant female mice are followed by hCG injections, ovulations do not occur; instead, the hypoplastic follicles differentiate into corpora lutea with trapped oocytes inside. Surprisingly, LH receptor signaling appears normal, because induction of *COX2* and progesterone receptor mRNAs occurs. Interestingly, the trapped oocytes from mutant follicles, when manually released and cultured in vitro, can develop to the blastocyst stage *(104)*.

Many human granulosa and testicular germ cells have upregulated mRNA levels of cyclin D2. Similarly, human mammary carcinomas have high-level expression of cyclin D1, and cyclin D1–deficient female mice have impaired cell proliferation in the mammary glands *(104)*. Thus, these mutant models reinforce the notion that a cyclin that is required for normal growth of a tissue, when overexpressed in the same tissue, may contribute to its neoplastic growth.

HSP70-2

The G2/M-phase transition during the mitotic and meiotic cell cycles is dependent on cyclin B–dependent CDC2 kinase activity. CDC2 is present constitutively in most cells; however, its activity oscillates during the mitotic cell cycle. *Cdc2* transcripts are abundantly expressed in late pachytene and diplotene spermatocytes prior to the first meiotic division *(101)*. Cyclin B1 and CDC2 proteins are readily detected in pachytene spermatocytes, and in naturally occurring mutant, germ cell-deficient mice, they are reduced and/or completely absent. In addition, CDC2 kinase activity is present mainly

in pachytene spermatocytes and in undetectable levels in somatic cells and early testicular germ cells.

Heat-shock proteins (HSPs) are protein-associated molecular chaperones important for folding, transport, assembly, and disassembly of polypeptide complexes. Mouse testes contain at least four HSPs; one of them, HSP70-2, is spermatocyte specific and developmentally regulated *(105)*. Its synthesis begins in early meiotic prophase I, paralleling with expression of cyclin B/CDC2. Mice deficient in HSP70-2 have been generated, and these mice proved invaluable to test the hypothesized roles of HSP70-2 protein in G2/M phase transition during meiosis in male germ cells *(105)*. Male mutant mice are infertile. The spermatocytes in the testis do not progress through the G2/M phase checkpoint of meiosis I. Immunoprecipitation experiments using testicular extracts from wild-type and mutant mice confirm that HSP70-2 acts as a chaperone for CDC2 in presenting to and forming a complex with cyclin B1. There is no detectable CDC2 kinase activity in the testis of HSP70-2-deficient mice. Recombinant HSP70-2, when added in vitro, reconstitutes the CDC2/cyclin B1 complexes and CDC2 kinase activity. The pachytene spermatocytes in the mutant testis also undergo apoptosis. In addition, the desynapsis or disassembly of the synaptonemal complex is disrupted in pachytene spermatocytes, consistent with the appreciation of HSP70-2 in the synaptonemal complex *(105)*. These results have thus identified HSP70-2 as a key factor in three important processes: meiosis, apoptosis, and synaptonemal complex function in pachytene spermatocytes.

Recently, cyclin B1- and cyclin B2-deficient mice were generated. Whereas cyclin B1 mutant mice die *in utero,* cyclin B2 mice develop normally and are fertile, although they produce small litters. Histologically, no differences are found in cyclin B2–deficient mice compared to wild-type controls. In wild-type mice, the expression pattern of cyclins B1 and B2 overlap, and both are expressed in comparable levels during meiotic prophase in spermatocytes. Therefore, cyclin B1 can compensate for cyclin B2 in meiosis in the spermatocytes *(106)*.

$p27^{Kip1}$

During the G1/S-phase transition in mammalian cells, the cyclin D-CDK4 and cyclin E-CDK2 complexes are catalytically active. A family of proteins known as cyclin-dependent kinase (CDK) inhibitors that consist of two major classes of proteins inhibit this critical transition *(101)*. Members of this family all block the kinase activity of cyclin-CDK complexes. The Cip/Kip family members p21, p27, and p57 have broader blocking specificity whereas the Ink family members p16, p15, p18, and p19 are CDK4/CDK6-specific inhibitors. When transferred into and overexpressed in cells, all these CDK inhibitors cause G1 arrest *(101)*. Growth factors such as TGF-β act via these CDK inhibitors and cause cells to exit the cell cycle and drive them to differentiate *(101)*.

$p27^{Kip1}$ is associated mainly with cyclin D-CDK4, but has the ability to block other complexes as well. Several studies support the hypothesis that $p27^{Kip1}$ plays an important role in the negative regulation of cell growth in multiple tissues. The $p27^{Kip1}$-deficient mouse model has been generated by three independent groups *(107–109)*. Although these mice have multiple defects including thymic hyperplasia and pituitary intermediate lobe tumors, we will describe only the reproductive defects.

$p27^{Kip1}$ is highly expressed in the testis, and $p27^{Kip1}$-deficient mice are fertile and

have enlarged testes with marked hyperplasia. $p27^{Kip1}$-deficient female mice are infertile. These mutant females have disordered estrous cycles *(107–109)*. Only primary and secondary follicles, not antral follicles, are present in the ovary. Granulosa cells exhibit hyperplasia and no corpora lutea are formed, whereas thecal cells appear normal. PMSG/hCG injections cause superovulation of the mutant mice, but uterine implantation does not occur. The endometrium and glandular cells in the uterus also exhibit hyperplasia. In wild-type mice, $p27^{Kip1}$ is undetectable in ovarian granulosa cells but abundantly expressed when they differentiate into the corpus luteum. This is supported by a reciprocal BrdU staining pattern, the highest in granulosa cells and the lowest in luteal cells. The granulosa cell hyperplasia and corpus luteum defects in $p27^{Kip1}$ knockout mice, therefore, indicate that this regulator plays a crucial role in granulosa-luteal cell transition during ovarian folliculogenesis *(107–109)*. It remains to be seen if and how gonadotropins and various factors that regulate this step of folliculogenesis mediate their effects via $p27^{Kip1}$.

Bax

Members of the Bcl2 family include both cell death effectors and repressors. Typical of many regulators, these members exert their effects as homo- or heterodimers. Depending on the specific combination of the selective partners that they form complexes, and the cell context, they may have either positive or negative effects leading to cellular hypo- or hyperplasia *(110)*. Bcl2 is a well-characterized cell death negative regulator, and Bcl2-deficient mice have increased apoptosis in selected tissues. Another member of this family, Bax, heterodimerizes with either Bcl_2 or Bcl_{XL} (in addition to forming heterodimers) and promotes cell death, and therefore acts as an antagonist to Bcl/Bcl_{XL}. In some instances, when more of Bax in a given cell heterodimerizes with either Bcl_2 or Bcl_{XL}, apoptosis is repressed *(110)*.

Bax-deficient mice have been generated, and these are externally indistinguishable to control mice. These mice demonstrate selective hyperplasia of lymphoid tissues and, in contrast, hypoplasia and atrophy of the testis leading to infertility *(110)*. The epididymis and vas deferens are completely devoid of sperm. Histologically, several abnormalities are apparent within the mutant testis. Many tubules exhibit abnormal mitotic or meiotic figures and contain multinucleated giant cells and pyknotic cells between the basal lamina and pachytene spermatocytes *(110)*. Ultrastructural analysis indicated that these premeiotic cells have atypical characteristics of spermatogonia or preleptotene stage spermatocytes. There is a reduction in pachytene cell number; round spermatids with acrosomes are rarely seen, and elongated spermatids are completely absent. There is an enhanced apoptosis in the mutant testis with clusters of germ cells, and in the most severe cases, the tubules are partially or completely devoid of germ cells. In Bax-deficient female mice, germ cell development is unaffected and the oocytes appear normal. However, there is an unusual accumulation of atretic follicles with atrophic residual granulosa cells that presumably fail to undergo apoptosis *(110)*. Thus, Bax-deficient mice provide a model for male infertility owing to enhanced apoptosis leading to absence of spermatogenesis in the testis. Some of the histological characteristics in the testes of these mice are reminiscent of those seen in p53-deficient mice. Taken together, these mouse models suggest that apoptosis plays a significant role in monitoring the male gametogenesis.

Bclw

Male reproductive phenotypes are also observed in Bcl2-mutant mice generated not via homologous recombination, but by a random proviral integration with the *lacZ* expression gene-trap approach in ES cells *(111)*. Bclw is a death-protecting protein that is expressed in multiple tissues. In the testis, Bclw is expressed in step 10 elongating spermatids and continues until the step 16 stage; low level expression is also seen in Sertoli cells. The homozygous (for the insertion) mutant mice do not express any immunoreactive Bclw protein in testicular extracts, confirming that the proviral insertion abolishes the expression of Bclw transcripts. No significant defects are observed in many cell types. Male mice exhibit progressive infertility. The major defect in young adults is a block during spermiogenesis. By 6 mo of age, these male mice exhibit additional testicular phenotypes. These include gradual depletion of all stages of germ cells and localized Leydig cell hyperplasia. Subsequently and characteristically, Sertoli cells are lost from the seminiferous tubules with greatly reduced Leydig cell number *(111)*. Although the molecular mechanisms of these defects are not yet known, the Bclw-deficient mouse model is unique, with interrelated defects in both somatic and germ cell lineages. These data reemphasize that cell-cell interactions within the testis are essential for normal spermatogenesis.

DNA Repair Enzymes

Both mitotic and meiotic cell divisions involve dynamic chromosome structural remodeling events accompanied by an accurate DNA replication. These processes require a coordination of several "proofreading" mismatch repair enzymes, the failure of which results in an error-prone DNA repair system that has consequences as severe as tissue-specific or more global tumorigenesis *(101)*. Several mouse homologs of yeast or *E. coli* DNA repair enzymes have been identified and cloned, and mutant mice deficient in some of the components of these pathways have been generated. These mouse models are important primarily for studying radiation, mutagen, and carcinogen-induced DNA repair pathways. Because of the intrinsic complex meiotic recombination programs that operate during male and female gametogenesis (which also must be safeguarded to result in the production of errorproof and functionally efficient gametes), these mice may be valuable models to study male and female infertility.

A close survey of reproductive phenotypes in DNA repair enzyme-deficient mice reveals a striking feature of meiotic prophase I arrest in the gonads during gametogenesis. In Atm (ataxia telangiectasia, a member of the phosphatidylinesite 3-kinase-like kinase)-deficient male and female mice, the absence of mature gametes results in infertility in both sexes *(112,113)*. The gonads are distinctly small. Female mutant mice are devoid of primordial and growing follicles and oocytes. The interstitial stromal cells are vacuolated. The uterine morphology and histology is consistent with the absence of estrous cycles in the mutant female mice. In the testis of adult Atm-deficient male mice, Sertoli and Leydig cells are present, but the spermatogenic cells undergo degeneration. This is owing to increased apoptosis of the spermatocytes at meiotic prophase I. This stage, which requires the proper assembly of a repair enzyme known as Rad51, onto the chromosomal axial elements, is abnormal in Atm-deficient males and results in chromosome fragmentation. During normal meiosis, at this phase, the homologous chromosomes "search" for a pair (synapse) and later exchange DNA through synaptone-

mal complexes (meiotic recombination). This process involves DNA strand-breaks and involves repair mechanisms *(107,113)*.

Meiotic pachytene stage-specific arrest resulting in male and female infertility is observed in mice deficient in a mammalian homolog of the yeast MLH1, a DNA mismatch-repair enzyme *(114)*. Female mice produce oocytes and breed normally, but oocytes fail to develop beyond the one-cell stage following fertilization with sperm. These early stage one-celled embryos never complete the second round of meiosis. Mutant male mice have small testes. Spermatogenesis is arrested at the pachytene stage, although chromosomal synapses are normal. At this stage, they undergo apoptosis resulting in eventual depletion of spermatids and spermatozoa in the seminiferous tubules *(114)*.

In contrast to male and female infertility in MLH1-deficient mice, only male infertility results in mice deficient in PMS2, another DNA mismatch-repair gene, in addition to impaired microsatellite instability in many tissues *(115)*. The epididymis in mutant males contains a reduced number and abnormal spermatozoa with misshaped heads and truncated irregular flagella *(115)*. Further analyses indicate chromosome synapsis defects similarly seen in Atm-deficient mice. Because mature spermatozoa are observed, although with abnormal head morphology, this suggests that meiosis proceeds until completion and that normally PMS2 enzyme functions at a later stage than MLH1.

Recently, mutant mice deficient in the DMC1 (disrupted meiotic cDNA) gene also demonstrate defects in meiotic prophase arrest and chromosome synapsis leading to male and female sterility *(116,117)*. Unlike other DNA repair enzymes, Dmc1 in yeast and mice appears to be germline specific. *Dmc1* is a mammalian homolog of *E. coli RecA* gene.

Only male infertility is the characteristic feature of mice deficient in the HR6B (homolog of yeast RAD6/ubiquitin-conjugating enzyme, UBC2) ubiquitin-conjugating DNA repair enzyme, a mammalian homolog of the yeast hHR6B *(118)*. The ubiquitin pathway in mammals is involved in the selective removal of a number of short-lived cell-cycle regulator molecules, transcription factors, and cell-surface receptors. In the HR6B-deficient male mouse testis, there are defects in the postmeiotic condensation of chromatin spermatids. Similar to PMS2-deficient male mice, sperm with abnormal head morphology are present in the epididymis. There is accelerated apoptosis in the spermatogenic cells with vacuolated cytoplasm *(118)*. The HR6B pathway is implicated in the histone degradation and subsequent replacement by protamines that are critical for postmeiotic chromatin remodeling during spermatogenesis. This may well be the step that is affected in the testis of HR6B-deficient male mice.

Clearly, analyses of DNA repair enzyme-deficient mouse models suggest that male and female meiotic recombination programs are distinct (Tables 2–4), although they share some common themes. Many clinically documented human infertility cases are owing to chromosome structural anomalies. These mouse models will be extremely useful to explore the molecular basis of these clinical cases.

General Enzymes

Enzymes are biological catalysts, control metabolic pathways in the body, and have distinct patterns of subcellular localization and substrate specificity. Most often they are expressed in more than one isoform, each of which subserves a specialized function.

In this section, mouse mutations in some specific and general enzymes, which affect the reproductive axis, are described.

Enzymes Affecting Sperm Function

Angiotensin-Converting Enzyme and Acrosin. The two isoforms of angiotensin-converting enzyme (ACE), the somatic and the testis-specific forms, are encoded by a single gene. The testis ACE is generated by a differential use of a 91-bp testis-specific promoter located within intron 12 *(119)*. The somatic ACE is membrane bound in vascular endothelial and epididymal epithelial cells, and an additional soluble form is present in blood. The testis ACE is expressed in spermatogenic cells, particularly in developing spermatids and mature sperm. The ACE protein is first detected in haploid spermatids, and the transcription of the mRNA begins in late pachytene spermatocytes or postmeiotically *(119)*. Although the substrates for somatic ACE have been identified as angiotensin I and bradykinin (whose C-terminal dipeptides are cleaved by ACE), the substrates for testis ACE are not known. To understand the biological functions of ACE in male reproduction, two different ACE-deficient mouse models have been generated *(120)*.

Mice that are deficient in somatic ACE but have intact testis ACE are fertile and do not show any defects, suggesting that the somatic ACE isoform is not essential for male and female fertility. Whereas female mice deficient in both somatic and testis ACE are fertile, male mutant mice are subfertile *(120)*. Detailed quantitative analyses and functional assays indicate that sperm from double mutant mice fertilize eggs in vitro at a reduced frequency. Sperm number, viability, motility, and the ability to undergo capacitation and acrosome reaction are all indistinguishable from results obtained from sperm of wild-type control male mice. But when compared to wild-type sperm, mutant sperm have defects in oviduct transport. A significant number of these sperm fail to reach the extramural uterotubal junction and the lower and upper isthmus regions of the oviducts, and very few reach the ampulla *(120)*. The mutant sperm have decreased capacity to bind to zona pellucida of eggs in vitro. The molecular mechanisms of these sperm defects are not yet known, but one possibility is that the catalytic activity of ACE is required at capacitation for proteolysis of a specific substrate when sperm membrane binds oviduct epithelium, and this enzymatic reaction is impaired in the mutant sperm. At least the normal substrate of somatic ACE, angiotensin II, is not this substrate, because mice deficient in angiotensinogen (the precursor molecule that generates angiotensin I, which is later converted to angiotensin II by ACE) have no male fertility defects.

Some combinations of mutations in the *Drosophila* ACE gene result in approx 90% lethality and male sterility. Therefore, the ACE pathway in male fertility seems evolutionarily conserved. Another important observation is that gametes from double heterozygous mice (for somatic and testis ACE isoforms) do not have any functional differences that might affect gamete function, and both wild-type and mutant alleles are transmitted to offspring at equal frequencies *(120)*.

Acrosin, another major enzyme present in sperm acrosomes, is a 417 amino acid containing serine protease. It is synthesized as a proacrosin precursor protein following a specific peptide bond cleavage *(121)*. Although acrosin was believed to be a recognition protein for sperm-egg binding, and for sperm penetration of ovum zona pellucida, male mice deficient in acrosin are fertile and penetrate the zona pellucida of eggs in vitro.

Of the two groups that reported the characterization of acrosome-deficient mice, at least one group has in vitro data suggesting that acrosin-deficient sperm have reduced fertilizing capacity and a selective disadvantage in penetrating zona pellucida of eggs when they are in competition with sperm containing acrosin *(121)*.

Men with reduced acrosin activity in their spermatozoa have fertility problems. Both ACE- and acrosin-deficient mouse models will therefore prove useful in delineating the molecular mechanisms of some forms of human male infertility owing to sperm function defects.

Prohormone Convertase 4 (PC4). The mammalian convertases are structural homologs of bacterial subtilisms and yeast kexin. These are serine proteases, and they cleave many important precursor polypeptides at specific dibasic residues. Seven distinct members of this family are known to date. PCA is a testicular germ cell–specific prohormone convertase *(122)*. It is exclusively expressed in spermatocytes and round spermatids, suggesting its probable role in male reproduction. Consistent with this expression pattern, male mice deficient in PCA have drastically reduced fertility and a reduction in their sperm-fertilizing ability to zona pellucida similar to that seen in ACE and acrosin mutant mice. In addition, some of the mutant sperm do fertilize the eggs, but these fertilized eggs fail to grow to blastocyst stage in vitro *(122)*. Histology of the mutant mouse testes show no defects in the spermatogenic cells. The null mutation in the *Pcsk4* locus (encodes PC4) was created by an insertion of *lacZ* sequences, which disrupt the locus. In mutant female mice, ovarian *lacZ* expression is obvious in the steroid-producing thecal-interstitial and luteal cells *(122)*. Whether this results in an altered steroidogenic function of these cells and, as a consequence, impairs female fertility is unknown. Several potentially important substrates for PC4 within the male reproductive system could be enkephalins, pituitary adenylate cyclase–activating peptide, growth hormone-releasing hormone–related peptide, and nerve growth factor. All these polypeptides are synthesized as precursors within the male germ cells and require proteolytic processing to function.

Enzymes Affecting Female Reproduction

Caspase-2. The 12 members of the caspases are the mammalian homologs of *Caenorhabditis elegans* cysteine protease death effector, CED-3. Active forms of this class of enzymes are synthesized from their inactive precursors by cleavage at specific aspartic acid residues *(123)*. Caspase-2 is highly expressed in embryonic mouse brain and is downregulated in the adult brain. It is expressed in ovarian germ cells also *(123)*. Several in vitro and in vivo lines of evidence implicate caspase-2 as an important enzyme in the programmed cell death pathway. Multiple tissues express two distinct isoforms of the enzyme—some the short and others the long isoform owing to alternate splicing *(123)*. Mice deficient (both long and short isoforms) in caspase-2 have multiple defects, including accelerated motor neuron death during development and defects in B lymphoblast development and apoptosis, but reach adulthood with no gross abnormalities *(123)*.

Caspase-2-deficient female mice have normal ovarian growth. However, histologically, the ovaries of these mutants contain an increased number of primordial follicles containing oocytes compared with control female mice. This increase is apparent by p 4 by which time, there is a wave of massive apoptosis in germ cell population, by almost one-half to two-thirds, in the normal ovary *(123)*. This suggests that in caspase-

2-deficient female mice, the fetal ovarian germ cells lack the apoptotic pathway. The lack of apoptotic pathway is further confirmed by in vitro experiments. When caspase-2-deficient oocytes are treated with an anticancer chemotherapeutic drug called doxorubicin, a known inducer of human germ cell death, they are completely resistant to apoptosis. Preimplantation blastocysts normally also express caspase-2 in high levels. Preimplantation blastocysts obtained from caspase-2-deficient female mice show an equal sensitivity to doxorubicin, suggesting that other caspases (at least caspase-3 is expressed in blastocysts) may compensate in blastocysts *(123)*. Thus, these studies with caspase-2-deficient female mice have identified caspase-2 as an important mediator for both normal and pathophysiological apoptosis in the female germ cell lineage.

5α-Reductase. Testosterone, in both males and females, is converted either into estrogen by aromatase or to a more potent androgen, dihydrosterone, by 5α-reductase. Dihydrotestosterone is further catabolized to 5α-androstatan-3α, 17β-diol (3α-Adiol), which has no well-defined hormonal activities *(1,2)*. 5α-Reductase is a membrane-bound enzyme and has two isoforms: 5α-reductase type I and type II enzymes. The type I and type II isoforms both convert testosterone to dihydrotestosterone, but differ in their cell- and tissue-specific expression patterns and their biochemical and pharmacological properties. Mutations in the type II gene in humans cause male pseuohermaphroditism with normal internal but female external genitalia, whereas no known mutations exist in the type I gene. To study the biological roles of the type I enzyme, mice deficient in this enzyme were generated *(124,125)*. Mutant male mice develop all the male sex organs normally in the absence of type 1 5α-reductase enzyme. Female mice also develop intact reproductive tracts and can become pregnant, but they have fetal survival and parturition defects. These pregnant mutant female mice fail to synthesize 3α-hydroxysteroid dehydrogenase in the uterus and fail to produce 3α-Adiol, which normally peaks during the late gestation period *(124,125)*. Supplementing the pregnant female mice with 3α-Adiol reverses this effect. Fetal loss in mutant mice occurs between gestation E 10.75 and E 11.0 as a result of elevated androgen and estrogen levels in the serum. Either aromatase inhibitors or ER antagonists prevent this fetal death in mutant mice and protect the embryos against estrogen toxicity *(124,125)*.

In human amniotic fluid, high levels of estrogens are present owing to a highly active placental aromatase and yet the human fetus is safeguarded from estrogen toxicity. There are clinical cases in midgestational miscarriages reported in women, and these perhaps could be owing to mutations in the 5α-reductase type I gene accompanied by excess estrogen levels. 5α-Reductase type I enzyme-deficient mice thus will help us understand this estrogen toxicity in midpregnancy.

Copper-Zinc Superoxide Dismutase. Three forms of antioxidant enzymes belonging to the superoxide dismutase (SOD) family protect almost all cells from reactive oxygen species (ROS) that are generated by normal metabolism. SOD1, the Cu-Zn SOD, is a cytosolic enzyme whereas the other two (SOD2, the manganese-SOD and SOD3, an extracellular form) are either localized to mitochondria or one secreted form. The role of these enzymes and their ROS-detoxifying mechanisms in neurodegenerative diseases have been under intensive investigations for several decades *(126)*.

In the rodent gonads, all three SOD isoforms are expressed. SOD3-deficient mice have no reproductive defects. SOD2-deficient mice die perinatally, and therefore the effects on the reproductive function in these mutant mice could not be studied *(126)*. SOD1-deficient mice develop normally and male mutant mice are fertile with no obvious

gonadal phenotypes. Female mutant mice, by contrast, are either sub- or infertile *(126)*. They have hypoplastic ovaries and uteri, suppressed serum gonadotropin levels, and a folliculogenesis block at the secondary follicle stage. No antral follicles or corpora lutea are present in the mutant ovaries *(126)*. An independently generated SOD1 null mutation in females leads to normal ovarian folliculogenesis, estrous cycles, ovulation, and fertilization but a marked postimplantation embryonic loss of fetuses *(127)*. Although the mechanisms of this defect in these two strains of SOD1-deficient female mice are not clear, the potential target tissues may be the pituitary, gonads, and uterus. The consequences of excess ROS and its metabolites, in particular, free radicals, in normal reproductive physiology need to be further investigated.

Cyclooxygenases and Prostaglandin F Receptor. Prostaglandins are products of arachidonic acid metabolism. They are well-known mediators of several biological processes, notably the inflammatory response. They act through G-protein-coupled receptors including prostaglandin F receptor and four subtypes of the prostaglandin E_2 receptor. There are two isoforms of the rate-limiting enzyme, cyclooxygenase (COX), involved in the production of prostaglandins in multiple tissues including the female reproductive axis. COX1 is a constitutively active enzyme localized in the endoplasmic reticulum whereas COX2 is a nuclear envelope-associated, inducible enzyme *(128)*.

COX1-deficient female mice have normal fertility but have limited defects in parturition *(129)*. Prostaglandin F receptor–deficient female mice have normal estrous cycles, ovulation, fertilization, and implantation. But these mice have parturition defects associated with an absence of oxytocin receptor induction owing to persistently elevated serum progesterone levels that normally decline preceding parturition *(129)*. Ovariectomy of the mutant mice, which abolishes luteal cell progesterone production, rescues the parturition defects and results in successful delivery of the pups *(129)*. Therefore, prostaglandin F receptor signaling normally is required for luteolysis and parturition in mice.

In contrast to the implantation and parturition defects in COX1- and prostaglandin F receptor–deficient mice, COX2-deficient female mice have multiple reproductive defects. Both ovulation and fertilization are defective or completely absent in these mice, and superovulation by gonadotropins does not restore these defects *(129)*. COX2 transcripts are present in cumulus cells surrounding the oocyte in antral follicles and ovulated eggs in control female mice, and therefore altered oocyte maturation may be one reason for these defects. In addition, although the prostaglandin receptor subtypes and implantation-specific genes are completely expressed with unaffected steroid hormone responsiveness in mutant females, their uteri fail to implant wild-type eggs and to exhibit a uterine decidualization response *(129)*. Partial restoration of decidualization response occurs following supplementation of prostaglandin analogs to the mutant females. In addition, COX2- but not COX1-specific inhibitors block implantation in wild-type female mice. Collectively these observations identify previously uncharacterized multiple roles of COX2 in female reproduction and conclusively prove that the defects are the direct result of target organ–specific COX2 deficiency but not of impaired hormone levels or their responsiveness in target tissues *(129)*.

Telomerase. Whereas the preceding mouse models deficient in enzymes have male- or female-specific reproductive defects, mice deficient in telomerase, a critical enzyme that maintains the stability of chromosome termini during each round of replication, have defects in both male and female reproduction, in addition to defects in multiple

tissues *(130)*. By the sixth generation of passage of the mutation, the mutant male mice become infertile. Their testis size is dramatically decreased, with germ cell depletion, increased apoptosis, and decreased replication rates of spermatogenic cells, whereas the mitotically less inactive Sertoli and Leydig cells are unaffected *(130)*. Telomerase-deficient female mice have variable size decreases in uteri and ovaries. In the mutant uterus at the sixth generation, the myometrial thickness is reduced and there is an increase in smooth muscle cell atrophy. Ovarian histology appears normal with fully grown antral follicles and corpora lutea. However, the number of eggs released is decreased and the fertilized eggs fail to develop in vitro into blastocysts *(130)*.

These findings suggest that a propagation of the null mutation in telomerase gene until late generations (sixth and onward) have consequences in the genomic integrity of organ systems that exhibit high-renewal of cells, such as the spermatogenic cells. In addition, telomerase-deficient cells will be invaluable in studying the long-term consequences of genomic stability during cancer and senescence.

Miscellaneous

Mouse models in which mutations created in genes encoding other factors not described herein also have reproductive defects.

A heterozygous mutation in apolipoprotein B, a lipoprotein metabolic pathway component, causes severe reduction in sperm motility, survival time, sperm count, and a failure to fertilize eggs *(131)*.

Mice deficient in centromere protein B, a constitutively present protein on chromosomal centromeres, have grossly normal mitotic and meiotic cycles but lower body and testes weights. No consequences of male/female reproduction are observed *(132)*.

Male mice deficient in the recently discovered testis-specific ER chaperone, calmegin, a calnexin homolog, are infertile, have normal stages of spermatogenesis, and have normal male reproductive behavior, but the mutant sperm fail to adhere to zona pellucida of the eggs *(133)*.

Female mice deficient in ZP3, an important glycoprotein of the egg extracellular matrix, are infertile. These mice have "zona-free" oocytes within the ovarian follicles, the germinal vesicle appears intact, but corona radiata is disorganized. Germinal vesicle breakdown occurs with disrupted cumulus-oocyte complex formation, prior to ovulation *(134,135)*.

Female infertility is also seen in mice deficient in connexin 37, an ion channel present in the gap junctions between oocyte and granulosa cells *(136)*. In the ovarian follicles, oocytes are present but developmentally arrest and fail to acquire meiotic competence. In the ovaries of the mutant mice, mature follicles are absent, ovulation is impaired and premature, and inappropriate corpora lutea-like small structures develop *(136)*.

Infertility in male and female mice is observed in two mouse models in which RNA-binding protein-encoding genes located on Y chromosome, TIAR, or Dazla are mutated. In both these mouse models, the primordial germ cells fail to survive and proliferate, leading to complete absence of germ cells *(137,138)*.

Finally, a small percentage of surviving mice deficient in basigin, an immunoglobulin superfamily member, have implantation defects in females, and male sterility owing to spermatogenesis block at metaphase of the first meiosis with only few spermatocytes that differentiate into step 1–stage spermatids *(139)*.

SUMMARY

In this chapter, we have described several mouse models generated by ES cell technology to study the development and function of the hypothalamic-pituitary-gonadal axis. Tables 1–4 summarize the reproductive phenotypes in these and in naturally occurring mouse mutants.

Although a number of mouse models demonstrate reproductive defects, several questions—what are the early events in sex determination and gonad differentiation? What are the roles of several neuropeptides in reproduction? What are the mechanisms of intracellular signaling and transcriptional regulation within the pituitary gonadotropes?—remain unanswered. The definitive roles of a number of known and unknown intra- and extragonadal factors in spermatogenesis and folliculogenesis still remain elusive.

Future work will surely focus on a more-detailed characterization of the already available mutant mice. Generation of mice with double and multiple combinations of mutations will be generated, and synergistic and redundant roles of various factors involved in a given pathway will be established. In addition, the conventional transgenic gain of function mice complement these models *(140).* The second-generation gene-targeting approaches will be useful in determining the effects of controlled spatiotemporal gene expression in the reproductive axis by generating cell tissue–specific and conditional knockout mouse models. Large-scale mouse genome mapping and sequencing, identification of novel genes and defining their in vivo function by creating mutant mice via N-ethyl-N-nitrosourea mutagenesis schemes *(141),* or random gene trap or random gene deletions in ES cells *(142,143)* will be feasible in the near future. Knowledge gained by this functional genomics approach will eventually facilitate a better understanding of the regulation of the hypothalamic-pituitary-gonadal axis. Ultimately, this understanding will help unravel the mechanisms of infertility and may lead to the design of novel contraceptive strategies that will control the global population.

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11 Transgenic Approaches to Study Developmental Expression and Regulation of the Gonadotropin Genes

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Contents

INTRODUCTION

Applications of Transgenic Technology

In the past decade, transgenic animals have evolved as key tools for studies of the development, molecular biology, and function of the reproductive system. Probably the single most important contribution of transgenics to the investigation of gonadotrophs and the gonadotropin genes has been the development through targeted oncogenesis of differentiated pituitary gonadotroph cell lines, specifically the α-T3 *(1)* and LβT2 *(2)* cell lines. This chapter reviews the application of these cell lines and in vivo transgenic expression systems to the understanding of the physiological regulation of the glycoprotein hormone α-subunit, luteinizing hormone β (LHβ), and follicle-stimulating hormone β (FSHβ) genes, and the detailed analysis of promoter elements involved in the complex integration of signals for α-subunit gene transcription. Other advances that have been possible as a direct result of the α-T3 cell line include the cloning of LH-2, a novel LIM/homeodomain transcription factor *(3),* and the gonadotropin-releasing hormone (GnRH) receptor cDNAs *(4,5),* as well as a detailed

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understanding of the intracellular signaling pathways involved in GnRH action (for a review see ref. *6).*

Focusing on development of the reproductive system, ontological studies of the pituitary have utilized two different transgenic strategies: targeted cell ablation and expression of transgenic reporter genes for lineage analysis. Gene-deletion experiments have also made significant contributions to our understanding of development and tumorigenesis in the reproductive system and are discussed in Chapters 9 and 10 of this volume. Finally, transgenic models have contributed to the understanding of diseases of the human reproductive system including pituitary null-cell adenomas, polycystic ovary syndrome, and the potential role of gene therapy in human infertility.

Advantages of Transgenic Expression Systems

The development of transgenic technology has offered an extremely powerful tool for the analysis of gene expression. However, there are both scientific and practical advantages and disadvantages to transgenic expression systems compared to transient gene expression in transfected primary cultures or immortalized cell lines. Transgenic expression in vivo may be "truer" to endogenous expression because it requires the stringency of chromatin structure and the potential role of imprinting *(7).* In addition, transgenic expression avoids the obvious problem of differences between immortalized cells and their normal counterparts. These alterations may include expression of a different array of transcription factors or down- or upregulation of intracellular signaling pathways involved in growth and differentiation. For example, regulation of GnRH mRNA and receptors in response to GnRH, estrogen, and nonspecific activation of protein kinase C (PKC) has been shown to be different in α-T3 cells compared to primary pituitary cultures *(6,8,9).* In addition, although initial GnRH receptor–stimulated and depolarization-induced calcium currents are similar, α-T3 cells do not exhibit the intracellular calcium current oscillations and frequency-modulated calcium signaling observed in primary gonadotrophs *(6,10,11),* and they differ in their calcium response to PKC activation *(11).*

Transgenic approaches have been particularly advantageous in the study of hypothalamic-pituitary interactions because complex hormonal feedback loops as well as paracrine and autocrine signaling pathways remain intact. In vitro perifusion models using dissociated pituitary cells have been able to circumvent this problem only partially *(12,13)* and are technically complicated.

Despite these advantages, there are problems with transgenic expression. First, differences in chromosomal integration sites can affect transgene expression *(14)* by disrupting endogenous regulatory elements, or causing transposition of transgene promoters to the location of distal endogenous suppressors and/or enhancers. These limitations can usually be circumvented by the development of multiple independent transgenic lines. However, this raises one of the major limitations of a transgenic approach, which is the cost and time involved in producing the multiple lines necessary to evaluate each transgene construct adequately. A "founder" analysis may be useful to eliminate a portion of these costs by avoiding the breeding of F1 mice, but it does not allow any further characterization of the line that would be produced from that founder and may underestimate the true extent of transgene expression given the up to 30% rate of mosaicism in the founders *(15).*

Second, transgene constructs usually do not include noncoding DNA sequences that may be important for expression. Examples of this include locus control regions similar to those described in the β-globin gene cluster *(16)* and the metalliothionin gene *(17)* or full intronic sequences, as in the metallothionin gene *(18)*. Additionally, the presence of sequences within transgene constructs may confer expression inappropriately. In one case, the presence of cryptic sequences within the 3′ flank of the human growth hormone (hGH) gene directed ectopic expression of transgenes containing either cytomegalovirus (CMV) or metallothionin promoter elements to gonadotrophs *(19–21)*.

Although transient transfection introduces artifacts compared to the expression of endogenous genes, transfection experiments may still be superior to in vivo transgenic expression in terms of quantitation of responses. Transfection methods can be standardized to provide consistent transfection efficiency and normalization with a second reporter construct. In contrast, transgenic expression may vary from line to line depending on integration sites or transgene copy number and is much more difficult to standardize. There are examples of transgenic experiments that have allowed quantitative comparison of gross magnitudes of response; but, even these did not allow measurement of fine changes in expression *(22)*. The main advantage of transfection experiments, however, is the ability to quickly screen multiple gene constructs in vitro. Subsequently, accurate expression of key constructs must be confirmed in transgenic animals to evaluate complex feedback systems adequately.

Given the strengths and shortcomings of each of these techniques, it is likely that a combination of in vitro transfection in immortalized cell lines and transgenic expression will continue to be used to evaluate gene expression. Further, the ever-expanding availability of transgenic animals from academic core facilities and private companies as well as advances in techniques such as gene replacement will likely make the contribution of transgenics increasingly more important.

REGULATION OF PITUITARY GONADOTROPIN GENES

α-*Subunit*

α-Subunit Promoter Mapping

The glycoprotein pituitary hormones, thyroid-stimulating hormone (TSH), LH, and FSH are structurally related heterodimers consisting of a common α-subunit and receptor-specific β-subunits. The subunits are all encoded by separate genes and are regulated individually. Consequently, the α-subunit gene is expressed in two pituitary cell types, gonadotrophs and thyrotrophs, as well as in the primate and equine placenta (but not in other mammalian species), where it forms species-specific heterodimers with chorionic gonadotropin β (CGβ). Not surprisingly, α-subunit gene expression is regulated by multiple hormonal pathways (see ref. *23* for a recent review). Some of the initial studies demonstrating cell-specific expression utilized transgenic expression systems. The earliest experiments with transgenic mice *(24)* addressed the intriguing question, Is human α-subunit gene expression in placenta determined by species differences in *cis*- or *trans*-acting elements? Using human or bovine α-subunit promoter elements linked to chloramphenicol acetyltransferase (CAT), pituitary-specific expression in transgenic animals was demonstrated *(25,26)*. Promoter elements fused to the bacterial *lacZ* gene encoding β-galactosidase allowed detection of gonadotroph-specific expression *(25)* and

also allowed colocalization by immunostaining with other pituitary hormone antisera. Together, these studies determined that distinct promoter elements controlled expression of the α-subunit gene in thyrotrophs compared to gonadotrophs and suggested that expression in the placenta required a functional diad of repeated cyclic adenosine monophosphate (cAMP) response elements (CREs) *(25,26)* as well as species-specific *trans*-acting elements *(24)*.

More detailed mapping of promoter elements that confer gonadotroph specificity and hormonal responsiveness has utilized the α-T3 cell line. The α-T3 cell line was derived from a pituitary tumor induced by targeted oncogenesis using the mouse α-subunit promoter fused to the large T-antigen coding sequence from simian virus 40 (SV40) *(1)*. This line was determined to be of gonadotroph origin based on expression of the mouse α-subunit gene *(1)*, expression of GnRH receptors *(8)* and intracellular responses to GnRH (for a review, see ref. *6*), and expression of pituitary gonadotroph–selective transcription factors including steroidogenic factor-1 (SF-1) *(27)*. These cells have served as the primary in vitro model of gonadotroph cells. A current view of the promoter structure of the mouse and human α-subunit genes is illustrated in Fig. 1 and is detailed in the following paragraphs. Notably, all the gonadotropin genes contain TATA boxes and putative CAAT boxes; for clarity, these have been omitted. Putative transcriptional start sites are shown, although evaluation of the endogenous transcriptional start site has not been conducted in all genes.

The critical importance of the CRE in placental expression in transfection experiments utilizing mouse, bovine, or human α-subunit promoter reporter constructs in the JEG-3 and BeWo cell lines (both human choriocarcinoma lines) has been compared to the minor contribution of the CRE in α-T3 gonadotroph cells *(26,28)*. Lack of expression of α-subunit in the placenta of nonprimate (or equine) species is believed to be owing to the lack of a CRE or the presence of a variant CRE in the nonprimate genes *(29)*. However, CRE-binding protein (CREB) still binds the variant CRE in vitro as a heterodimer *(30)* and has been shown to bind other members of the bZIP family, specifically ATF2 and c-Jun *(31)* raising questions about its functional significance.

A comparison of gene expression in placental cell lines and the α-T3 cell line has revealed several additional differences among *cis*-elements conferring tissue specificity. In addition to the CREs, placental expression of the α-subunit gene requires the presence of a second strong regulatory element from −182 to −142 in the human gene, the upstream regulatory element (URE) *(32)*. This region contains three overlapping sites: a GATA element also termed the α-activator (α-ACT) element from −161 to −142, and the trophoblast-specific element (TSE) and URE1 from −182 to −159, which bind both TSE-binding protein (TSEB) and URE1-binding factor *(33,34)*. There are single base pair differences in two of these other elements, the TSEB and α-ACT, between the mouse and human genes. These differences eliminate binding of these elements to their respective transcription factors *(34)*, and these elements are also probably involved in the species-divergent placental expression.

In addition to differences in regulatory DNA sequences, further restriction in gene expression may be imposed by tissue-specific expression of transcription factors. Comparisons have been made using the consensus α-subunit CRE and variant CRE probes (which express in gonadotroph and trophoblast cells, respectively) in gel-shift assays *(31)*. Although the consensus CRE showed identical binding of transcription factor heterodimers, the variant CRE showed decreased affinity for factors identified as c-Jun

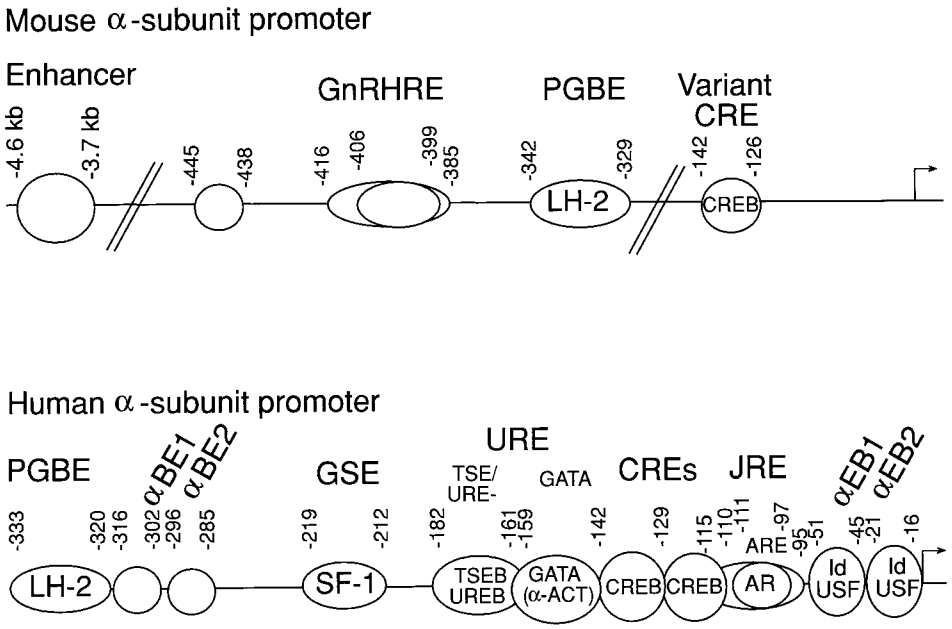

Fig. 1. Promoter structure of the mouse and human α-subunit genes. Illustrated are the base pair positions of elements that confer pituitary- and placental-specific expression and their cognate binding proteins (see text for details). Elements important for placental expression in the human gene include the URE, which includes a GATA element (also called α-ACT) and the TSE, two CREs, and the JRE. Pituitary expression involves the PGBE, GSE, and α-BEs in addition to the GATA element and the CREs; additional DNA sequences important for gonadotroph expression have been demonstrated, but the proteins binding to them have not been identified and are not shown here. A far upstream enhancer has been demonstrated to confer thyrotroph and gonadotroph specificity. kb, kilobase; GnRHRE, gonadotropin-releasing hormone response element; PGBE, pituitary glycoprotein binding element; LH-2, Lim-homeodomain 2; CRE, cAMP response element; CREB, CRE-binding protein; α-BE, α-basal element; GSE, gonadotroph-specific element; SF-1, steroidogenic factor-1; TSE, trophoblast-specific element; URE, upstream regulatory element; TSEB, TSE-binding protein; UREB, URE-1-binding factor; GATA, GATA-containing element; α-ACT, α-activating protein; JRE, juxtaregulatory element; ARE, androgen response element; AR, androgen receptor; αEB, αE-box; Id, an ubiquitous bHLH protein; USF, upstream stimulatory factor, a bHLH leucine zipper protein. Arrow indicates transcriptional start site. Sizes are disproportionate to show details of promoter areas.

and ATF2 by antibody shifting of the DNA-factor complex mobility. In addition, it is postulated that there are lower concentrations of ATF2 and c-Jun observed in BeWo cells *(31)*. Finally, it has been shown that the array of GATA proteins expressed in these two cell types differs *(34)*. Thus, tissue specificity appears to involve divergent expression of transcription factors as well.

In contrast to placental expression, pituitary expression of α-subunit involves at least four different elements, three of which (all except the CRE) are not required for placental expression. The first is the pituitary glycoprotein basal element (PGBE) from −333 to −320 in the human gene (and −344 to −300 bp in the mouse gene) that binds LH-2, a member of the LIM/homeodomain transcription factor family *(35)*. Downstream from the PGBE in the human gene is the gonadotroph-specific element (GSE) from −219 to −212 *(36)* that binds SF-1 *(27)*. In close proximity are the α-basal elements 1 and

2 (α-BE1 and α-BE2) at −316 to −302 and −296 to −285, respectively, that bind two similar proteins of molecular weight 54 and 56 kDa that have not been further characterized *(37)*. Analysis of transfected mutated human α-subunit promoter constructs in α-T3 cells has demonstrated that these elements cooperate in promoter activation in several different ways. The GSE appears to act independently of the PGBE, α-BEs, or CREs. The PGBE, however, is dependent on the α-BEs, suggesting synergistic interaction among the proteins binding these elements. By contrast, the PGBE and α-BEs have a compensatory relationship with the CREs, which has been found to be more critical in constructs containing the variant CRE *(31)*. The details of potential protein-protein interactions involved in these dependent and synergistic relationships and how they ultimately increase transcriptional activity remain to be elucidated.

Separate from these tissue-specific elements, the human α-subunit promoter contains two E-boxes. One of these elements (αEB-2) was shown to bind a basic helix-loop-helix (bHLH) bZIP protein immunologically similar to upstream stimulatory factor (USF) that is important for cell-specific activation of many genes (see ref. *38* for a review). Furthermore, mutations of this site or overexpression of Id, a bHLH protein that binds and inactivates the activity of USF, resulted in a reduction in basal activity of the human α-subunit promoter in α-T3 and α-TSH cells *(38)*. In other tissues, bHLH proteins are important for conferring tissue specificity; whether and how these elements interact with the other known tissue-specific elements in the α-subunit gene remains to be studied.

Other elements that have been shown to be important for expression in α-T3 cells include regions in the mouse gene from −445 to −438 bp *(39)* and from −215 to −156 bp *(40);* however, proteins that bind to these regions have yet to be identified. GnRH and cAMP responsiveness in the mouse gene map to two other regions, −406 to −399 and −416 to −385 bp, respectively *(40)*. In addition, further transgenic and transfection experiments have identified an 859-bp enhancer far upstream of the promoter elements (at −4.6 to −3.7 kb in the mouse gene) that directs high-level expression to both gonadotrophs and thyrotrophs in the pituitary gland and α-T3 and α-TSH cells (a thyrotroph cell line) *(39)*. Cell specificity of the enhancer action was lost with deletion of sequences from −341 to −297 bp, probably related to loss of the PGBE and LIM and/or α-BE1 binding, confirming the importance of these elements. In the aforementioned and in previous studies *(8,22,35),* comparison of expression in α-T3 and α-TSH cells has allowed delineation of elements conferring gonadotroph vs thyrotroph specificity. The elements that confer gonadotroph specificity are shown in Fig. 1; these and additional elements conferring thyrotroph specificity have been extensively reviewed by Schoderbek et al. *(35)* and Brinkmeier et al. *(39)*.

Hormonal Responsiveness of the α-Subunit Gene

Hormonal responsiveness of the α-subunit gene was initially demonstrated in primary pituitary cultures (for a review see ref. *41*). GnRH responsiveness has been confirmed by multiple groups in transgenic animals and in transfection experiments utilizing α-T3 cells *(1,40,42,43)*. More recently, detailed studies of the intracellular signaling pathways involved in GnRH action have been performed utilizing the α-T3 cell line (see ref. *6* for a review). To summarize briefly, these studies have demonstrated a role for cAMP mediated through Gq and involving both PKC and inositol phosphate activation

of mitogen-activated protein kinase pathways and have suggested a mechanism for desensitization of transcriptional stimulation of the α-subunit gene by continuous rather than pulsatile GnRH *(43)*. The α-T3 cell line was also used to demonstrate that GnRH increased α-subunit mRNA levels not only by activating transcription but also by increasing mRNA stability *(44)*.

Mechanistic studies of the regulatory effects of gonadal steroids and peptides are not as extensive as those involving GnRH. Direct negative feedback regulation by androgen has been shown in transgenic and transfection experiments and maps to −97 to −111 in the human gene *(45)*. Estrogen repression of the α-subunit gene has been shown in transgenic experiments but not in α-T3 cells, and probably involves an indirect effect on GnRH secretion rather than direct DNA binding of the estrogen receptor to the α-subunit gene *(25,45,46)*. Interestingly, in α-T3 cells, the activation of α-subunit expression by GnRH stimulation was recently shown to be synergized by hCG and LH *(42)*, suggesting a potential mechanism of the ovulatory LH surge. Finally, repression of transcription of mouse and human α-subunit constructs by activin was found in α-T3 cells *(47)* and mapped in the mouse promoter to the PGBE, but may also involve other sequences. No effect of inhibin or follistatin (gonadal peptides that primarily regulate FSH) was demonstrated.

β-*Subunits*

LHβ-Subunit

Although knowledge of the physiological regulation of the β-subunits is extensive from in vivo studies, the details of promoter activation are very poorly understood compared with what is known for the α-subunit. Although a number of putative consensus sequences for transcriptional activators, such as CRE, glucocorticoid receptor, and Pit-1, have been identified *(48,49)*, only elements and factors that have been demonstrated to bind DNA in gel-shift assays and/or have functional significance are shown in Fig. 2. The lack of information about these promoters compared to the α-subunit can be attributed primarily to the lack of an endogenous β-subunit-producing cell line and lack of expression of transfected β-subunit constructs in α-T3 cells *(50)*. Only recently has the paradigm of targeted oncogenesis of gonadotrophs with an rLHβ promoter-SV40 T-antigen transgene resulted in development of the LβT2 cell line *(2)* that expresses α-subunit and LHβ and GnRH receptors *(51,52)*. These cells appear to be hormonally regulated in a fashion similar to gonadotrophs with increased LH secretion in response to pulsatile GnRH. LH secretion is further potentiated by estradiol, probably related to estradiol-induced increases in GnRH receptor gene transcription. Promoter analysis in this line, similar to that performed for the α-subunit gene in α-T3 cells, should yield detailed information about the factors regulating the LHβ gene. Because of the previous lack of a β-subunit-expressing cell line, studies of the LHβ gene have been conducted primarily with transgenic animals, utilizing bovine, ovine, or rat LHβ-promoter-luciferase or CAT reporter-gene constructs.

Experiments with −776 bp of bovine *(50)*, −1.9 kb of ovine *(53,54)*, or −2 kb of rat *(55)* LHβ promoter sequence with luciferase or CAT reporters in transgenic mice have demonstrated physiological hormonal regulation similar to that of the endogenous LHβ gene. GnRH-responsive and gonadal steroid-regulated transcription were demonstrated in each study by an increase in reporter expression after gonadectomy that was prevented

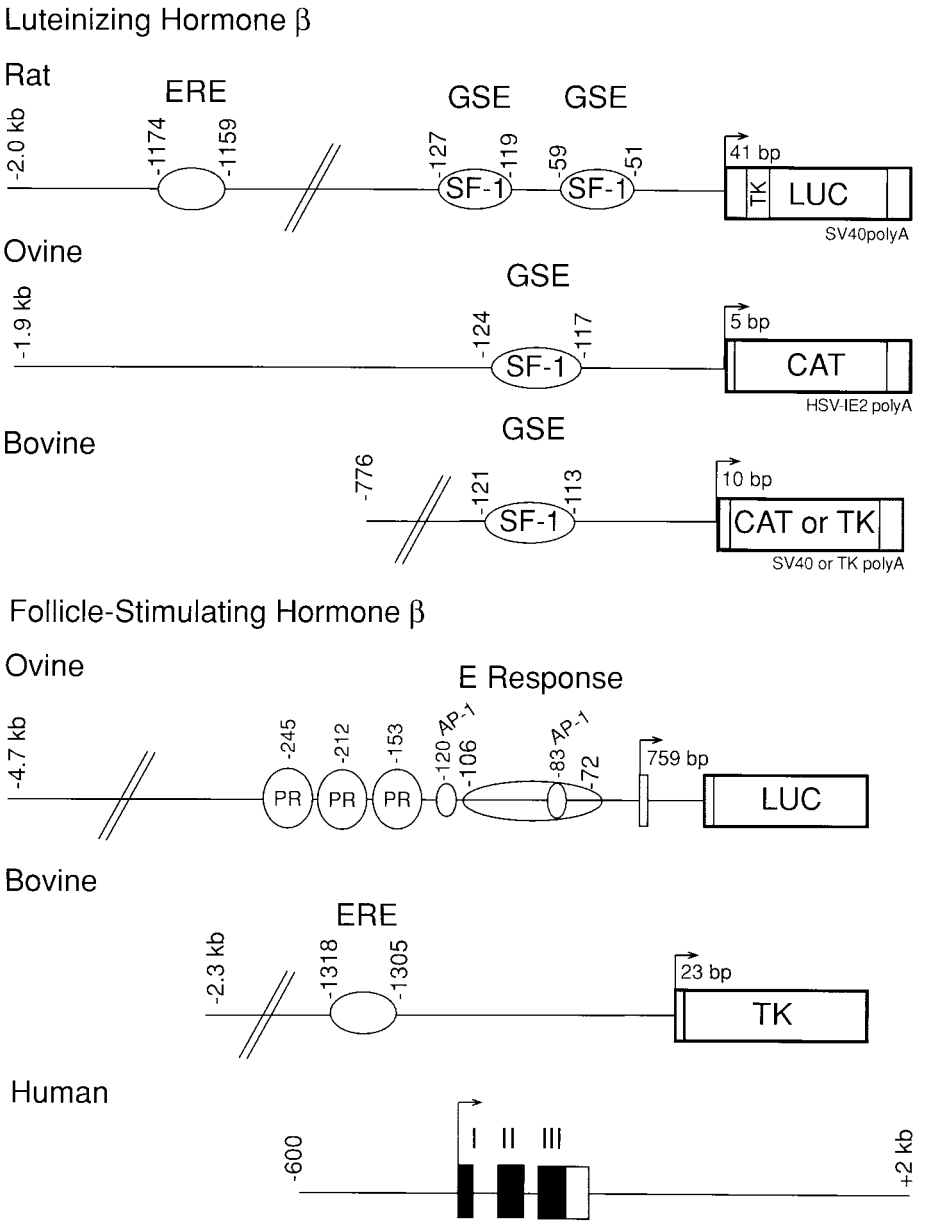

Fig. 2. Structure of LHβ and FSHβ transgenes. Shown are transgene constructs from various species that have been demonstrated to confer pituitary- and/or gonadotroph-specific expression in transgenic mice and transfection experiments. Limited mapping of promoter elements has been done (see text for details); shown are elements and factors that have been demonstrated to bind DNA in gel-shift assays and/or confer functional regulation. kb, kilobase; ERE, estrogen response element; GSE, gonadotroph-specific element; SF-1, steroidogenic factor-1; bp, base pair; TK, thymidine kinase; LUC, luciferase; SV40 polyA, simian virus 40 polyadenylation sequences; CAT, chloramphenicol acetyltransferase; HSV-IE2 polyA, herpes simplex virus IE2 polyadenylation sequences; PR, progesterone response element; E response, area mapped to estrogen responsiveness; AP-1, activating protein-1; jun, jun kinase. Arrow indicates transcriptional start site. Exons are shown by boxes; untranslated areas are shaded. Sizes are disproportionate to show details of promoter areas.

by treatment with the appropriate sex steroid. Whether this is a direct effect of gonadal steroids on promoter activation or an indirect effect on GnRH has not been demonstrated. The −2 kb of rat promoter sequences used have been shown to contain an estrogen response element at −1173 to −1159 *(56);* however, the −766-bp bovine sequence contains no high-affinity binding sites for either androgen or estrogen receptors *(50).* Treatment of mice carrying ovine or bovine LHβ reporter transgenes with GnRH antisera or agonists *(54),* or antagonists *(50,55),* resulted in decreased reporter activity, confirming GnRH responsiveness. Low-level ectopic expression in the ovary, testis, and hypothalamus conferred by the bovine promoter constructs *(50)* is unexplained, although the hormonal responsiveness was found only in pituitary-expressed constructs.

The LHβ genes from most species do contain consensus GSEs and the physiological significance of this element for LHβ expression and its potential interaction with SF-1 has recently been demonstrated. In α-T3 cells, cotransfection of −776 bp of the bLHβ-luciferase reporter with a CMV-driven SF-1 expression vector resulted in five-fold activation of the promoter, which was dependent on the integrity of the GSE consensus sequence *(57).* Expression of a −776-bp bLHβ-CAT reporter with a mutated GSE in transgenic mice was prevented even after gonadectomy, suggesting that increased GnRH cannot compensate for reduced activity at this site *(57),* unlike the analagous situation in the α-subunit gene. By contrast, a −209-bp rLHβ promoter-luciferase reporter gene that contains a consensus GSE at −127 to −119 was not expressed in α-T3 cells even when cotransfected with a constitutively active SF-1 construct *(58).* However, this rat promoter fragment was active when cotransfected with an SF-1 expression vector either in CV-1 cells (56-fold activation) or GH3 cells (15-fold activation) that lack endogenous SF-1. Confirming that this SF-1-stimulated expression was related to the GSE, no activation was seen when the consensus sequence was mutated. An evaluation of other *cis*-elements in either the bovine or rat LHβ promoters has not yet been reported.

FSHβ Subunit

Attempts to express FSHβ gene constructs in a number of established pituitary cell lines including the α-T3 line have been unsuccessful *(59,60).* Initial reports suggested that the LβT2 line does not express endogenous FSH *(2).* These data were interpreted to be consistent with the hypothesis that immortalization of gonadotrophs by T-antigen in early stages (embryonic [E] d E11.5 for α-subunit or d E16.5 for LHβ) "freezes" that cell in a primitive state of differentiation *(61).* Recently, however, we demonstrated expression of FSHβ by RNase protection assay and FSH secretion in LβT2 cells *(62),* disputing this hypothesis and suggesting an alternative hypothesis that lack of β-subunit expression is a consequence of dedifferentiation as a result of the immortalization process. Attempts to produce an FSH-expressing cell line by targeted oncogenesis with transgenic expression of hFSHβ regulatory sequences fused to a temperature-sensitive T-antigen has resulted in transgenic mice that develop differentiated gonadotroph tumors. Consistent with our hypothesis, these tumors demonstrate robust FSH expression in vivo and immediately on culture. However, FSH expression decreases with increasing tumor size, and the cell lines that have been immortalized from these tumors fail to express FSH after prolonged culture *(63).*

Transient expression of oFSHβ reporter constructs in heterologous cells (HeLa,

Chinese hamster ovary, JAR, BeWo, T47-D, and COS-7 cells) has been successful *(49,64,65);* however, the utility of these studies for evaluating gonadotroph-specific and hormonally regulated expression is obviously limited. Results of expression of reporter genes in primary pituitary cultures have also been mixed (*[66,67];* K. E. Graham, unpublished observations). Because of these limited in vitro paradigms, transgenic expression has been the primary approach of our laboratory for study of the regulation of the FSHβ gene.

Transgenic expression of a 10-kb hFSHβ genomic clone spanning the three exons was detected by dot-blot or RNase protection assay (RPA) of total RNA as well as by radioimmunoassay of the heterologous mouse α-subunit-hFSHβ hormone heterodimer *(68–70).* RPA allowed simultaneous analysis of both the endogenous mFSHβ gene and the hFSHβ transgene *(59).* These studies with the hFSHβ gene demonstrated gonadotroph-specific expression that was hormonally regulated with the sexually dimorphic pattern of higher FSH expression in males *(68),* similar to the characteristic pattern in rodents *(41,71).* Additional studies revealed inhibitory responses of both the mouse and human FSHβ genes to androgen replacement after castration *(69),* in contrast to the reported stimulation of rFSHβ mRNA levels *(71).* This androgen response was at least partially GnRH independent, suggesting a direct inhibitory action of androgens at the level of the pituitary *(69).* A more detailed analysis of promoter elements using transgenic expression of truncated promoter constructs has localized gonadotroph-specific, hormonally regulated expression to within −600 bp of 5′ flanking sequences and also demonstrated the requirement of 3′ flanking sequences in the hFSHβ gene for expression *(59).* In the oFSHβ gene, sequences in the first intron enhance expression in transfection experiments *(49).* The second intron has been shown to be nonessential for expression of the hFSHβ gene.

Transgenic constructs containing 4.7 kb of 5′ flanking sequences of oFSHβ can also drive expression of a luciferase reporter to the pituitary of transgenic mice but have not been further characterized for gonadotroph specificity or hormonal regulation (W. Miller, personal communication). This same construct confers estrogen and progesterone responsiveness in primary ovine pituitary cultures and heterologous cells. Although no estrogen response element has been found, a series of functional near-consensus progesterone receptor elements at −245 to −230, −212 to −197, and −153 to −139 has been identified. The potential for interaction of these elements with activator protein-1 (AP-1) sites at −120 and −83 in the oFSHβ gene has been postulated. Although these AP-1 sites have been demonstrated to be responsive in heterologous cell transfection experiments, and there is strong conservation of the element at −120 bp across species (human, rat, bovine, porcine, and rabbit) *(64),* the physiological significance of this element for expression in vivo has not yet been demonstrated. Finally, gonadotroph-specific expression has been demonstrated with 2.3 kb of 5′ flanking sequence from the bFSHβ promoter driving herpes simplex virus thymidine kinase (HSV-tk) *(72,73).*

Unfortunately, the molecular mechanism of GnRH-independent androgen responsiveness in the hFSHβ gene, identification of additional specific promoter/enhancer elements within the regions demonstrated to be required for expression and determination of the physiological significance of various consensus sequence elements in FSHβ genes will likely be elucidated only when an FSH-expressing cell line is eventually developed.

ADDITIONAL ASPECTS OF HYPOTHALAMIC AND GONADOTROPH FUNCTION

Transgenic mice and immortalized cell lines developed by targeted oncogenesis have contributed to the studies of several other important reproductive genes that warrant mentioning, although a comprehensive discussion is beyond the scope of this chapter. The α-T3 line provided a source of enriched mRNA and was instrumental in cloning of the GnRH receptor cDNA and gene *(4,5),* and of LH-2, an LIM/homeodomain transcription factor *(3)*. Detailed studies of the GnRH receptor gene promoter have been performed in α-T3 cells using an approach similar to that described for the α-subunit gene *(6,45,74,75)*.

This cell line has also been critical for studies elucidating the intracellular signaling pathways involved in GnRH receptor action as alluded to in the Introduction and reviewed recently by Kaiser et al. *(6)*. In addition, intracellular signaling of the activin receptor has been characterized in a similar fashion *(12,76),* and studies of other hormones putatively involved in the regulation of gonadotropins, such as pituitary adenylate cyclase-activating polypeptide *(77–80),* have been advanced by use of these clonal cells. The LβT2 cell line, in addition to providing a tool for the study of the regulation of the LHβ and FSHβ genes, has provided a second in vitro model of gonadotrophs. Experimental studies of this line have provided insight into the mechanism of GnRH-induced exocytosis in single-cell studies *(51)*.

Transgenic reporter constructs including both luciferase and CAT have been used for transcriptional regulation studies of the GnRH and GnRH receptor genes *(81,82)*. This approach has led to detailed analyses of promoter structure and function of the GnRH gene (reviewed in refs. *83* and *84*) and the elucidation of mechanisms underlying changes in pituitary responsiveness to GnRH during the ovulatory surge *(82)*.

The strategy of targeted oncogenesis was also used to produce both the GT1-1 *(85)* and GN *(86)* cell lines, two independent hypothalamic neuronal cell lines that express GnRH. Although the GN cell line has not been extensively studied, the GT1-1 and GT1-7 cell lines have characteristics indicating a close similarity to endogenous GnRH neurons. They show spontaneous, pulsatile secretion of GnRH with a pulse frequency similar to endogenous GnRH neurons. They also demonstrate stimulus-induced secretion that closely patterns endogenous GnRH neurons (reviewed in ref. *87*). However, there are differences in regulation of GnRH secretion and/or gene transcription in response to certain agents. In addition, GnRH mRNA levels are 10-fold higher in brain tissue than in the cell lines, and there appear to be differences in the primary modality of regulation, with posttranscriptional changes observed more commonly in animal studies, and transcriptional regulation dominating in GT1 cells *(87)*. Despite these small differences compared to endogenous GnRH neurons, these cells appear to be an excellent in vitro model. They have been used to study the promoter structure and function of the GnRH gene *(83),* GnRH processing *(88),* and mechanisms of LH inhibition of GnRH release and of the LH surge *(13)*. In addition, coculture experiments of GT1-7 cells with various other cells, including astroglial cells and α-T3 gonadotroph cells, have provided novel insights into the mechanisms of axonal targeting and intercellular communication involved in neuronal control of the reproductive system *(88)*.

DEVELOPMENTAL STUDIES

Transgenic Expression of Reporters

In addition to analysis of promoter elements important for cell-specific and hormonal regulation of the α-subunit gene, transgenic expression of reporters has aided in the localization of DNA regions important for developmental regulation *(22)*. Transgenic mice expressing 4.6 kb of 5′ flanking sequence of the mouse α-subunit promoter conferred expression of a β-galactosidase reporter gene on d E9.5 in the developing mouse Rathke's pouch, comparable to d E11 in the rat, when α-subunit expression is first observed *(89)*. Stronger expression was seen at d E12.5 in the developing pituitary gland and was localized to cells in the ventral and anterior portions of Rathke's pouch, in the rostral tip, a pattern similar to that observed by *in situ* hybridization for mouse α-subunit sequences *(90)*. In contrast to the transgenic β-galactosidase expression, expression of endogenous α-subunit was first detected at d E11.5 in Japón et al.'s study *(90)* and at d E12.5 but not at d E10.5 in Sheng et al.'s study *(91)*. Additional areas of transgenic β-galactosidase reporter expression in the developing mouse embryo were observed at d E9.5 in the trigeminal area in the region of condensing mesenchyme and in low levels in the umbilical region. At d E12.5, low levels of expression were seen in multiple areas including the regions forming the extraocular muscle, the first brachial pouch, the trigeminal area, the vestibular and cochlear apparatus, the marginal zone of the spinal cord, the genital tubercle, and the pancreatic primordium. The significance of this putative extrapituitary α-subunit expression in ontogeny is uncertain at this time, and the discrepancy in temporal expression remains unresolved. This finding, however, is intriguing given the suggestion of α-subunit as a modulator of differentiation in in vitro studies *(92)*.

Transgenic Ablation of Gonadotrophs

Targeting Utilizing α-Subunit Sequences

As a complement to experiments with reporter constructs, which provided insights into the timing of expression of specific gene targets, targeted ablation of specific cells have further extended developmental studies of the pituitary gland. The α-subunit gene provides one example of this paradigm; expression of rat α-subunit early in development in the oral placode prior to the development and invagination of Rathke's pouch suggests a potential role in differentiation of the rest of the pituitary gland. Indeed, in vitro studies suggest that expression of α-subunit can induce differentiation in lactotrophs *(92)*. Targeted ablation of cells expressing α-subunit have provided further elucidation of the potential role of α-subunit in pituitary gland development.

One strategy for targeted ablation used cell-specific expression of either wild-type or attenuated diphtheria toxin, which resulted in lethality of individual cells expressing the transgene, even at low levels of expression. Ablation of gonadotrophs was achieved by transgenic expression of diphtheria toxin targeted with 313 bp of the bovine α-subunit gene *(93,94)*. As expected, the transgenic mice demonstrated impaired gonadal development and function accompanied by low levels of sex steroids. Development of the pituitary gland was normal, although there was decreased function of lactotrophs. However, this may have been owing simply to the lack of estrogen secondary to the severe hypogonadism.

Cell-specific ablation has also been achieved using targeted expression of HSV-tk

(95). This method has the theoretical advantage of a lack of toxic effects in the absence of antiherpetic nucleoside analogs (most commonly gancyclovir) that are specific substrates for the viral thymidine kinase. This strategy allows for temporally controlled ablation of the targeted cells. However, ablation of gonadotrophs with this method utilizing the same bovine α-subunit sequences used to direct diphtheria toxin expression has not been successful *(7)*. Nonspecific toxicity was observed with the doses of gancyclovir required for ablation. Although an alternative nucleoside analog, 1-(2′-deoxy-2′fluoro-β-D-arabinofuranosyl)-5-iodouracil, was better tolerated, effective ablation was still not obtained. Other transgenic studies have indicated that HSV-tk may also have toxic actions on certain mammalian cells independent of the nucleoside analogues *(95,96)* or cell proliferation *(97)*.

The most definitive evidence regarding the role of α-subunit in pituitary development has come not from transgenic experiments, but, rather, from knockout mice utilizing targeted disruption of the α-subunit gene in embryonic stem cells *(98)*. These mice demonstrated normal sexual differentiation in fetal development but had severe hypothyroidism and hypogonadism. The gross development of the pituitary gland was normal and all cell types were represented; however, there was thyrotroph hypertrophy and hyperplasia and underrepresentation of somatotrophs and lactotrophs. A direct dependence on α-subunit as opposed to the absence of sex steroids or paracrine or autocrine effects in this phenomenon is not clear, although these experiments do argue against an indispensable role for early cell-cell interaction mediated by α-subunit in pituitary development.

Gonadotroph Targeting Utilizing FSHβ Sequences

Partial ablation of pituitary gonadotrophs has also been achieved utilizing 2.3 kb of the bFSHβ promoter fused to HSV-tk *(72,73,99,100)*. After treatment with gancyclovir, these transgenic animals generally demonstrated moderate reductions in serum FSH, pituitary FSH content, and ovarian weight, with some variability in the pattern and degree of decrease depending on the age and sex of the animal *(72,73)*. Because the onset of gonadotroph ablation can be temporally controlled, these animals may serve as a complement to mice that completely lack FSHβ *(101)* in studies of the role of gonadotropins in the development of the reproductive system. Like the α-subunit knockout mice *(7)*, these mice also demonstrated normal pituitary gland development.

An intriguing observation in the bFSHβ-HSV-tk transgenic mice was that of testicular expression of thymidine kinase and endogenous gonadotropin subunits. Although in this case thymidine kinase expression was reported to be owing to transcription from the FSH promoter sequences *(99)*, the presence of a cryptic promoter in the thymidine kinase gene that has been demonstrated previously to direct testicular expression independently of promoter elements *(95,102)* offers a more compelling explanation. Additionally, endogenous FSHβ expression reported in the testis was of low level and unknown physiological significance.

TRANSGENIC MODELS OF HUMAN DISEASE

Pituitary Tumorigenesis

Human null-cell adenomas, which do not express immunoreactivity for any pituitary hormones, comprise a significant proportion of pituitary macroadenomas and have no

targeted treatments (in contrast to the use of dopamine agonists for prolactinomas or somatostatin for GH-secreting tumors). This is partially related to the uncertainty regarding their cellular origin. The use of targeted oncogenic strategies to induce pituitary tumors for the purpose of developing differentiated gonadotroph cell lines has provided new evidence that human null-cell adenomas are derived from gonadotrophs *(63)*. Specifically, transgenic expression of an hFSHβ-SV40tsTAg construct resulted in gonadotroph hyperplasia progressing to slowly growing nodular adenomas that gradually lost immunoreactivity for the gonadotropins. When evaluated by electron microscopy, these tumors had ultrastructural characteristics identical to those observed in human null-cell adenomas. These data support the hypothesis that human null-cell adenomas are derived from gonadotrophs. These tumors, as well as the gonadotroph adenomas induced by targeting with α-subunit and LHβ promoter sequences, provide an animal model for further study of this disease. In addition, these transgenic pituitary adenoma models could potentially lead to the development of pharmacological interventions for macroadenomas as well as mechanistic insights into the development of pituitary adenomas. Indeed, studies of the α-T7 line of transgenic mice demonstrated an unusual feature of ingrowth of neural tissue in the anterior pituitary gland concentrated around transformed gonadotrophs and provided strong evidence for the role of basic fibroblast growth factor as a neurotropic factor *(103)*.

One unresolved issue in pituitary tumorigenesis is the concept of the role of hypothalamic-releasing factors in tumor development. This has been studied in other pituitary cell types utilizing transkaryotic (corticotrophs) *(104)*, transgenic (somatotrophs *[105]* and corticotrophs *[106,107]*), and gene disruption strategies (lactotrophs) *(108)*, but has not been reported in detail for gonadotrophs. One study of transgenic overexpression of GnRH using a CMV promoter failed to induce gonadotroph proliferation *(7)*; likewise, overexpression of corticotropin-releasing hormone resulted in hypercortisolemia and features of Cushing's syndrome, but no corticotroph hyperplasia *(107)*. This result is in contrast to the overexpression of GH-releasing hormone that resulted in dramatic somatotroph hyperplasia and adenomas *(105)*. Likewise, the absence of dopamine D2 receptors was shown to be sufficient to initiate a programmed development of diffuse lactotroph hyperplasia and prolactinoma formation in mutant mice *([108,109]*; M. J. Low, unpublished observations). The mechanisms underlying these different responses, and whether they are related to inherent differences in the target cells, is of considerable interest.

LH Hypersecretion in Polycystic Ovary Syndrome

Hyperandrogenism can cause significant problems in women, including polycystic ovaries, oligomenorrhea, infertility, hirsutism, and, if severe, masculinization. One feature of the polycystic ovary syndrome is hypersecretion of LH; however, the exact role that elevation of this gonadotropin plays in the etiology of the syndrome is not clear. The development of transgenic mice that overexpress LH has provided a model to ascertain the role of LH and androgens in this syndrome *(110,111)*. A modified bLH construct was made by fusion to C-terminal peptide (CTP) sequences from hCG, which are involved in prolonging hormone clearance from the vascular compartment, and was targeted to the pituitary gland using bovine α-subunit promoter sequences. Transgenic mice were found to oversecrete bLH-CTP, with the predicted prolongation in half-life, and demonstrated markedly elevated testosterone levels, precocious vaginal

opening, and enlarged, polycystic ovaries with thecal hypertrophy. Exogenous androgen treatment produces evidence of masculinization of the hypothalamus *(111);* it is not clear whether the presence of elevated LH and androgens in this transgenic model represents the same phenomenon, altered regulation of the transgene with insensitivity to negative feedback, or abnormalities of ovarian steroidogenesis related to the thecal hypertrophy. In any case, these mice now provide an excellent mechanistic model of polycystic ovary syndrome.

Gene Therapy

The techniques of targeting gene expression are likely to be key in future developments of gene therapy for medical applications. The reproductive system has already provided two animal models demonstrating the potential of this approach. Transgenic expression of the mouse GnRH gene was shown to restore fertility in the hpg hypogonadal mouse, which contains a spontaneously disrupted GnRH gene *(113).* Similarly, reproductive function was restored in the FSH knockout mouse by transgenic rescue with the human FSH transgene *(114).* Further refinements in techniques of cell-specific gene targeting and gene delivery are required to make such therapies feasible for medical application.

SUMMARY

Transgenic animals have contributed significantly to studies of the reproductive system. Key experimental paradigms in the study of α-subunit promoter function have been transgenic expression and transient, in vitro expression of reporter constructs in α-T3 cells, immortalized gonadotroph cells derived from oncogene-induced pituitary tumors in transgenic mice. The α-T3 cell line continues to be important for the study of mechanisms of gonadotropin regulation, including the subcellular events involved in GnRH signaling and the interaction of the multiple hormonal signals that characterize the complex regulation of the reproductive system. Although studies of the β-subunits are less advanced, with the recent development of the LβT2 cell line, a similar level of understanding about these genes should soon be possible. In addition to the basic studies of gonadotropin hormone gene regulation, studies of the ontogeny of the pituitary gland have been advanced by transgenic techniques. This has included the use of transgenic reporters to study temporal activation of gonadotropin subunit gene expression as well as transgenically targeted ablation experiments to study lineage relationships and cell-cell dependence. Finally, the development of transgenic models of human disease such as pituitary tumors and polycystic ovary syndrome has contributed to the understanding of the mechanisms of these diseases, with the potential for novel therapies. Although human gene therapy is considerably more complex than current mouse transgenic technology, the concepts of targeted gene expression will be key for future gene therapy applications.

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12 Molecular Events Defining Follicular Developments and Steroidogenesis in the Ovary

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CONTENTS

INTRODUCTION

The Ovarian Follicle

The ovarian follicle assembles to nest and prepare the oocyte for ovulation. Figure 1 shows that some follicular cells even escort the extruded egg to the site of fertilization in the oviduct. By means of its steroid hormone secretion, the follicle also secures the chances of the embryo to launch a successful implantation in the uterus. To this end, the ovarian follicle undergoes two fundamental processes: a dramatic growth in size to create a minimal cellular mass of secretory cells, followed by acquisition of the functional capacities, the most prominent of which is the making of steroid sex hormones (steroidogenesis). The important ovarian sex hormones are progesterone and estradiol. Both hormones support follicular growth and function, as well as prime the cells of the uterine wall for proper future implantation of the embryo. Ovarian androgens are also essential, as both aromatizable estrogen-precursors and modulators of gonadotropin action. But for the initial phases of follicular development, the pituitary gonadotropins, follicle-stimulating hormone (FSH) and luteinizing hormone (LH), control most of the follicle's life span, acting as trophic agents and inducers of steroidogenesis *(1)*. After ovulation, the ruptured follicle transforms into a powerful endocrine gland, the corpus luteum, which secretes progesterone and estrogen serving to maintain a healthy pregnancy. Whereas in humans, the endocrine role of the corpus luteum is assumed by the

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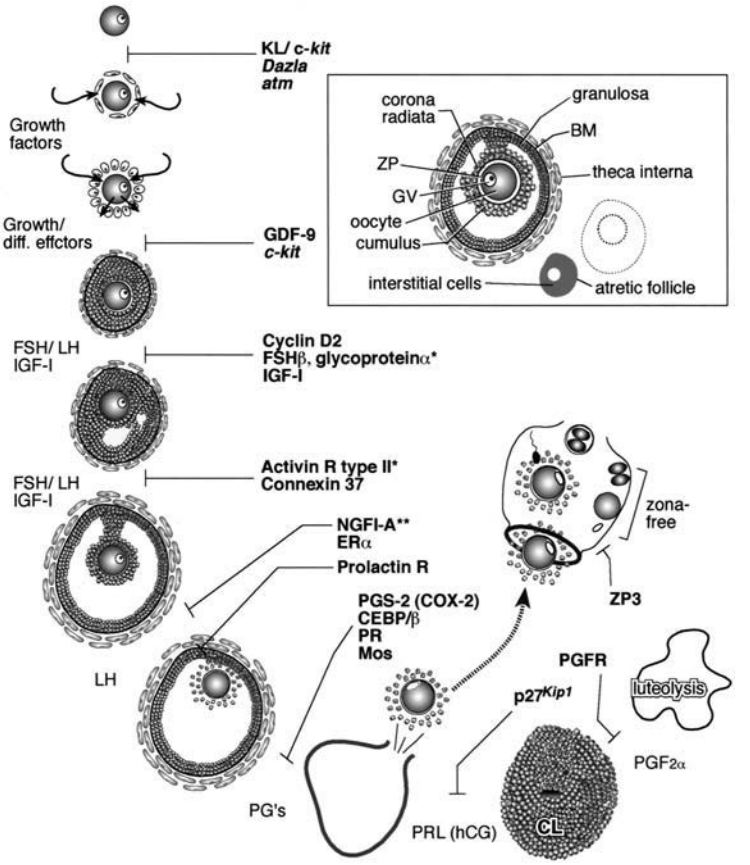

Fig. 1. Critical checkpoints during ovarian folliculogenesis are revealed by the disrupted gene technology. The process of folliculogenesis is illustrated from top to bottom. **(Left)** Factors involved in control of follicular development. **(Right)** Targeted gene disruptions (or mutations) known to arrest folliculogenesis. Gametes: Onset of folliculogenesis requires the arrival of the female germ cells **(top)** at the fetal gonad site *(103,182)*. This process can be interrupted by disruption of *KL*/c-*kit, Dazla,* or *atm* genes. Lack of KL/c-*kit, Dazla,* or *atm* gene products will consequently prevent the formation of the primordial follicle. **(A)** Primordial follicle: In most mammals, before birth oogonia are transformed into primary oocytes at meiotic prophase, which become surrounded by a layer of flattened granulosa cells (second from top) *(103)*. **(B)** Primary follicle: The granulosa cells attain a unilamellar cuboidal morphology. To the best of our knowledge, the factor(s) responsible for the morphological transformation of the granulosa cells is not known. **(C)** Secondary follicle: A series of mitotic divisions of the granulosa cells attaining four layers, and recruitment of theca cells, mark the formation of this preantral secondary follicle. This formation is still gonadotropin independent. Loss of growth factors or growth-factor receptors, such as growth-differentiation factor 9 (GDF-9) or c-*kit,* respectively, can inhibit further development of this follicle. **(D)** Tertiary follicle: This follicle is marked by the formation of the antrum and acquisition of meiotic competence of the oocyte. It depends on growth from FSH, LH, and IGF-1. Disruption of the following genes inhibits further development of this follicle: cyclin D2, FSHβ, and α-subunit of the pituitary glycoprotein hormones and IGF-1. **(E)** Preovulatory Graafian follicle: FSH, LH, and IGF-1 also control the formation of this follicle, whose granulosa cells produce high levels of estrogens and express LH receptors. **(F)** Periovulatory follicle: In response to LH surge, the granulosa cells initiate a process of luteinization that is inhibitable if a disruption of the NGFI-A transcription factor results in a marked attenuation of the pituitary LH production. At 2–4 h after LH surge, this follicle extinguishes expression of P450arom and P45017α (Fig. 5A), as well as transiently upregulates expression of prostaglandin H synthase-2 (PGHS-2, COX-2), C/EBPβ transcription factor, and progesterone receptor (PR). Gene disruption of the latter three inhibits ovulation, but allows the mucification of the cumulus and resumption of meiosis (GVB). Lack of ovulation is also observed in ER-α-deficient

placenta at the onset of the second trimester, in rodents the corpus luteum is indispensable until the end of the pregnancy *(2)*.

Three of the four steroidogenically active cell types in the ovary compose the follicle:

1. The cumulus oophorus/corona radiata are the only cells that make a physical contact with the oocyte, thereby providing the means to keep the oocyte in meiotic arrest until the time of ovulation commences (for a review, *see* ref. *3)*. The LH surge triggers mucification of the cumulus cells, which thereafter leave the ovary with the ovulated oocyte. Following fertilization of the egg in the ampullar end of the oviduct (Fig. 1), these cells are absorbed by the oviduct wall while the denuded embryo starts migrating toward the uterus.
2. The granulosa cells are the major somatic cell component of the follicle and determine, more than any other cell type, the final size of the preovulatory follicle in each species. Ovarian cues, including that of insulin-like growth factor-1 (IGF-1), induce expression of FSH receptors in granulosa cells at early stages of follicular organization. If the follicle is further selected for ovulation, FSH priming will also induce new LH receptors (Fig. 2), thus making the granulosa cells functionally ready for the LH surge.
3. The cells that delineate the follicle on the outer side of the basement membrane are the theca interna cells. Unlike the granulosa cells, the theca interna cells are endowed with only LH receptors and produce androgens as their final steroid product (Fig. 2).

The last cellular compartment, which is semantically regarded as nonfollicular, is the secondary interstitial cells scattered in between the follicles. Interestingly, the interstitial cells are, in fact, reminiscent of the corpus luteum since both tissues are postfollicular structures, highly steroidogenic, and present LH receptors. Whereas the corpus luteum originates from the ruptured follicle, the secondary interstitial tissue develops from atretic follicles that do not ovulate and undergo a degeneration process based on programmed cell death of the granulosa cells. Eventually, the only cells surviving the atretic process are the theca interna cells, which become part of the ovarian interstitium. Their functional trait as androgen-producing cells is understandable in light of their theca cell lineage. Inequitably, these cells are perceived as "conceptual orphans" of unclear physiological role in many texts describing the principle of follicular function. In this chapter, I discuss at least one experimental model proposing that the

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ovaries, where excessive response to LH causes hemorrhagic cyst formation; most females develop ovarian teratomas due to parthenogenetic activation of oocytes prior to ovulation. On ovulation, the extruded oocyte (first polar body is observable) and accompanying cumulus cells enter the oviducal ampulla, where fertilization commences, as illustrated. In the absence of the zona pellucida (disrupted ZP3), the oocyte and/or the embryo are absorbed by the oviduct wall. *Corpus luteum* (CL); in rodents, the development and maintenance of this secretory gland during pregnancy depends on pituitary prolactin (PRL) and uterine PRL-like substances *(158,181),* whereas the trophoblast-made human chorionic gonadotropin (hCG) plays this role in humans. Terminal differentiation of the granulosa cells necessitates a complete arrest of proliferation mediated by the cell-cycle inhibitor $p27^{kip1}$. Luteolysis: degeneration of the corpus luteum is induced by $PGF_{2\alpha}$. Therefore, loss of PGF-receptor expression in CL cells does not allow the critical downregulation of progesterone production preceding labor. **(Inset)** The cellular composition of a follicle includes the oocyte, the corona radiata, and the cumulus, granulosa, and theca interna cells. A basement membrane (BM) divides the compartments of the latter two cell types. In the rat, small atretic follicles give rise to the LH-responsive interstitial tissue, which exhibits high androgenesis capacity. GV, germinal vesicle; ZP, zona pellucida.

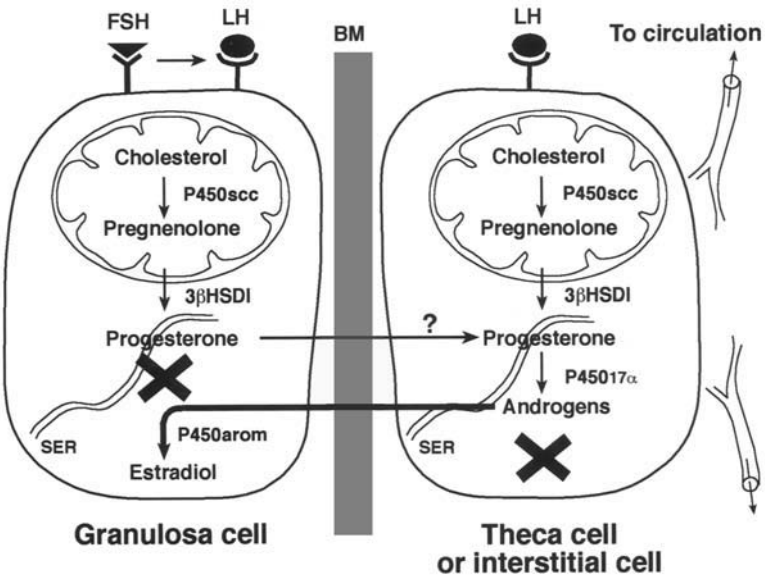

Fig. 2. Cellular organization of steroidogenic genes and hormone receptors in granulosa and theca cell types. Granulosa and theca cells rest on both sides of the follicular basement membrane (BM). The interstitial cells (not shown) are functionally identical to the theca cells, but localize in between the follicles. The following description portrays an extended view of the "two-cell, two-gonadotropin theory" *(7). De novo* synthesis of progesterone is initiated in the mitochondria (arrowhead) of both smooth endoplasmic reticulum (SER) cells by P450scc converting cholesterol to pregnenolone. Pregnenolone diffuses to the cytosol, where both cell types convert it to progesterone by 3βHSD action. 3βHSD, and the other downstream enzymes of the steroidogenic cascade are anchored to the smooth endoplasmic reticulum (SER). Progesterone has two potential fates in the follicle: either diffuse to the theca cell compartment and enter the circulation via the blood vessels, or serve as substrate for P45017α and 17β-HSD, which convert it to androstenedione and testosterone (androgens). The latter two reactions commence only in the theca-interstitial cells, where the androgens are end-steroidogenic products. Then, these androgens can diffuse over the BM to the granulosa cell compartment, where the avascular environment facilitates their efficient aromatization, by P450arom, to active estrogens (estradiol). Since the granulosa cells are P45017α deficient **(X),** and the theca cells do not express aromatase **(X),** the two cell types perform a functional complementation (large arrows) to overcome their missing capacities. Expression of the steroidogenic enzymes is triggered by FSH or LH signaling. Granulosa cells express FSH receptors (solid triangle) during early follicular development. Also, FSH can induce LH receptors (solid oval) in granulosa cells of a preovulatory (Graafian) follicle. By contrast, theca cells are endowed with only LH receptors. More aspects of the two-cell, two-gonadotropin theory are discussed under "Steroid Hormone Synthesis."

physiological role of the secondary interstitial cell is indistinguishable from that attribute to its parental theca cells. However, I do not discuss in depth the function and regulation of the corpus luteum *(4),* nor do I dwell on apoptosis and follicular atresia *(5).*

As well expressed by Greenwald *(6),* a primordial follicle has three potential fates: to remain quiescent, to initiate growth, and later to drop the race to become atretic, or survive to the finish line, mature, and ovulate. What factors control, then, the decision-making checkpoint during follicular development? In recent years, more and more such factors regulating folliculogenesis have been discovered, largely thanks to the impact of molecular biology and the powerful techniques of transgenic and knockout mice.

Interestingly, some of the discoveries were serendipitous, resulting as by-products of various creations that did not necessarily intend to study reproductive endocrinology; after all, sterility of a mutant mouse missing any disrupted gene is a readily observable feature of the manipulated animal. In view of the past and future potential to unveil new factors controlling follicular development by the knockout gene technology, Table 2 and Fig. 1 provide an introductory survey of this experimental approach, listing the various null-mice mutants associated with female infertility.

Ovarian Steroidogenesis: The Overall Picture

Conforming with the "two-cell, two-gonadotropin" theory *(7),* the exchange of steroid products between the granulosa and the theca cells, aiming to produce estradiol, is an intriguing example for a "paracrine trade" of products between two follicular compartments that are physically divided by basement membrane (Fig. 2). Such a joint effort is essential in face of the fact that the androgen-secreting theca cells are unable to express aromatase (P450arom) required to produce estradiol. Conversely, the granulosa cells, which do not express P450 17, 20-lyase ($P450_{17\alpha}$), are incapable of producing estradiol *de novo* either, unless supplied with aromatizable androgens made by the theca cells across the basement membranes (Fig. 2). It was also suggested that the granulosa cell compartment can reciprocate by supplying ample amounts of progesterone substrate for androgen production *(8,9).* New insights related to the relevance of this exchange of steroid commodities will be addressed in light of the latest findings related to the ovarian expression of steroidogenic acute regulatory (StAR) protein. StAR was recently discovered *(10)* as an indispensable component of the steroidogenic machinery, serving to facilitate cholesterol supply for the key and rate-limiting enzyme in steroidogenesis, cholesterol side-chain cleavage cytochrome P450 (P450scc).

Experimental Models of Ovarian Steroidogenesis in Rodents: Are These Small Animals Worth the Effort?

In the past three decades, in vitro models using primary cells in culture served for the majority of the studies related to the molecular basis of ovarian function. The history of those studies reflects the general progress made through the years in the field of tissue culture. In the late 1960s, pioneer adrenal and ovary endocrinologists realized that primary steroidogenic cells could be isolated and prepared for cultures in which their typical characteristic functions could be maintained *(11,12).* Yet, those studies used serum-containing nutrient media that still suppressed many functional aspects of ovarian cell differentiation *(13).* Therefore, it was not until the end of the 1970s that the use of hormone-supplemented serum-free medium allowed the first demonstration of FSH induction of LH receptors in cultured rat granulosa cells *(14).* Since then hormone-supplemented serum-free approaches, largely inspired by the conceptual breakthrough made by Gordon Sato *(15),* have become dominant *(16,17)* and promoted our understanding of ovarian cell function as we perceive it today. Rat and porcine granulosa cells are the most widely used models, thanks to their robust responses to hormone administration and the nonproteolytic ease of preparation for culture. Moreover, in the rat, one can obtain five- to sixfold more granulosa cells if 25-d-old immature animals are primed in vivo with estradiol prior to pricking the follicles to release their granulosa cell content.

The rodent ovary places obvious difficulties related to the limited source of cellular

Table 1
Disrupted Genes in Null-Mice Models Compromising Female Fertility hr[a]

Targeted Gene Cellular Origin	*F and M Sterility/ Fertility Physiological Impairment*	*Effect on Growth and/or Function*		*Reference*
		Follicular Growth	*Steroid Synthesis, Receptors*	
Dazla cytoplasmic germ cell protein	Loss of germ cells; sterile F and M**	No folliculogenesis		*100*
atm gametes	Absence of mature gates; apparently no estrous cycle; sterile F and M	No primordial or higher follicles	NT; reported stromal and interstitial cells	*101*
c-*kit* and KL gametes and somatic cells	Sterile (*Sl/sl*^{t}) F, arrest of folliculogenesis; fertile (*Sl/Sl*^{t}) M	Arrested at primary follicle	FSH-inducible aromatase but no P45017α activity	*107*
GDF-9 oocytes	Sterile F, unovulation; fertile M	Arrested beyond primary one-cell layer; absence of theca cell	Probably reduced; normal FSHR and LHR	*108*
Cyclin D2 granulosa	Sterile F, unovulation; fertile M, low sperm count	Hypoplastic follicles arrested at four layers of granulosa cells	Normal; unaffected P450scc, P450arom, FSHR, LHR, PGS-2	*110*
FSHβ pituitary	Infertile F, arrest of folliculogenesis; fertile M	Arrested at preantral stage	NT	*76*
Glycoprotein pituitary	Infertile F, arrest of folliculogenesis; infertile M, no developed sperm	Arrested at preantral stage	NT	*114*
IGF-1 granulosa	Arrested onset of puberty; infertile F and M	Arrested at preantral stage	Reduced FSHR (50%); ablated (P450arom)	*75, 115*
Connexin 37 oocytes	Sterile F, unovulation; Male*	Lack of Graafian follicles	NT	*127*
ActRcII pituitary	Impaired estrous cycle owing to suppressed FSH synthesis; fertile M	Absence of CL and increased atresia	Apparently normal	*123*
NGFI-A (Egr-1, Krox-24, Zif/268) pituitary	Loss of estrous cycle owing to decreased levels of LH-β; fertile M	Lack of CL, otherwise normal follicular development	Normal in response to administered gonadotropins	*130*

continued

Table 1
Continued

Targeted Gene Cellular Origin	*F and M Sterility/ Fertility Physiological Impairment*	*Effect on Growth and/or Function*		*Reference*
		Follicular Growth	*Steroid Synthesis, Receptors*	
ERα granulosa	Infertile F; reduced but not abolished fertility of M	Arrested at Graafian stage	Normal steroidogenesis; unaffected PR, AR	*118, 184*
PRL-R granulosa	Failure of embryonic implantation; sterile F and half of M	Arrest of GVB in 40–60% of ovulated eggs; numerous CL (atretic follicles?)	NT, apparently normal	*134*
PGS-2 (COX-2) granulosa	Sterile F, unovulation; Male*	Lack of CL	Apparently normal	*139*
C/EBPβ granulosa	Sterile F, inefficient ovulation; fertile M	Arrested beyond preovulatory stage, no CL	Improper sustainment of post-LH levels of P450arom and PGS-2	*144*
PR granulosa	Unovulation, lack of CL, lack of follicular rupture; fertile M	Normal, including GVB and cumulus mucification	Lack of granulosa cell luteinization	*147*
Mos oocyte	Infertile F, oocyte parthenogenesis, ovarian teratomas; fertile M	Not affected Failure to arrest oocytes at 2nd metaphase of meiosis	NT	*119, 120*
ZP3 oocyte	Female infertility owing to loss of zona-free oocyte or embryo in the ampulla; Male*	Normal follicular development	Normal	*149*
$p27^{kip1}$ granulosa	Sterile F, failure to maintain pregnancy; fertile M	No CL	Arrest of CL differentiation	*151, 152, 183*
$PGF_{2\alpha}$ receptor lutein cells	Sterile F, lack of parturition; fertile M	Normal	Normal, sustained progesterone secretion at term caused by lack of luteolysis	*154*

[a]NT, not tested; F, female; M, male; GVB, germinal vesicle breakdown; CL, corpus luteum; *c-kit* and KL, tyrosine kinase receptor and its ligand; GDF-9, growth differentiation factor-9; LHR, LH receptor; FSHR, FSH receptor; ERα, alpha-type estrogen receptor; IGF-1, insulin-like growth factor 1; NGFI-A, PGS-2, prostaglandin H synthase-2; C/EBPβ, CCAAT/enhancer-binding protein β; PR, progesterone receptor; ZP3, zona pellucida glycoprotein 3; Male*, lack of information on sterility/fertility of males; M**, additional disrupted genes that cause selective male infertility in null-mice models are *Zfy, MIS, HR6B, Bax, CREM, RXRb,* and *Hsp70-2*.

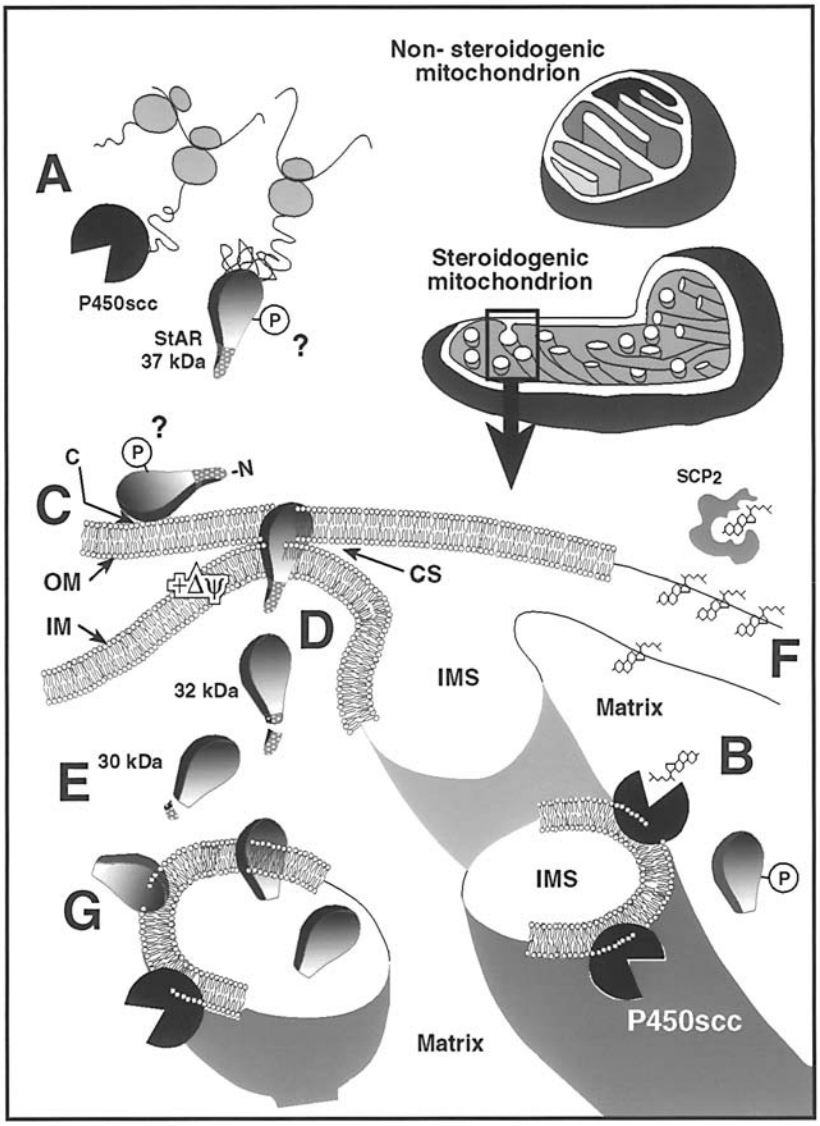

Fig. 3. Illustrated organization of P450scc and StAR in a steroidogenic cell. **(Top right)** On steroidogenic differentiation of ovarian and adrenocortical cells, the mitochondrial plated-cristae (nonsteroidogenic) transform to tubular and vesicle-like structures *(32)*. **(Bottom)** A scale-up of the framed area (large arrow) in the steroidogenic mitochondrion. **(A)** Both P450scc and StAR proteins are translated on free ribosomes and contain N-terminal signal peptide that guides them into the mitochondrion. On P450scc import, the enzyme is anchored to the cristae membrane *(32)* facing the matrix as shown in **B.** StAR is synthesized as a 37-kDa precursor (A) that may undergo post- or cotranslational phosphorylations on various consensus motifs for protein kinases, such as protein kinase A (PKA) *(48)*. **(C)** For the sake of simplicity, it is hypothesized that the C-terminal portion of StAR (c), which is essential for bioactivity, interacts with a putative component of the mitochondrial surface (*see* "StAR" under "Hormone Biosynthesis"). Thereafter it is assumed that termination of StAR activity is facilitated by import into the organelle via its N-terminal signal peptide **(D). (E)** A two-step proteolytic processing yields the mature form of 30-kDa StAR *(43)*. Import of StAR precursor probably occurs in the area of contact sites (CS) between the outer- and the inner-mitochondrial membranes and depends on membrane potential (+$\Delta\psi$) across the inner membrane *(54)* and ATP in the matrix *(53)*. The latter mitochondrial qualities are still needed for execution of StAR activity

material from the tiny organs. Yet, as will be discussed, serum-free cultures readily allow the study of molecular steroidogenesis using fewer cells in miniature culture wells. For example, cells can be treated with gonadotropins to induce the enzymes of the steroidogenic pathways, whereas less than 5×10^4 cells are enough for determination of mRNA by quantitative reverse transcriptase polymerase chain reaction (RT-PCR) *(18),* Western enhanced chemiluminescence (ECL) assessment of proteins *(18),* and promoter analysis studies *(19).* Even fewer cells are enough for characterization of enzyme levels by fluorescence *(20)* and immunoelectron microscopy *(21).* Furthermore, because of their amphipathic nature, metabolism of the steroid hormones can be studied by simple incubations of cells with radioactive hormones *(8,22–24),* which rapidly exchange with the cell interior ($t_{1/2}$ = 20 s).

Adult cycling females are obviously the most physiologically relevant animal models to study. In the rat, however, a combination of practical difficulties in obtaining animals at the same stage of the cycle and the variability of the follicles in the adult tissue caused many investigators to prefer to use the gonadotropin-treated immature rat model. Thus, following the administration of pregnant mare serum gonadotropin (PMSG) *(25)* or individual gonadotropins to 25-d-old females *(26),* a forced precocious onset of puberty initiates the growth of a synchronized and size-controlled cohort of follicles toward ovulation. It is most rewarding to monitor the dramatic progress of follicular maturation in such ovaries, which may ovulate between half a dozen *(26)* to 30 eggs *(23).* Unlike the cycling gonad, which consists of corpora lutea occupying more than 90% of its tissue volume, the entire body of the immature ovary is dedicated for the boosted onset of first ovulation, so that any potential event we might wish to study is tremendously amplified in the immature rat model.

FOLLICULAR STEROIDOGENESIS

Hormone Biosynthesis: The Cellular Level

P450scc, 3βHSD, P45017α, and P450arom

In most of the classical steroidogenic cells, trophic hormones stimulate the synthesis of steroid hormones through the intermediary cAMP *(27,28).* The cyclic nucleotide triggers the expression of P450scc *(29,30),* which catalyzes the first and key reaction in the steroidogenic cascade. P450scc is a nuclear encoded mitochondrial protein, and electron microscopy studies have shown that its 54-kDa mature form is embedded in the inner membranes of the mitochondrion facing the mitochondrial matrix (Fig. 3; *[31,32]*). This orientation is probably favorable for converting cholesterol to the first steroid molecule, pregnenolone. To this end, P450scc utilizes atmospheric oxygen and

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even if StAR cannot be imported in the absence of its signal peptide (*see* "StAR" under "Hormone Biosynthesis" and refs. *53* and *54).* **(F)** StAR bioactivity has been shown to facilitate transfer of cholesterol from the outer mitochondrial membranes to the inner membranes of the organelle *(50).* **(G)** Immunoelectron-microscopy studies of ovarian and adrenal cells have shown that StAR mainly occupies the matrix or associates with cristae membranes facing the matrix *(18,21,51).* OM, outer membrane; IM, inner membrane; CS, contact site; IMS, intermembrane space; SCP_2, sterol carrier protein-2 *(10),* which mobilizes free cytosolic cholesterol from lipid droplet reservoirs (not shown) to other cellular destinations.

reducing power provided by electron transport intermediates consisting of ferridoxin (a mitochondrial-matrix soluble protein), ferridoxin-reductase (membrane bound), and $NADPH^+$ *(29)*. Consequently, a complex catalysis produces pregnenolone by removing a six-carbon unit from the cholesterol side chain. For most of the classical steroidogenic tissues including the ovary, pregnenolone is further metabolized to progesterone by the endoplasmic reticulum enzyme 3β-hydroxysteroid dehydrogenase/isomerase type I (3βHSDI) *(33,34)*. Unlike the ovary, recent studies have unequivocally shown that in the cortical cells of the adrenal, about 40% of 3βHSDI resides inside the mitochondria, which probably facilitates a local production of progesterone inside the organelles *(34)*. The physiological significance of this phenomenon is not clear. Further production of androgens from progesterone is catalyzed by P45017α, which is anchored to the endoplasmic reticulum of the ovarian theca and interstitial cells (Fig. 2). The membranes of the endoplasmic reticulum are also the docking site for P450arom, which catalyzes the aromatization of androstenedione and testosterone to form estrogens *(35)*.

StAR

Is the P450scc protein complex the only limiting factor determining the rate of steroidogenesis? Apparently not. Early studies conducted by the first adrenal biochemists in the early 1960s led to the hypothesis that, in addition to existing P450scc, the rate of ACTH-induced steroidogenesis in the cortical cells is dependent on *de novo* protein synthesis *(36,37)*. Later it was shown that the transfer of cholesterol from the outer mitochondrial membrane to the inner membranes of the organelle was probably the critical step that was dependent on such *de novo*–synthesized factor *(38,39)*. Not until the mid-1980s was a correlation made between the appearance of a 30-kDa mitochondrial phosphoprotein and ACTH-induced steroidogenesis *(40–42)*. Recently, this long-sought protein regulating the acute response of steroidogenesis was isolated and cloned *(43)*. The novel protein was designated StAR for *St*eroidogenic *A*cute *R*egulatory protein. Figure 3 provides a summary of a proposed model of StAR's fate in a steroidogenic cell. Murine StAR is a 284 amino acid protein, the first 47 residues of which compose a mitochondrial signal peptide. Consequently, StAR is rapidly imported to the mitochondria and on a two-step cleavage process yields its 30-kDa mature form *(43)*. Perhaps the most compelling evidence for the critical role of StAR in steroidogenesis was the discovery that deleterious mutations in the human StAR gene *(44,45)* cause a syndrome known as congenital lipoid adrenal hyperplasia *(46)*. Affected individuals die shortly after birth in the absence of adrenal steroids unless treated with steroid hormone replacement therapy. The cortical adrenal cells of affected infants are filled with surplus cholesterol, and in the absence of glucocorticoids the tissue hyperplasia results from lack of the normal negative feedback downregulation of ACTH secretion *(46)*. Also, owing to impairment of gonadal steroidogenesis during fetal development, XY males are born with female external genitalia.

The exact molecular mechanism by which StAR facilitates steroidogenesis is still obscure. It is clear, however, that in various model and authentic cell systems, a marked increase in steroid hormone synthesis is correlated with a rapid hormone-dependent *de novo* synthesis of StAR *(47);* a cAMP induction of post- or cotranslational phosphorylation of StAR on serine 194/195 *(48);* and a StAR-promoted increase of cholesterol mobilization from the outer mitochondrial membranes to the inner membranes of the organelle *(49)*. Most intriguingly, deletion of the first 47 amino acids from the amino-

terminal end of StAR did not result in the expected loss of its biological activity *(49,50)*. Confocal and immunoelectron microscopy studies have shown that in the absence of the mitochondrial signal peptide, StAR deletion mutant indeed remained in the cytosol, but yet was fully active *(49,50)*. On the other hand, the carboxy-terminal domain of StAR is critical for its bioactivity. One of the severe impairments of StAR function in patients with lipoid CAH was the result of a premature stop codon deleting 28 residues from the C-terminal portion of StAR *(45)*. In accordance, COS cells expressing the C-28 deletion mutant of StAR lost their steroidogenic capacity *(49,50)* owing to the inability of the mutant protein to mediate cholesterol mobilization into the mitochondria *(50)*.

In view of the facts that inactive C-28 StAR is readily imported into the mitochondrion *(50)*, the nonimportable N-47 StAR mutant is fully active, and only 2–4% of wild-type StAR molecules reside at any time on the outer mitochondrial membranes *(51,52)*, it became apparent that the critical events associated with StAR bioactivity must precede its translocation into the mitochondria. Thus, it was proposed that the mitochondrial import of StAR is no more than a turning-off mechanism to terminate StAR action by removing it from the organelle surface *(49,50)*. Consequently, steroidogenesis cannot be sustained unless *de novo*-synthesized StAR molecules replaced the imported ones. Nevertheless, an intact mitochondrial inner-membrane potential and adenosine triphosphate (ATP) are still required for StAR activity *(53,54)*, suggesting the involvement of additional co-StAR factors. Furthermore, in the absence of current knowledge on potential ligands that can interact with the functionally essential carboxy-terminal domain of StAR, it is not clear whether a putative "functional platform" for StAR action is physically associated with the mitochondrial surface, or resides in the cytosol. Whatever such a putative molecular entity might be, the successful reconstitution of steroidogenesis in double-transfected COS cells expressing both StAR and the P450scc complex *(50)* indicates, however, that the cell's ability to respond to StAR is an ubiquitous cellular feature and does not necessarily characterize steroidogenic cells.

Steroid Hormone Synthesis: The Follicular Level

Spatiotemporal Expression of the Steroidogenic Machinery

A few introductory comments aim to highlight key aspects related to hormonal control of steroidogenic gene expression in developing follicles. First, growth and differentiation phenomena probably determine many regulatory patterns, particularly in the granulosa cell compartment, which, more than any other constituent of the follicular structure, determines the potential follicular size in each species *(185)*. Second, for many years FSH was thought to be the sole hormone that initiates follicular development toward ovulation. It is now accepted that in the rat, sheep, and more species other than primates, low-dose administration of LH can support the selection of small antral follicles that will enter the preovulatory stage *(1)*. However, the presence of FSH bioactivity is also essential for the selection process, as we learn now from studies of the FSHβ knockout ovaries (*see* "Folliculogenesis").

If LH is truly critical to initiate follicular development, one must assume that two cell types bearing LH receptors can receive this hormonal cue—the theca cells and the interstitial cells. Many studies (reviewed in ref. *17*) have suggested that prior to the onset of puberty in rodents, the low levels of circulating LH maintain a sizable population of steroidogenic interstitial cells *(55)*, which develop during atretic processes

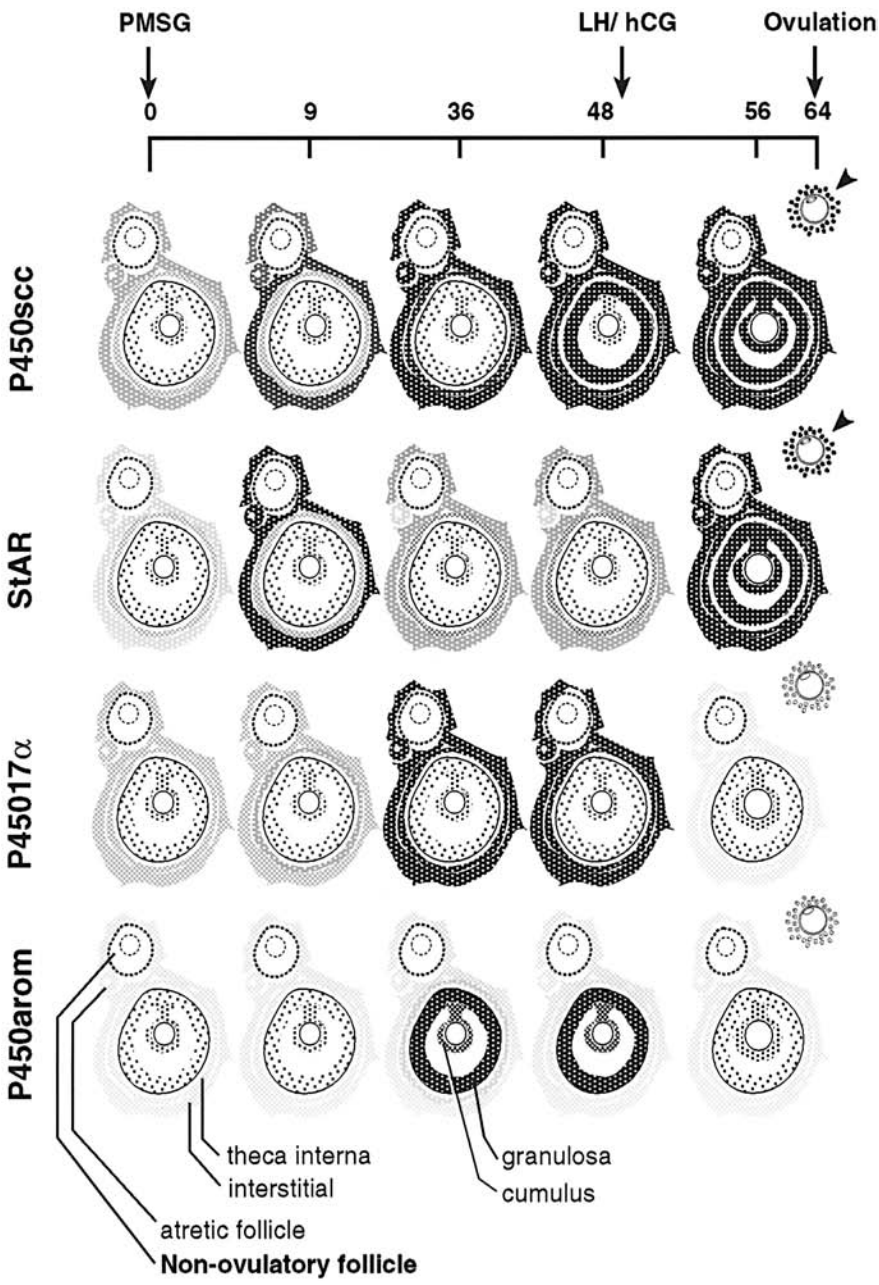

Fig. 4. Schematic representation of spatiotemporal expression of P450scc, StAR, P45017α, and P450arom during follicular development. Twenty-five-day-old immature rats were injected with 15 IU of PMSG at time zero and 4 IU of hCG was administered 52 h later. Immunofluorescence localization studies and quantitative biochemical analyses *(52,55,56,58,95)* are schematically summarized by a gray level–coded illustration of the steroidogenic proteins as they are expressed in each of the ovarian cell types (darker shaded areas denote higher levels of expression). For complementary detail, see Fig. 5 and under "Steroid Hormone Synthesis." **P450scc**, a vectorial progress of P450scc expression proceeds from the follicular periphery to the center of the periovulated follicle. First, more P450scc is expressed in the already differentiated interstitial cells (9 h); later, P450scc-positive cells develop in the theca interna layers (36 h), granulosa cells (48 h), and cumulus cells (56 h). The latter cells leave the follicle with the ovulated egg (arrowhead). **StAR** is expressed in biphasic

in the juvenile female. These cells outnumber the follicular theca interna cells *(56)* and raise the question, for what physiological purpose does the ovary support a persistent reservoir of nonfollicular androgenic capacity? The following spatiotemporal patterns of P450scc, StAR, P45017α, and P450arom (Figs. 4 and 5) may propose attractive working hypotheses addressing this question. First, Fig. 4 schematically illustrates a summary of our studies showing cell-specific expression patterns of the key steroidogenic gene products we and others have determined throughout follicular development by use of immunohistochemistry, Western blot analyses, *in situ* hybridizations, and RT-PCR assays.

P450scc Pattern

In the superovulated rat model, administration of PMSG generates a time-dependent "tidal wave" of P450scc expression proceeding from the periphery of the follicle to the center, where the oocyte rests (Fig. 4). The first cell types to respond within 9–24 h are the nondividing interstitial and theca cells, which are partially differentiated even prior to the administration of gonadotropin *(55)*. As maturation progresses, the granulosa cells are next to express P450scc shortly before the expected timing of LH surge. Finally, the cumulus cells acquire P450scc during the few hours preceding ovulation (Fig. 4; *[58]*). The factors controlling this programmed expression of P450scc in a timely orchestrated fashion are still obscure. Do cell-cycle elements cause an attenuation of P450scc expression in dividing granulosa cells and/or resting follicles? Earlier studies of primary granulosa cells in culture have indicated that mitotic activity and steroidogenesis cannot occur simultaneously *(57)*. More in vivo studies of the infertile cyclin $D2^{-/-}$ female mice, as well as female $p27^{Kip1}$ knockout mice lacking a functional corpus luteum owing to loss of growth restraints do seem to support such a possibility (*see* under "cyclin D2" and "$p27^{Kip1}$").

StAR Pattern and Physiological Ramifications

The functional "knockout" of human StAR (lipoid CAH; *[45]*) and the recent creation of StAR-deficient mice *(59)* showed beyond any doubt that StAR is essential for adrenal and testicular steroidogenesis. Yet, these studies have not provided evidence to show the importance of StAR for the female reproductive organs. Since StAR is not expressed in human placenta *(44)*, and pregnancy is not interrupted in StAR-deficient mice, it is assumed that StAR is not functionally important for placental steroidogenesis. Interestingly, recent studies have shown that a less efficient StAR homolog, the MLN64 protein,

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peaks. The first wave of high StAR level is restricted to the interstitial cells responding immediately after PMSG administration. StAR level is then partially attenuated (36–48 h) and rises again in both the theca-interstitial and granulosa cells, responding to the LH surge in ovulatory follicles (56 h). The peak levels of **P45017α** and **P450arom** rise together at 36–52 h (*see* Fig. 5). The expression of both genes is also coordinately extinguished by LH surge (56 h), as shown in Fig. 5. Note that P45017α is exclusively expressed in the theca-interstitial cells and P450arom is restricted to the granulosa cells compartment. In the granulosa cells, expression of P450scc, StAR, and aromatase is clearly limited to those cells confined to ovulatory follicles *(52,55)*. Accordingly, granulosa cells of nonovulatory follicles do not express steroidogenic capacities. This rule does not apply to the pattern of the genes involved in androgen production. At least in the PMSG-induced rat model, P450scc and StAR are elevated in the entire interstitial-theca tissue.

can potentially replace StAR functions in the human placenta *(60)*. Since the ovarian steroids are not important during fetal development of the female, the knockout studies were not very helpful for assessment of the importance of StAR for ovarian steroidogenesis. Examination of the corpus luteum formation provided the first examples stressing a tight correlation between ovarian steroidogenesis and the presence of StAR. Evidently StAR expression is high in steroidogenically active mid-to-late cycle corpora lutea of numerous species, including human *(61)*, bovine *(62)*, mouse *(47)*, and rat *(63)*. Moreover, StAR expression is clearly attenuated concomitantly with onset of luteolysis *(64)*.

Further support for the central role of StAR in ovarian steroidogenesis was recently obtained from studies of the superovulated rat model *(18)*. Figure 5A illustrates the unexpected pattern of StAR expression during follicular development; PMSG/human chorionic gonadotropin (hCG) administration upregulated StAR during two relatively narrow time intervals. The first wave of StAR expression rises immediately after hormone treatment and predominantly occurs in the nonfollicular androgenic interstitial tissue *(18)*. The second burst of StAR expression responds to the LH surge, generating a concerted crescendo of StAR expression in the granulosa and theca interna compartments of the dominant follicles *(18)*. StAR is not expressed in granulosa cells of nonovulatory follicles, which never express P450scc. Therefore, these observations are in accordance with the rationale assuming that a preceding expression of P450scc is mandatory for onset of StAR expression in the cell, which otherwise is not ready to use the functional "service" StAR can provide. However, the surprising lack of StAR expression in the Graafian follicle prior to LH surge indicates that existing P450scc expression is probably a necessary, but not the only, requirement for StAR expression (reviewed in ref. *186*).

StAR patterns have more interesting physiological ramifications. The absence of StAR in the granulosa cells of the Graafian follicle prior to the LH surge suggests that the only source for *de novo* synthesis of androgens serving in the capacity as estrogen precursors are the theca-interstitial cells. However, Fig. 5A shows that prior to hCG treatment (simulating the LH surge), StAR is expressed at submaximal levels (20% of maximum), whereas P45017α and P450arom transcripts soar and provide a proper estrogen output required for the onset of LH surge (Fig. 5B). One should therefore conclude that a submaximal rate of *de novo* progesterone and androgen synthesis, most likely determined by the limited StAR content, is still high enough to support maximal estrogen synthesis. This assumption is reasonably accepted since the serum levels of estrogen are always 2 to 3 orders of magnitude lower than those of progesterone (Fig. 5B). This notion is indeed supported by the fact that, inversely to the levels of estrogen in the cycling rat, serum progesterone is low prior to LH surge (1400 h of proestrus) (*see* Fig. 5B). Then, as a result of LH surge, progesterone level acutely soars six- to sevenfold within less than 2 h of LH release *(65)*. It is highly likely that the dramatic and fast upregulation of StAR expression determines this elevated rate of steroidogenesis.

Finally, what is the relevance of the first rise of StAR expression in the interstitial cells of the prepubertal ovary? Obviously, shortly after PMSG administration there is no need to provide androgen substrate for estrogen production, as that P450arom has not been induced, as yet, in the granulosa cells (Fig. 5A). Instead, we already know that during this early stage of follicular growth the interstitial cells produce mainly nonaromatizable 5α-reduced androgens *(56)*. Therefore, it is worth recollecting a previously proposed notion, advocating the fact that androgens can synergistically potenti-

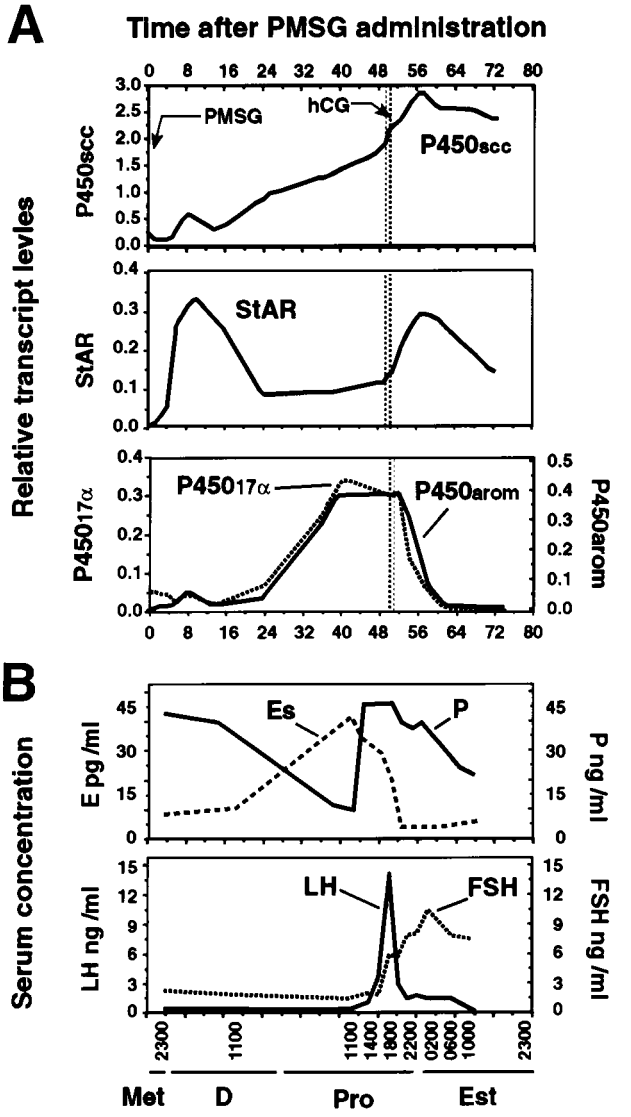

Fig. 5. Time-dependent levels of steroidogenic gene products and steroid hormones during follicular development. **(A)** Messenger RNA levels of the steroidogenic cytochromes and StAR were determined by quantitative RT-PCR using RNA extracts from whole ovaries prepared from PMSG/hCG-treated immature rats *(52)*. Unlike the seemingly gradual increase of P450scc during the maturation process of the follicle, StAR expression is characterized by biphasic peaks: first, an acute response to PMSG administration, and later a robust response to the LH-surge/hCG administration. The spatial characteristics of StAR expression are illustrated in Fig. 4. The levels of P45017α and P450arom transcripts rise prior to the onset of LH surge, in accordance with the production of estrogen, which is essential to generate the LH surge (B). **(B)** Levels of serum concentrations of progesterone (P), estradiol (E), LH, and FSH during a 4-d cycle of the adult rat. This illustration is based on data reported by Park and Mayo *(65)*. The timing of LH surge in the cycling animal was aligned with that of hCG administration in the rat model to demonstrate the well-fitted expression patterns of the steroidogenic genes and the ovarian steroid hormone output.

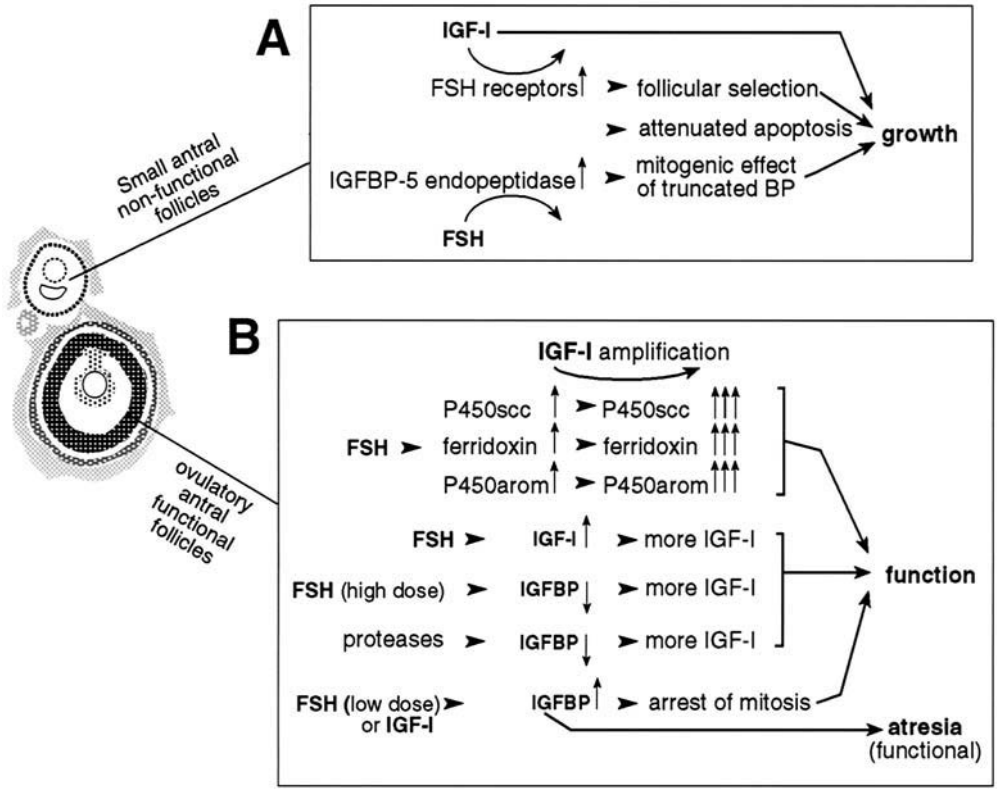

Fig. 6. Potential actions of IGF-1 on growth and differentiation at different phases of follicular development. This figure mingles a variety of observations made on the ovarian IGF-1 complex in the rat, mouse, and porcine experimental paradigms. Therefore, because of potential inconsistencies among the expression patterns of the IGF-1 components in the different species, this hypothetical working model might not be fully compatible with each of the individual paradigms. Additionally, quite a few of the observations made in the ovarian IGF-1 system were inspired by preceding findings noted in the nonovarian systems. Therefore, we took the liberty of making some speculations, borrowing current concepts that have not necessarily been tested or proven relevant for the ovarian system. The central theme emerging from the following summary is focused on the mutual interactions between FSH and IGF-1 action. **(A)** (from top to bottom) Preantral and small-antral follicles are not functional *(55)* and require further growth. In these follicles, IGF-1 may support follicular selection by elevating FSH receptors *(75,76)* and/or increasing the pool of ovulatory follicles by attenuating follicular apoptosis *(159)*. IGF-1 can also directly promote mitosis of granulosa *(78,160)* and theca cells *(79)*. Alternatively, a targeted proteolysis of an IGF-1-binding protein, such as the mouse IGFBP-5 *(161)*, can yield a truncated product, which might have a direct mitogenic effect *(162)*. Specific proteolytic cleavage of such a putative "prohormone" molecule, e.g., the IGFBP-5, is an FSH-inducible event in the rat ovary *(161);* in accordance, the mouse IGFBP-5 is expressed in preantral follicles *(163)*. **(B)** (from top to bottom) In antral follicles destined for ovulation, IGF-1 supports mainly cytodifferentiation, although a limited effect on cell proliferation is still effective *(164)*. IGF-1 affects differentiation by amplifying FSH-induced expression of steroidogenic genes. The latter include the granulosa and thecal P450scc *([85,165];* S. Eimerl and J. Orly, unpublished data), ferridoxin *(84)*, and P450arom *(160,166)*. In the pig cells, FSH also induces IGF-1 production *(167)* to later serve in the capacity of FSH "cohormone." For further assessments, the concentration of FSH was critical for discerning the effects of the FSH on the IGF-1 system. A high dose of FSH prevents sequestration of active IGF-1 by attenuating the level of IGF-1-binding proteins (IGFBPs; *[168–170]*). Even higher IGF-1 levels can be sustained in preovulatory follicles responding in hormone-induced proteolysis of IGFBP by serine- and metallo-proteases *(171–173)*. On the other

ate FSH-induced actions in the granulosa cells *(66,67)*. Although the mechanism of this "nonsubstrate" action of androgens still remains ill-defined, thorough studies have demonstrated that aromatizable, as well as nonaromatizable (5α-reduced), androgens can markedly augment FSH induction of granulosa cell P450scc, P450arom, LH receptors, and prolactin receptors (PRL-Rs) *(67,68)*. It is therefore tempting to speculate that in the prepubertal animal, the early acute induction of StAR in the steroidogenically active interstitial tissue supports folliculogenesis at the onset of first ovulation. Hours later and prior to the first LH surge, a rapid and irreversible loss of ovarian 5α-reductase occurs to allow manifestation of estrogen production in the pubertal ovary *(69)*.

FINE TUNING OF FOLLICULAR FUNCTIONS: THE IGF-1 EXAMPLE

Intraovarian IGF-1 System: Expected Relevance

The enhancing effect of somatomedin-C (IGF-1) on steroidogenesis performed in cultured granulosa cells was first observed over 15 yr ago *(70–72)*. Since then, an ever-growing body of evidence has mushroomed to constitute the intraovarian IGF-1 system, including the ligand, a receptor (type I), binding proteins, and binding-protein protease(s) *(73)*. This complex interaction serves as an autocrine/paracrine mechanism regulating follicular growth and steroidogenesis, as described subsequently. Most of the information suggests that more than any other follicular cell type, the granulosa cells serve as the predominant site of IGF-1 production, reception, sequestration, and action *(73,74)*. The rat and porcine granulosa cell–culture models have been most constructive for many lateral studies, aiming to characterize the various components of the IGF-1 system. Yet it seems somewhat frustrating that attempts to resolve the molecular mechanism underlying the bioactivity of this growth/differentiation factor are still in their infancy. Furthermore, in face of the difficulty of selectively ablating the ovarian IGF-1 system by transgenic technology, there is no compelling evidence to indicate that the impact of IGF-1 on folliculogenesis is indispensable for the ovarian function in vivo. However, targeted disruption of IGF-1 in mice revealed that female infertility associates with lack of puberty owing to arrest of follicular development at the preantral/early antral stage (*[75];* Table 1; Fig. 1). Furthermore, granulosa cells of those IGF-$1^{-/-}$ preantral follicles express fewer FSH receptors *(75)* and therefore resemble the phenotypic characteristics of FSH ablated females *(76)*. Hence, at least in rodents *(77),* the in vivo relevance of IGF-1 for ovarian physiology is fairly substantiated.

◄

hand, low levels of FSH elevate the expression of IGFBPs *(90,168)*. Addition of IGF-1 also triggers upregulation of IGFBPs *(170)*. Two possible consequences could evolve under elevated IGFBP status. The first is increased atresia of antral follicles owing to sequestration of active IGF-1 *(171,172)*. Intriguingly, such atretic follicles later become highly steroidogenic while they develop into the interstitium tissue *(55,174–176)*. The second optional role for IGFBPs stems from an inspiring concept *(177)* demonstrating a direct inhibition of growth elicited by IGFBP-3 interacting with its own putative receptors, independently of IGF-1 action *(178)*. As a result, it is likely that a follicle committed for ovulation can improve its functional performances thanks to a concurrent arrest of mitosis in the granulosa cell layers *(179)*. In support of this notion, we already know that IGFBP-3 is highly expressed in the rat interstitial cells and the corpus luteum *(180),* both of which are nonproliferative tissues and the best steroid hormone producers.

IGF-1 System and Folliculogenesis

Although IGF-1 may act in its own right to promote growth of follicular cells *(78–81),* the most important role of this growth factor appears contingent on the ability to synergize with gonadotropins and to amplify their impact. Figure 6 attempts to integrate the intricate effects of the IGF-1 components on both growth and function of the granulosa and theca-interstitial cells during folliculogenesis. Although the mechanism by which IGF-1 promotes DNA synthesis and cell proliferation *(82)* probably follows that of insulin action *(83),* the mechanism subserving the observed synergism between IGF-1 and gonadotropin-boosted steroidogenesis is not entirely clear. Earlier studies with pig granulosa cells and recent data using rat cells (*[24];* S. Eimerl and J. Orly, unpublished data) suggest that IGF-1 enhances the expression of the steroidogenic genes at the level of mRNA and protein (*[24,84,85];* S. Eimerl and J. Orly, unpublished data). However, the fine details of the effect of IGF-1 on gene transcription are still enigmatic. Recently it was proposed that IGF-1 can stimulate a ligand-independent activity of estrogen receptor *(86,87).* Since estrogen is required for proper follicular performance, it is not unlikely that the addition of IGF-1 to the medium of granulosa cell cultures results in a marked synergistic effect exerted by the cross talk between the IGF-1 and the ER pathways. Additionally, by using IGF-binding protein and neutralizing antibodies against IGF-1, Adashi and colleagues *(24,73)* reached the conclusion that progesterone production by granulosa cells consists, in fact, of a modest intrinsic response to FSH, which is synergistically augmented (two- to fourfold) by the autocrine action of granulosa-made IGF-1 *(88).* To date, this model has not been challenged by any alternative mechanism.

The differences among the experimental conditions used by individual laboratories in the field, as well as species-dependent variations of IGF-1 action, probably made it more difficult to resolve the molecular events associated with IGF-1 synergism with FSH actions. For example, whereas IGF-1 alone substantially elevates the porcine granulosa cell content of the P450scc transcript and protein *(84,85,89),* the rat granulosa cells show hardly any stimulatory effect of IGF-1 on P450scc mRNA levels *(24).* Furthermore, whereas FSH elicited a dramatic increase of P450scc transcript in the rat granulosa cells, the addition of IGF-1 did not synergize at this level at all *(24).* These findings are not coherent with another rat cell model, in which RT-PCR analysis clearly demonstrates a 25-fold synergism between IGF-1 action and FSH-induced P450scc mRNA (S. Eimerl and J. Orly, unpublished data). Figure 7 depicts similar results obtained with forskolin-induced P450scc and P450arom transcripts. Whether the culture conditions (i.e., serum-free vs. serum-containing media, serum coating of the plastic substrate, use of estrogen-primed ovaries, or use of androstenedione-fed cells) may have variably altered the nature of the cellular responses to IGF-1 is yet to be determined.

The (unresolved) Mechanism of IGF-1 Action

As expected, FSH modulates the levels of the IGF-1 components via cAMP as a second messenger *(90,91).* Also, a reciprocal argument was put forward suggesting that IGF-1-augmented steroidogenesis simply results from boosting the FSH-driven cAMP production *(92).* It is highly likely that this perception is an oversimplification of what should be a complex cross-talk mechanism coupling the FSH/cAMP/A kinase and the IGF-1 receptor tyrosine kinase (RTK) signaling pathways *(82).* Circumstantial clues in support of such a potential cross-talk were provided by showing an in vitro

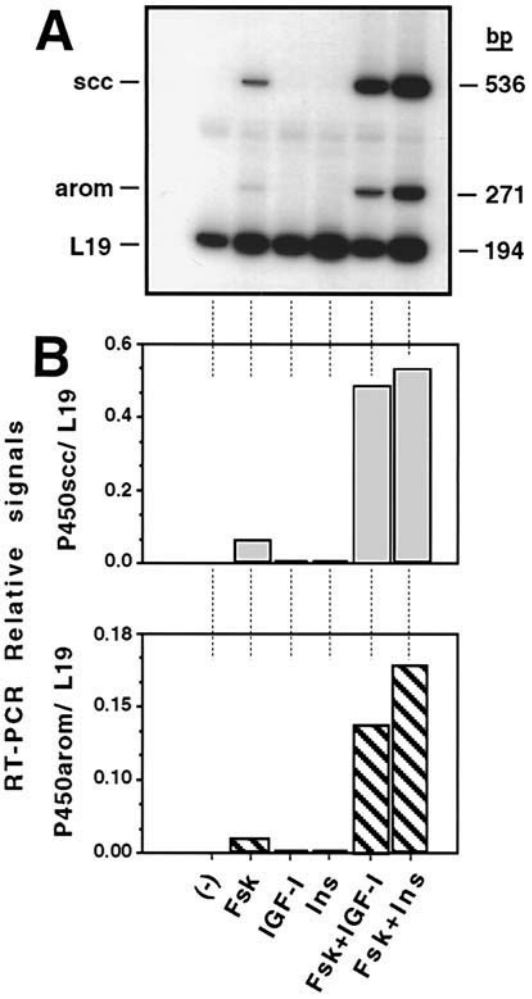

Fig. 7. IGF-1 and insulin markedly augment forskolin-induced accumulation of P450scc and P450arom transcripts in granulosa cells. Rat granulosa cells were prepared from immature, estrogen-primed rat ovaries as previously described *(19)*. Cells were grown on serum-coated plasticware in serum-free DMEM:F-12 nutrient medium *(19)*. One day after seeding, forskolin (Fsk) (10 μ*M*) was added with or without IGF-1 (100 ng/mL) or insulin (Ins) (2 μg/mL). After a 40-h incubation, total RNA was extracted and a quantitative RT-PCR assay *(96)* was applied to assess the relative transcript levels of the P450scc (scc) (536-bp PCR fragment), P450arom (arom) (271 bp), and ribosomal protein L19 (L19) (194 bp). An equivalent of ~5×10^4 cells (50–80 ng of RNA) was used for each mRNA determination. **(A)** An autoradiogram depicting the specific PCR products shows a marked IGF-1 and insulin-mediated amplification (up to 13-fold) of the forskolin-induced gene products. **(B)** Phosphorimager quantification *(18)* of the results shown in (A). The level of the ribosomal protein L19 PCR product, which is not affected by hormone treatments (A), served as quantitative normalization of the target-gene products. Note a characteristic lack of IGF-1 and insulin effect when added alone to the rat cells. At 2 μg/mL, insulin probably acted via the IGF-1 receptors since that moderately lower insulin concentration (700 ng/mL) resulted in a marked loss of the hormone effect (S. Eimerl and J. Orly, unpublished data).

inhibition of FSH-induced granulosa cell transcription of P450scc and P450arom by tyrosine kinase inhibitor, tyrphostin AG18 (also known as tyrphostin A23) *(19,96)*. These findings strongly suggest that tyrosine kinase–dependent events, potentially involving IGF-1R or cytosolic protein tyrosine kinases *(97),* are necessary for the expression of the steroidogenic cytochromes. On the other hand, in luteinizing granulosa cells known to express the highest steroidogenic capacity, IGF-1 responsiveness becomes redundant and is lost *(93)* once their regulation of P450scc turns out to be cAMP-independent *(94,95)*.

No less intriguing is the question, What potential promoter elements of the steroidogenic genes could respond to IGF-1 action downstream of the signal transduction events? To date, only one example has successfully addressed this question. Using pig granulosa cells, Urban and colleagues *(98)* identified a GC-rich domain of the porcine P450scc gene that imparts IGF-1 regulation through a distinct *trans*-acting protein complex. The relevance of these findings for the rodent cell system is currently under investigation (S. Eimerl and J. Orly, unpublished results); however, it is not unlikely that the rodent cells fundamentally differ from their pig counterparts, in which IGF-1 alone can induce P450scc expression *(85,89)* and the nominal synergism between FSH and IGF-1 is less prominent *(89),* or even does not exist at the promoter activity *(98)* and mRNA/protein levels *(85,89)*.

FOLLICULOGENESIS: THE KNOCKOUT LESSON

In recent years, targeted gene disruption technology has provided a powerful approach to recover critical checkpoints determining follicular growth and function *(99)*. Figure 1 and Table 1 extract the hallmark consequences of each mutation. Table 1 furnishes additional information regarding the effect of the disrupted genes on male fertility. This chapter does not cover those knockout mice models that led to selective male infertility (listed in Table 1), or female sterility inflicted owing to defective uterine response to implantation (e.g., interleukin 11 receptor, or leukemia inhibitory factor [LIF]).

Gametogenesis: Dazla and atm Genes

The creation of the *Dazla*- and *atm*-deficient mice models *(100,101)* represents a typical example of how rewarding the gene-targeting approach can be, even if not necessarily intended for the study of reproduction. *Dazla* encodes a cytoplasmic protein, which is probably engaged in the control of mRNA fate and function. A completely different phenotypic background was observed in female mice lacking the *atm* gene product. In humans, mutated *ATM* causes the ataxia telangiectasia disorder with pleiotrophic phenotyes, including neuronal degeneration, immune dysfunction, premature aging, and increased cancer risk. Later it was revealed that *ATM* and its mouse *atm* homolog are putative lipid- or protein-kinase involved in cell-cycle control and DNA repair *(102)*. Yet, no matter how unrelated these two mutations may seem to be, both *Dazla*- and *atm*-deficient females were infertile, secondary to a total loss of germ cells, and their prepubertal and adult ovaries did not contain follicular structures *(100,101)*. Although *Dazla* is expressed in some of the somatic cells of adult ovary, the high expression of the protein in the cytosol of fetal oogonia suggests that *Dazla* is essential for survival and differentiation of germ cells *(100)*. Together, the loss of *Dazla* and

atm serves best as an unambiguous reminder that the ovarian somatic cells require the presence of a mature oocyte to organize a new follicle around it.

Early Phases of Follicular Organization

Once gametes reach the fetal ovary and properly develop, they attract the somatic cells to form the primordial follicle, in which a single layer of flattened granulosa cells organizes around it, as illustrated in Fig. 1. The next phase of follicular organization ensues when a unilaminar layer of cuboidal granulosa cells (primary follicle) is observable. The third step of follicular development, which is still independent of gonadotropin action (for a review *see* ref. *103*), results from a limited proliferation of the granulosa cells to form up to four layers in the secondary follicle (Fig. 1). Critical mutations and gene manipulations in mice have provided convincing evidence to support the notion that a dynamic cross talk between the oocyte and the somatic cells is mediated by growth factors and their cognate receptors to control these phases of follicular development critically.

c-*kit*

The situation in which the oocyte presents an RTK, c-*kit,* and the somatic cells produce its cognate ligand KL *(104)* demonstrates the potential effect of the ovarian stroma on oocyte growth and early folliculogenesis. The loci encoding the c-*kit* receptor and its KL ligand are known as the *White spotting (W)* and *Steel (Sl),* respectively. Mutations at these loci cause infertility owing to a variety of deficiencies. For example, in the most severe phenotype observed for a double mutation at *W* and *Sl,* the primordial germ cells are generated normally but fail to populate the gonad *(105)* since during gonadal formation, the c-*kit* receptor is expressed in the premordial germ cells, whereas KL is expressed along the path of migration of the primordial germ cells to the gonadal ridge and into the fetal gonad *(106).* More relevant to our cause is one of the deleterious mutations of the *Steel* locus encoding a defective KL ligand; *Sl/Sl*^{t} mice are infertile owing to growth arrest of the cuboidal granulosa cells surrounding the growing oocyte in the primary follicle *(107).* Note that elegant analysis of these *Sl/Sl*^{t} follicles indicated that the arrest of follicular growth was not owing to an intrinsic defect in the granulosa cells, but rather defective KL production originating from the nonfollicular stromal cells *(107).*

Growth Differentiation Factor-9

Ablation of the growth differentiation factor-9 (GDF-9) provided a reciprocal example of an oocyte-derived growth factor, which modulates the somatic granulosa cell response in vivo *(108).* GDF-9 is a member of the transforming growth factor β family, which is synthesized in primary follicle oocytes, as well as spermatocytes and some nongonadal tissues *(109).* The loss of GDF-9 arrests follicular growth at the one-layer primary follicle *(108),* which is phenotypically similar to the infertile ovaries of the *Sl/Sl*^{t} KL mutant females. Collectively the GDF-9 and KL-deficient mouse models suggest that formation of the secondary follicle requires a complex signaling circuits among the ovarian mesenchymal cells (stroma), gametes, and epithelial-derived granulosa cells. Since chimera tests have shown that the oocytes and granulosa cells of *Sl/Sl*^{t} follicles are perfectly normal *(107)* but fail to function in the context of a defective stromal tissue, it is tempting to speculate that secretion of stromal KL upregulates GDF-9

synthesis by the oocyte, which, in turn, may stimulate the proliferation of the primary granulosa cells. It remains for future studies to address such a potential working hypothesis.

Tertiary Follicle Formation

The transition of the four-layer secondary follicles to the preantral/small antral stage is the first phase of follicular development that requires FSH. The variety of disrupted genes that can stall this phase transition testify not only for the complexity of the associated events, but also the importance of this developmental checkpoint.

Cyclin D2

Sterility of cyclin D2–deficient female mice *(110)* is an enlightening example for the unambiguous role of FSH as a trophic hormone, rather than inducer of granulosa cell differentiation. Activation of cyclin-dependent kinases (CDKs) regulates progression through critical steps of the cell cycle. The G1 CDKs, which can be distinguished into two groups, CDK4/CDK2 and CDK6, are composed of the CDK catalytic subunit and a regulatory subunit–designated cyclin. These G1 CDKs integrate mitogenic and antimitogenic signals that regulate progression through G1 into the DNA synthetic phase (S) until a commitment to continue the cell cycle can no longer be compromised *(111)*. The activation of CDKs is subjected to multiple levels of regulation, such as synthesis and degradation of cyclins, assembly or inhibition of assembly of the cyclins with the CDKs (see under "$p27^{Kip1}$"), and activation of the complex and modulation of its ability to phosphorylate downstream of substrates (reviewed in refs. *112* and *113*).

Key regulators of G1 progression in mammalian cells include no less than three D-type cyclins (D1–D3). However, targeted disruption of cyclin D2 demonstrated that these three D-type cyclins are not functionally redundant in the ovarian tissue. Thus, the cyclin D2–deficient females are sterile owing to the inability of the granulosa cells in preantral follicles to express their growth response to FSH stimulus *(110)*. This notion was supported by studies showing that a single injection of FSH to normal mice resulted in an increase of cyclin D2 mRNA (not D1 or D3), which was shown to be a cAMP/PKA signaling event *(110)*. Yet, other than growth retardation, in vitro tests of the knockout follicles proved that the functional integrity of the cyclin D2-deficient granulosa cells remained unharmed, as well as the oocytes recovered from the growth-arrested follicles. Whereas follicles from cyclin $D2^{-/-}$ animals did not exceed the preantral stage, administration of FSH resulted in the formation of bizarre antral follicles with much fewer granulosa cells and only one layer of cumular cells. These follicles were, however, fully functional and further administration of hCG even showed luteinization of the follicles that never ovulated *(110)*. In sum, these observations provided the first unequivocal evidence that a critical threshold number of granulosa cells is certainly essential for the normal progress of follicular growth and ovulation, presumably due to production of a granulosa cell factor (as yet unknown) controlling an event in the ovulatory process *(183)*.

Follicle-Stimulating Hormone

In light of the events described for cyclin $D2^{-/-}$ ovaries, it is not surprising that infertility secondary to a similar phenotype was observed in animals with disrupted FSHβ, or deficient in the pituitary glycoprotein hormone α-subunit *(76,114)*. In this

regard, it could have been of interest to complete this series of null animals with an LHβ-deficient mouse (also see NGFI-A-deficient mice). Such an animal model could confirm, or negate, the current notion that low chronic LH levels are essential to accompany the FSH action promoting growth at earlier stages of follicular development *(26,28)*.

Insulin-Like Growth Factor

As already discussed, the role of IGF-1 as an intraovarian "fine-tuning" modulator of FSH action is supported by the IGF-$1^{-/-}$ mouse model. These mutant mice *(115)* are particularly valuable in light of the fact that IGF-1 receptor–deficient mice die at birth *(116)*. A new potential aspect of IGF-1 action was revealed by *in situ* hybridization studies *(117)* demonstrating the exact colocalization of FSH receptors (FSHR) and IGF-1 in a subset of follicles of the wild-type ovary. Also, a 50% reduction in the content of FSHR was observed in the IGF-1 null ovaries *(75)*. It was therefore suggested that normally the local production of IGF-1 is meant to support a proper level of FSHR expression in the activated cohort of developing follicles, which, in turn, facilitates a threshold level of P450arom and estrogen production essential for follicular growth beyond the early antral stage *(75)*. An unequivocal confirmation of such a hypothesis must await further development of the targeted gene technology to allow a selective ablation of IGF-1 at the level of the ovary.

Estrogen Receptor-α

The disruption of the mouse estrogen receptor-α (ER) gene (*ERKO*) causes female infertility owing to lack of ovulation *(118)*. Recent studies have shown that the ERKO adult ovary contains preantral and antral follicles expressing high levels of LH receptors, some of which develop to large hemorrhagic cysts *(184)*. Together with the fact that the circulating levels of LH and androgens are high in ERKO females, these features suggest that sterility of these animals is secondary to a hyperandrogenic response of the ovary and failure to ovulate *(184)*.

Loss of Mos

Earlier studies have suggested that in *Xenopus* the c-*mos* protooncogene product (Mos) is essential for the initiation of oocyte maturation, progression from meiosis I to meiosis II, and the second meiotic metaphase arrest of oocytes (*see* ref. *119)*. However, the loss of Mos in knockout mice pinpointed the critical physiological relevance of Mos better than any previous approach. In mice, the absence of Mos results in untimely completion of meiosis; none of the earlier events attributed to Mos action was hampered. Consequently, infertility of the c-*mos*-deficient females is secondary to the failure of the mature oocyte to arrest at the second metaphase of meiosis *(119,120)*. As a result, extrusion of the second polar body, and parthenogenetic development occurred in follicles–enclosed oocytes. Further symptoms include high frequency of ovarian teratomas and cyst formation. The major role of Mos is therefore to prevent the parthenogenetic activation of unfertilized eggs.

Large Antral and Graafian Follicle

The transition to small-antral and further development to Graafian follicle can be arrested by the loss of rather unexpected gene products, the activin receptor type II (ActRII) and connexin 37 (Cx37).

ActRcII-, Inhibin-, and Activin-Deficient Mice

In light of the fact that inhibin-null mice developed ovarian tumors at the age of 5 wk *(121)* and activin$^{-/-}$ mice died shortly after birth *(122),* the creation of the ActRcII null mice remained the only potential strategy allowing the question of what is the relevance of activins for follicular development to be addressed *(123).* The elegant in vivo and in vitro studies showing that activin can promote follicular assembly and growth *(124–126)* raised much expectation in this regard. Surprisingly, ActRcII-deficient mice were not a phenocopy of the activin-deficient mice. Moreover, these female mice did not testify in support of any significant intraovarian role for activin since normal follicular development commenced until the preovulatory stage. Instead, a dramatic suppression of FSH production was observed in the pituitary gonadotrophs, whereas the level of LH remained unaffected *(123).* Consequently, the lack of FSH caused infertility probably owing to insufficient preparation of the granulosa cells for the LH surge. Unlike the FSH-deficient mice, the lower FSH level (~35% of wild type) in the serum of the ActRcII$^{-/-}$ animals was apparently sufficient for supporting follicular growth requirements, but apparently not for the final follicular performances and ovulation. Like IGF-1, future tissue-targeted ablation of the ActRcII in the ovary will, hopefully, provide conclusive evidence for the role of activins in follicular development.

Connexin 37

A conceptually different interruption of ovarian functions was revealed in mice lacking the oocyte-specific gap-junction protein, Cx37 *(127).* Gap junctions are intercellular channels composed of connexins, a family of more than a dozen proteins *(128).* Cx37 gap junctions physically connect the oocyte with the nearest surrounding somatic cells, the cumular corona radiata. Such junctions allow the diffusional movement of ions, metabolites, and signaling molecules such as cAMP *(129).* Correlated with the formation of the zona pellucida, anti-Cx37 antibodies labeled the surface of oocyte from secondary follicles throughout the formation of large antral ones, but not in primordial follicles *(127).* The loss of Cx37 in knockout mice did not affect the staining pattern of connexin 43, which is the specific gap-junction protein dominant in the somatic cells of the follicle. Electron microscopy examination revealed that in the Cx37$^{-/-}$ females, the corona radiata cells still extended processes through the zona pellucida to reach and form adherent junctions with the oocyte membrane, but that gap junctions were absent *(127).*

Three follicular abnormalities probably underlay Cx37$^{-/-}$ female infertility. First, adult Cx37$^{-/-}$ follicles never complete their growth to attain the Graafian stage. Second, the follicles fail to ovulate but develop into small and apparently functional corpora lutea. Consistent with earlier studies showing that surgical removal of oocytes form rabbit Graafian follicles causes morphological luteinization of the ovum-freed follicles, the premature luteinization in Cx37$^{-/-}$ ovaries suggests that, normally, the junctional channels between the oocyte and the somatic cells mediate the transfer of an inhibitory substance(s) that probably prevents a premature luteinization of the granulosa cells. Such putative inhibition of corpus luteum formation is expected to be relieved in the wild-type ovary by the time the cumulus cells' response to LH surge and the loss of gap-junction communication allows resumption of meiosis and proper ovulation *(129).* Third, development of mutant oocytes is arrested and the cells remain smaller and cannot exhibit germinal vesicle breakdown (meiotically incompetent). It is not unlikely,

therefore, that a reciprocal junctional transfer of effector(s) originating from the somatic cells of the follicle may promote meiotic competence of the oocyte.

LH-Responsive Luteinizing Follicle

The LH surge generates a cascade of events *(95)* paving the road for ovulation. The high dose of LH also determines the onset of the luteinization process, rendering the granulosa cell steroidogenic capacities independent of cAMP action *(95)*. Several knockout mouse models highlight the critical checkpoints that permit the proper preparation of the follicle toward extrusion of the egg.

NGFI-A (Egr-1)

The working hypothesis that guided Lee and colleagues *(130)* in their attempts to ablate NGFI-A assumed that this immediate-early transcription factor (also known as Egr-1, zif/268, or Krox-24) would specifically attenuate LH synthesis, and indeed this occurred. It was shown that the LHβ promoter contained a conserved NGFI-A site required for synergistic activation by NGFI-A and steroidogenic factor-1 (SF-1). However, targeting NGFI-A remained the only potential strategy to specifically reduce LHβ since earlier studies of SF-1-deficient mice *(131–133)* had shown that ablation of this critical transcription factor did not leave much to study in the absence of the gonads and the adrenals of homozygous offspring, which died shortly after birth.

As expected, failure of estrous cyclicity secondary to a severe, but not an absolute, LHβ deficiency resulted in female sterility. The residual LH levels in the male allowed normal testicular function. In the mutant ovary, but for a marked absence of corpora lutea indicative of lack of LH surge and ovulation, perfectly normal follicles were observed at all stages of development. These findings confirmed that the chronic presence of LH, which is required for development of the dominant follicle to attain the Graafian stage, necessitates only very low levels of the hormone, the "leaky" expression of which was still available in the NGFI-$A^{-/-}$ mice.

PRL Receptor

The null mutation of the PRL receptor (PRL-R) *(134)* caused a wide range of reproductive abnormalities, including reduced mammary gland development, arrest of preimplantation development, and a complete failure of embryonic implantation. In addition, the high number of eggs still containing germinal vesicles after ovulation suggested that PRL-R is important for oocyte maturation and GVB. Interestingly, unlike the dramatic arrest of follicular development coupled with the gamete incompetence observed in the $Cx37^{-/-}$ ovary, the PRL-$R^{-/-}$ follicles develop normally down to ovulation of many eggs still containing germinal vesicles. Two conclusions can therefore be drawn from these observations: (1) follicular development and ovulation are not necessarily functionally coupled to resumption of meiosis in the oocyte; and (2) it is not unlikely that PRL can directly interact with the oocyte to mediate GVB, at least in a permissive fashion. The latter notion is strengthened by earlier studies showing that PRL increased the rate of GVB and subsequent fertilization of immature eggs recovered from wild-type mouse ovaries *(135)*.

Ovulation

The loss of three gene products caused an arrest of the very final events associated with follicular rupture and extrusion of the oocyte. These are cyclooxygenase-2 (COX-

2), CCAAT/enhancer-binding protein β (C/EBPβ), and the progesterone receptor (PR). These knockout models were grouped owing to the fact that normally the granulosa cell expression of the three genes peaks precisely 3–6 after the onset of the LH surge. At least for the C/EBPβ- and PR-deficient ovaries, the oocytes initiate resumption of meiosis observed by GVB, and the cumulus layers mucify properly. It is not clear whether similar characteristics were also observable for the *COX-2*-deficient mice.

Cyclooxygenase-2

COX-2 is the hormone-inducible isoform of COX (also known as prostaglandin endoperoxide H synthase-2, PGHS-2 *(136*), which catalyzes the first and rate-limiting step in the conversion of arachidonic acid to the prostaglandins (PGs), thromboxane, and prostacyclin. PGHS-2 is induced in granulosa cells of the periovulatory follicles by LH surge *(137),* and its products are long-known as local modulators essential for ovulation *(138)*. It was therefore more than expected that PGHS-2-deficient females would be infertile *(139)* with virtually identical symptoms as the NGFI-A$^{-/-}$ mice, i.e., lacking corpora lutea.

Why is PGHS-2 expression so important for ovulation? The exact role of PGs in ovulation still remains a mystery, but interestingly enough, the tales of the PGHS-2- and C/EBPβ-deficient mice mutants have provided some clues when combined with recent new information on PGHS-2 expression in various mammals. In the rat, LH induces a transient expression wave of PGHS-2 mRNA and protein, which peaks at 4 h post-LH surge (or hCG administration in the rat model); PGHS-2 mRNA and protein disappear 3 h later *(140)*. Ovulation occurs in this species 14 h after LH surge, i.e., 10 h after PGHS-2 levels have peaked. Recently, using the hCG-induced ovulation model for large animals, Sirois and Doré *(141)* showed that in the cow and mare, the post-hCG rise of PGHS-2 expression is remarkably delayed (18 and 30 h, respectively) when compared to the rat schedule. Yet, an exact time gap of 10 h remains constant between a follicular rupture and the granulosa cell expression of PGHS-2 *(141)*. This result strongly suggests that the timing of PGHS-2 expression "sounds the alarm" for ovulation occurring exactly 10 h after LH surge, invariably the length of the ovulatory process across species *(142)*. No less intriguing is the question, What species-specific mechanisms determine the observed time differences required for the induction of PGHS-2 by the same LH agonist? Moreover, do the transient characteristics of PGHS-2 expression, which lasts for only 3–6 h, have any physiological significance? The possibly affirmative answer to this question comes from quite an unexpected knockout model of the C/EBPβ-deficient mice.

CCAAT/Enhancer-Binding Protein β

The C/EBPβ transcription factor was first associated with the regulation of ovarian function when analysis of the PGHS-2 promoter revealed a functional recognition element for this factor *(143)*. Therefore, considering the importance of PGs for induction of ovulation described previously, it did not seem surprising that C/EBPβ-deficient female mice were sterile owing to being unable to ovulate *(144)*. However, cells lacking C/EBPβ did not lose PGHS-2 but, rather, failed to extinguish the expression of this gene. Consequently, PGHS-2 expression turned constitutively active *(144)*. The same phenomenon was repeated when the expression level of P450arom was examined; P450arom remained highly expressed instead of being turned off by the LH surge (*see*

under "P450scc"). These results proposed that (1) the transient nature of PGHS-2 expression is critically required *per se,* or as part of a plausible programmed sequence of events leading to proper ovulation; and (2) it seems likely that C/EBPβ is dispensable for the upregulation of PGHS-2. The latter conclusion eventually conformed with later studies showing that in the context of an intact PGHS-2 promoter region, an E-box binding element, rather than the upstream C/EBPβ site, is critical for hormonal activation of PGHS-2 *(145).* In light of these understandings, an alternative explanation for the role of C/EBPβ deserves closer examination. For example, it is not unlikely that a truncated form of *C/EBP*β*,* LIP, could function as a transcriptional repressor responsible for the observed downregulation of PGS-2 and P450arom in normal animals.

Progesterone Receptor

Apart from PGHS-2 and C/EBPβ, the rise and fall of the granulosa cell content of PR *(65,146)* is consistent with the notion that "a transient expression" is the critical name of a game applicable for a battery of post LH surge-induced genes. Unlike other tissues, the induction of PR in the ovarian follicle responding to LH is unlikely to be mediated by the ER responsive element in the PR promoter since follicular estradiol production precipitously drops before PR expression is upregulated following the LH surge *(65).* Therefore, the observed sterility secondary to lack of ovulation in the PR-deficient female mice *(147)* probably relates to net deleterious consequences of PR deficiency. The mechanism of the paracrine action of progesterone in the post-LH follicle is not clear. Yet, former studies have proposed that progesterone can modulate proteolytic enzymes involved in follicular rupture *(148),* which conforms with unovulation in $PR^{-/-}$ females. Whatever the mechanism of progesterone may be, it is clear that the transient peak of the follicular PR must be a critical component of the programmed events leading to ovulation and corpus luteum formation.

Zonaless Eggs: The Lost Zygote

The onset of the synthesis of zona pellucida by the mammalian oocyte commences on transition of the primordial follicle to form the primary and secondary structure (Fig. 1). The signaling cues for this transition turn on the oocyte expression of three genes encoding the zona pellucida glycoproteins, ZP1–ZP3. Ablation of ZP3 *(149)* is enough to inflict sterility of the homozygous females. $Zp3^{-/-}$ females have follicles with apparently normal germinal-vesicle oocytes but the missing zona matrix causes a disorganized corona radiata *(149).* Although electron microscopy or functional gap-junction assays were not provided, it is hard to believe that cell-cell junctional communication between the zona-free oocyte and the somatic cells was impaired since oocyte development and, hence, follicular development and ovulation proceeded normally. However, a marked loss of eggs and embryos observed in the oviduct ampulla probably accounted for the sterility of the animals. The following hypothesis may provide a plausible explanation for the disappearing gametes and zygotes. Earlier studies have shown that zona-free eggs can be fertilized in vitro and grown to blastocyst stage before a successful transfer to foster pseudopregnant mothers developed to a normal pregnancy and live birth *(150).* However, if zona-free embryos at the one-to-four cell stage were returned to the oviduct of a foster mother in a similar fashion, they adhered to the epithelium of the oviduct and no further cleavage of the immobilized embryo occurred; thereafter, the embryos disappeared within 24 h of transfer *(150).* It is therefore assumed

that a similar fate led to the absorption of the zona-free *Zp3*$^{-/-}$ embryos by the ampullar wall, reinforcing the notion that zona hatching should not occur before the embryo reaches the uterine lumen.

Corpus Luteum Formation: Is It a Growth and Differentiation Wrestling Match?

p27^{Kip1}

The p27^{Kip1}-deficient ovary *(151,152)* provides an impressive example showing that in order to allow the development of the corpus luteum, an efficient inhibition of the cell cycle must be operative to arrest proliferation and thereby encourage terminally differentiated cells to become highly steroidogenic. In other words, the p27^{Kip1}-deficient follicles may support the notion that in a tissue accommodating alternating growth processes replaced by differentiation, specific measures must be taken in order to relieve existing growth commitments that oppose the cellular switch to a terminally differentiative state.

Specifically, p27^{Kip1} belongs to the Cip/Kips family of proteins that act as negative regulators of G1 CDKs *(112)*. In contrast to the other family of inhibitors, Inks, which specifically target the CDK4 and CDK6 to prevent their assembly with their cyclins, the Cip/Kips proteins bind to any preformed cyclin-CDK complexes and either prevent their activation by the CDK-activating kinase or inhibit their kinase activity *(112)*. Other members of the Cip/Kip family are known as p21 and p57. By creating a mouse line bearing a disrupted kinase-inhibitory domain of the p27^{Kip1} protein *(151)*, a larger mouse was obtained without any increase of the growth hormone/IGF-1 hormonal system. Besides the impairment of luteal cell differentiation in this p27$^{Kip1-/-}$ ovary, a disordered estrous cycle was detected, indicative of prolonged diestrus–estrus phases. However, ovulation and fertilization were normal and embryonic day 3.5 morula-embryos could be transferred to the recipient mother and carried to term. Transfer to a foster mother was necessary since the p27^{Kip1} null females could not sustain pregnancy, apparently owing to a total lack of corpora lutea. Elegant examination of p27 expression and BrdU incorporation into mitotic nuclei of wild-type ovaries showed dramatic reciprocal patterns; low mitotic activity in the CL cells colocalized with high expression of p27, and vice versa, low p27 content was typical for dividing granulosa cells in growing follicles *(151)*. Thus, these results certainly merit the speculation proposing that "p27 acts as a true regulator of growth by exerting its actions on the decision of a cell either to proliferate, or to withdraw from the cell cycle" *(151)* and become devoted for the making of steroid hormones, which are mandatory to maintain pregnancy in mammals.

PGF Receptor: The Corpus Luteum Dies Hard

Earlier studies proposed that prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) is implicated in ovulation, luteolysis, and parturition *(153)*. Once again, the knockout lesson is much appreciated for its sharp focus on the critical relevance of the action of $PGF_{2\alpha}$ in mice. In homozygous females carrying the disrupted gene for the prostaglandin F receptor (PGFR), ovulation, fertilization, and implantation occurred normally *(154)*. These surprising observations suggested that $PGF_{2\alpha}$ is not critically involved in any earlier event until the time of parturition, when PGFR-deficient females failed to induce labor. The reason for this failure of parturition was even more unexpected.

It has been long believed that parturition in mammals is largely controlled by steroid hormones, prostaglandins, and oxytocin *(153)*. It is also known that progesterone can downregulate the expression of oxytocin receptors in rat myometrium *(155)*. These observations seemed physiologically compatible with the fact that parturition is preceded by a decline of progesterone concentration in the maternal plasma *(153)*, and a concomitant increase of oxytocin receptors and uterine contractility *(155)*. According to earlier understandings, it was assumed that $PGF_{2\alpha}$ acts in the uterine tissue downstream of oxytocin, which triggers uterine production of PG *(156)*. However, administration of oxytocin to PGFR-deficient mice at term did not result in induced uterine contractility, as evidently happens in wild-type mice *(154)*. Eventually, it was clearly shown that the critical site where the PGF receptors are required for normal induction of labor is the ovarian corpus luteum. This hypothesis was proved correct when 19-d pregnant mutant females were ovariectomized and consequently delivered their pups alive after 24 h *(154)*. Therefore, it was concluded that sustained production of progesterone in the $PGFR^{-/-}$ corpus luteum failing to undergo a typical $PGF_{2\alpha}$-induced luteolysis was the direct cause of arrest of labor in the PGFR-deficient females.

An even more confusing epilog is the fact that suppression of oxytocin receptors could not be the true cause for the observed lack of parturition in the $PGFR^{-/-}$ mice. At least in mice, the alleged requirement for oxytocin as an inducer of parturition was seriously challenged by a recent generation of oxytocin-deficient females, which are perfectly fertile and deliver normally *(157)*. Therefore, it is highly likely that the constitutive secretion of progesterone from the $PGFR^{-/-}$ corpus luteum inhibits parturition by a mechanism that does not necessarily involve oxytocin action.

SUMMARY

The collection of null mutations capable of compromising murine reproduction makes us realize three important lessons. First, the knockout mouse models stress vividly how different the male and female gonads can be with respect to their vulnerability to deleterious gene disruptions. Although I did not discuss aspects of male infertility, Table 1 lists no less than 14 null mutations that inflict female sterility without affecting the male. Conversely, less disrupted genes selectively cause male infertility without affecting female reproduction. Only three mutations caused both male and female infertility in *Dazla-*, *atm-*, and IGF-1-deficient mice. It is likely that more null mutations will provide enlightening information to determine how far apart the gonads divert while striving to properly produce fertilizable female and male gametes ready to form new life.

Second, it is almost frightening how powerful the knockout approach can be to discover new insights and disclose unknown roles of essential genes involved in the making of the female ovum. The meaning behind the dry facts is that scientists should be cautious in using their ability to mold the quality of living creatures.

Third, as stressed throughout this chapter, some of the more impressive knockout examples reinforce the fundamental importance of viewing the molecular basis of ovarian function through the prism of growth and differentiation processes. The molecular wrestling between the two cellular fates has inspired our current understanding of normal- and cancer-cell biology. It also provides ample incentives and instrumental tools to further study ovarian physiology from this perspective.

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13 Regulation of Inhibin Subunit Gene Expression by Gonadotropins and cAMP in Ovarian Granulosa Cells

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Contents

INTRODUCTION

During the mammalian reproductive cycle, the ovary undergoes continuous morphological and biochemical changes that result in the production of mature oocytes and the timed secretion of steroid and polypeptide hormones. The cyclical proliferation of specific cell types, followed by their differentiation, is essential for normal ovarian function. These cell proliferation and differentiation events are controlled by endocrine factors, such as the pituitary gonadotropins, and by paracrine factors produced within the ovary. Among the regulatory factors produced within the ovary are the related polypeptide hormones inhibin and activin. These dimeric proteins were initially isolated as endocrine regulators of pituitary follicle-stimulating hormone (FSH) secretion, but they also function as intraovarian regulatory factors. While the inhibin and activin

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subunit genes are under complex multihormonal regulation, a predominant aspect of this regulation is exerted by the pituitary gonadotropins FSH and luteinizing hormone (LH).

This chapter considers ovarian follicular development and inhibin gene regulation during the rodent estrous cycle. In the first part of this chapter, we review selected aspects of ovarian physiology, ovarian-specific gene expression, and the signal transduction pathways and transcription factors by which the pituitary gonadotropins regulate ovarian gene expression. With this as a foundation, we then consider the inhibin and activin subunit genes and their regulation in ovarian granulosa cells, focusing on recent mechanistic studies of how the gonadotropins modulate inhibin subunit gene expression.

OVARIAN FOLLICULAR DEVELOPMENT IN THE RAT

The ovarian follicle is a complex structure that includes the oocyte, as well as somatic granulosa and thecal cells that support maturation of the oocyte. The earliest, or primordial, follicles are composed of a single layer of granulosa cells surrounding the oocyte and a basement membrane enveloping the follicle. Some primordial follicles undergo growth earlier than others, and during this initial period of growth the oocyte enlarges to its maximal size to form a primary follicle, which still consists of a single granulosa cell layer. Subsequent proliferation of the granulosa cells to form multiple layers results in the formation of a secondary follicle, and at this stage the presumptive theca cell layer is formed around the follicle *(1)*. The secondary follicle enlarges to form a preantral follicle that now has a large number of granulosa cell layers surrounding the oocyte and a more defined theca layer surrounding the basement lamina. Once follicles have reached the preantral stage, fluid-filled spaces appear within the granulosa cell layer, presumably owing to the selective filtration of thecal blood through a molecular sieve. These individual cavities ultimately coalesce into one large fluid-filled cavity within the follicle, called the *antrum,* and follicles at this stage of maturation are termed *antral follicles*. At any time during the reproductive cycle, within the ovary a mixed population of follicles at these different stages of growth and maturation exists. A comprehensive review of follicular growth and maturation can be found in ref. *2*.

The prolonged secondary FSH surge in the morning of estrus recruits a group of antral follicles into a phase of rapid growth and differentiation *(3,4)*. The granulosa cells within recruited follicles divide rapidly, increasing the size of the granulosa cell layer surrounding the antrum, which also enlarges owing to continued filtration of thecal blood. Concomitant with the increase in size of these antral follicles, the granulosa cells undergo differentiation and become partitioned into two distinct compartments within the follicle. A few layers of granulosa cells surround the oocyte, and these cumulus cells connect the oocyte to the majority of the granulosa cells, the mural granulosa cells, which form a layer surrounding the antrum. Large antral follicles reach preovulatory status on proestrus afternoon and release the oocyte with its surrounding cumulus cells in response to the preovulatory surge of LH. Subsequent to ovulation, the granulosa and thecal cells of the follicle further differentiate to form the luteal cells of the corpus luteum. Figure 1 presents a schematic representation of follicular development.

GONADOTROPIN RECEPTOR EXPRESSION AND SIGNALING

The maturation, ovulation, and luteinization of ovarian follicles is tightly regulated by the actions of the pituitary gonadotropins FSH and LH. The ability of ovarian cells

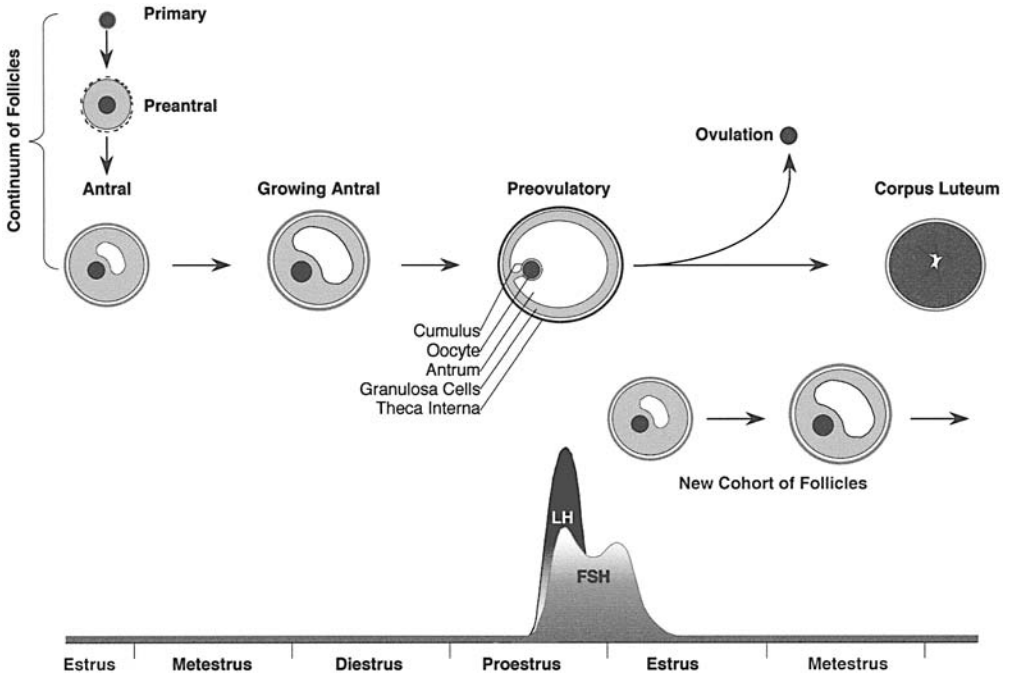

Fig. 1. Maturation and ovulation of ovarian follicles during the rat estrous cycle. **(Top)** Schematic representation of follicular architecture and follicular maturation and ovulation; **(Bottom)** Serum gonadotropin profiles during the 4-d rat estrous cycle.

to respond to gonadotropin stimulation is therefore a key determinant of ovarian function, and gonadotropin receptor expression and signal transduction have been intensively investigated. The gonadotropin receptors belong to the seven-transmembrane domain family of G-protein-coupled receptors *(5–7)*. The FSH and LH receptors, while having similar overall structures and sharing substantial sequence homology, confer specific FSH and LH or human chorionic gonadotropin (hCG) binding to target cells.

The expression patterns of the gonadotropin receptors in the ovary have been studied in substantial detail. Northern blot analysis demonstrates that multiple transcripts encoding these receptors are expressed in the rat ovary *(8–10);* however, some of these transcripts do not appear to encode full-length or functional proteins. *In situ* hybridization approaches have been used to localize receptor mRNAs in the ovary *(11),* and immunohistochemistry *(12)* and binding of radiolabeled ligand *(9)* have been used to localize functional gonadotropin receptor proteins. These studies indicate that FSH receptor mRNA and protein are expressed at very low levels in the granulosa cells of preantral follicles *(11,13)*. As follicles mature under the influence of FSH, both FSH and LH receptors are increased in larger antral and preovulatory follicles *(9–11),* while the preovulatory LH surge leads to a rapid downregulation of both FSH and LH receptors in the rat ovary *(11,14)*. The changes in FSH and LH receptor gene expression observed in a normal estrous cycle can be mimicked in immature rats by treatment with pregnant mare's serum gonadotropin (PMSG) followed by hCG. In the preovulatory follicle, the levels of LH receptor mRNA and protein far exceed those of the FSH receptor *(13),* and the LH or hCG-mediated downregulation of the LH receptor appears to be

more rapid and robust than the regulation of the FSH receptor. Whereas the FSH receptor is expressed only in granulosa cells, the LH receptor is also expressed in thecal and interstitial cells *(11,15)* as well as in the luteal cells of the newly formed corpus luteum *(6,11,15)*.

Both gonadotropin receptors are coupled to a G_s stimulatory G-protein and to the activation of adenylate cyclase *(16,17);* thus, ligand binding leads to an increase in intracellular cyclic adenosine monophosphate (cAMP) levels. Many of the effects of the gonadotropins are therefore thought to be mediated by the cAMP-dependent protein kinase, protein kinase A (PKA) *(16,18)*. The regulatory subunit IIβ (RIIβ) of PKA is in turn regulated by gonadotropins in the granulosa cell *(19)*. Moreover, FSH treatment induces a predominantly cytosolic A kinase anchoring protein (AKAP 79) *(20)* that interacts specifically with the type II PKA subunit RIIα, thereby causing a translocation of PKA from the membrane to the cytosol. Thus, FSH acts in a concerted fashion to induce PKA subunits, stimulate cytosolic localization of the kinase, and activate the kinase through the elevation of intracellular cAMP levels. In addition to the activation of PKA, the LH receptor can also activate the phospholipase C (PLC) signaling pathway in granulosa cells isolated from mature follicles *(21,22)*. When ectopically overexpressed in L-fibroblast cells, the murine LH receptor stimulates phosphoinositide metabolism, calcium mobilization, and protein kinase C (PKC) activation *(18,23)*. This might occur through coupling of the receptor to a $G_{q/11}$ protein or by stimulation of PLC by βγ subunits released from G_i. Figure 2 shows the predominant cAMP-dependent signal transduction mechanisms stimulated by gonadotropin receptor–ligand interactions.

Ovarian follicles at different stages of maturation vary in their ability to generate the cellular second-messenger cAMP in response to gonadotropin stimulation. The levels of cAMP generated in response to FSH in the granulosa cells of preantral follicles are much lower than those produced by FSH or LH in the granulosa cells of preovulatory follicles *(24–26)*. In general, LH receptors are much more abundantly expressed than FSH receptors. High pharmacological doses of FSH mimic many of the actions of LH, including the stimulation of ovulation *(27)*. These observations suggest that these two signaling systems differ largely in their ability to stimulate moderate (basal FSH) vs large (the LH surge) increases in intracellular cAMP levels in the granulosa cell. They further suggest that cAMP-dependent signaling pathways in the granulosa cell will be differentially activated at different stages of follicular maturation. These cAMP-dependent signaling pathways leading to changes in gene expression are discussed in the following section.

cAMP-RESPONSIVE TRANSCRIPTION FACTORS

Many of the effects of the cellular second-messenger cAMP are thought to be mediated by changes in gene expression. Target genes regulated by cAMP often contain *cis*-acting regulatory sequences called cAMP response elements (CREs). A large and growing family of transcription factors that bind to these elements has been characterized, and these proteins all belong to the bZip family of transcription factors *(28–30),* so named because the carboxy-terminal regions of these proteins contain a stretch of basic residues followed by a leucine zipper motif, the combination of which confers dimerization and DNA-binding ability to the proteins. The best characterized members

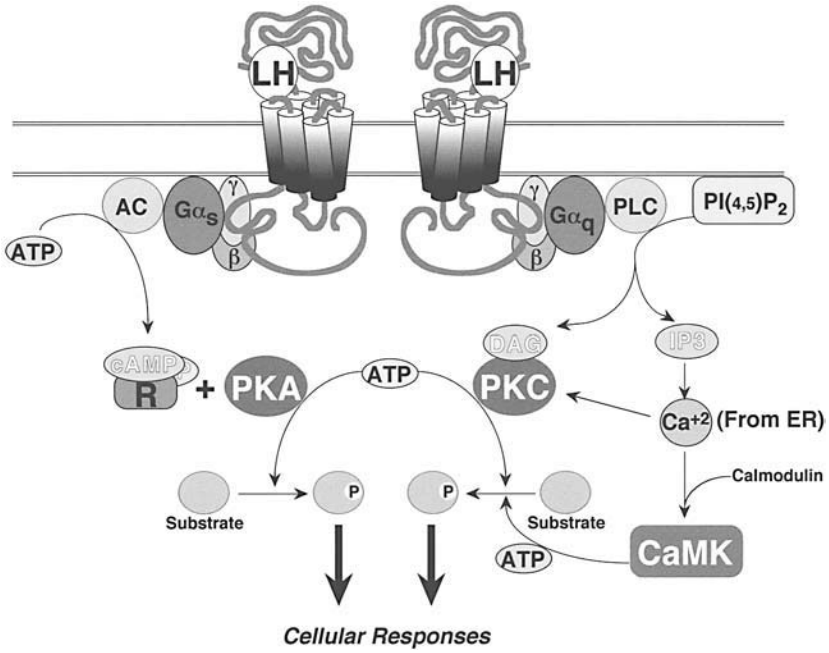

Fig. 2. Signal transduction by G-protein-coupled gonadotropin receptors in gonadal cells. Activation of the $G\alpha_s$ protein following hormone binding leads to the stimulation of adenylate cyclase (AC) and formation of cAMP. cAMP binds to the regulatory subunit (R) of PKA, leading to its activation. A second alternative pathway is shown in which activation of the $G\alpha_q$ protein stimulates PLC and the production of inositol triphosphate (IP_3) and diacylglycerol (DAG). IP_3 mobilizes calcium from intracellular sources such as the endoplasmic reticulum (ER). Ca^{+2} and DAG activate protein PKC. Phosphorylation of cellular substrates by PKA and PKC is likely to mediate many of the responses to the gonadotropins.

of this superfamily of transcription factors are the CRE-binding protein (CREB) *(31–33)* and the CRE modulatory protein (CREM) *(34–36)*.

The genes encoding the CREB and CREM family of transcription factors include multiple coding exons that correlate with specific structural domains of the proteins. A bZip DNA-binding dimerization domain (DBD) at the carboxy terminus allows these proteins to dimerize and bind DNA. Domains enriched in glutamine residues (Q) are essential for transactivation, and the kinase-inducible domain (KID) contains phosphorylation sites that are targets for numerous protein serine/threonine kinases *(37–42)*. Functional transcriptional activation proteins contain the DBD, Q, and KID domains, and the absence of any of these leads to a protein with altered regulatory function *(43)*. The combinatorial assembly of different exons of the CREB and CREM genes via alternative RNA processing leads to isoforms of these transcription factors that can act as transcriptional activators or repressors *(33,34,44–46)*. The switch between the transcriptional activator and repressor isoforms of CREM has been most intensively investigated in testicular cells *(33,44)*. Thus, alternative RNA processing provides a tremendous diversity of cAMP-dependent transcriptional regulatory proteins from the related CREB and CREM genes. Figure 3 presents a schematic diagram showing the

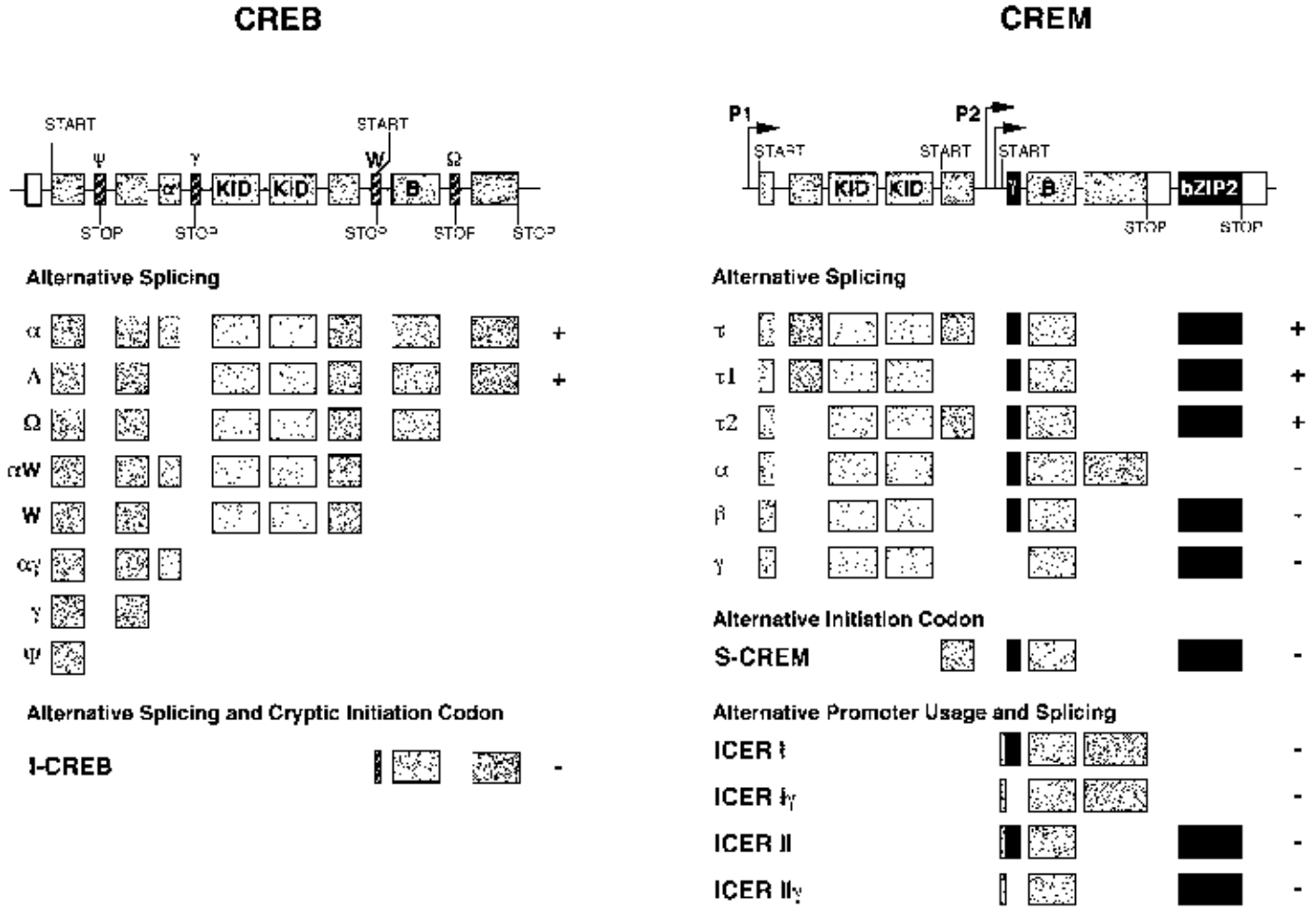

Fig. 3. Structure of the CREB and CREM genes and generation of protein isoforms. The gene structures are shown at the top, with the boxes representing exons. The domain structures of the multiple protein isoforms of each transcription factor are shown below the genes, and where known, + indicates a transcriptional activator and − a transcriptional repressor. The mechanisms used to generate these protein isoforms are also indicated.

structures of the genes encoding CREB and CREM, as well as the protein isoforms generated by alternative RNA processing or other related mechanisms.

In addition to alternative RNA processing, isoforms of the bZip transcription factors can be generated through regulated gene transcription. A well-characterized CREM isoform, the inducible cAMP early repressor (ICER), is generated by cAMP-regulated transcriptional initiation from an alternative intronic promoter of the CREM gene *(47)*. ICER acts as a cAMP-inducible transcriptional repressor and is thought to autoregulate its expression negatively leading to a transient expression pattern in cells that express ICER, which are found predominantly in the neuroendocrine system. CREB is a more ubiquitously expressed transcriptional activator. In most cases CREB is not induced at the transcriptional level, but there are reports that CREB can also autoregulate its expression, leading to a cAMP-responsive induction of CREB mRNA *(48)*.

The functions of many of the CREB and CREM isoforms are controlled by reversible phosphorylation within the KID *(49)*. CREB phosphorylation has been studied in great detail, and it is clear that for increased transactivation function, the CREB protein must be phosphorylated at serine residue 133 *(50,51)*. CREB can be phosphorylated at serine 133 by a number of protein kinases including PKA *(32,41)*, PKC *(42)*, pp90(RSK)-related RSK2 *(52)*, and mitogen-activated protein kinase *(53)*. Additional phosphorylation sites in CREB play roles in further modulating the activity of CREB that is phosphorylated at serine 133. Phosphorylation at serine 129 *(54)* or serine 142 *(55)* leads to an enhancement or repression of CREB activity, respectively. Although phosphorylation of CREB at serine 133 may enhance its ability to bind to DNA, especially at nonconsensus CREs, the predominant effect appears to be to allow interaction with the phospho-CREB binding protein CBP, a transcriptional coactivator that mediates the effects of multiple transcriptional regulatory proteins *(56)*.

CREB and CREM are able to form heterodimers with other bZip proteins, particularly the activating transcription factors *(45,57–59)*. There are also reports of interactions between these bZip proteins and other leucine zipper transcription factors such as fos and jun *(60,61)*. This finding introduces an additional level of complexity to cAMP-dependent transcriptional expression, in which the repertoire of transcription factors present in the target cell may dictate the regulation of cAMP-responsive genes.

GENE EXPRESSION IN OVARIAN GRANULOSA CELLS

During the rodent estrous cycle, many ovarian genes are subject to direct regulation by the pituitary gonadotropins FSH and LH. These include genes involved in proliferation and differentiation of the granulosa cell, steroidogenesis, ovulation, and luteinization. It is therefore not surprising that there are distinct temporal patterns of gene expression during the estrous cycle driven by the cyclical changes in gonadotropin levels and gonadotropin receptor expression.

Figure 4 illustrates several of these major temporal patterns of ovarian gene expression during the estrous cycle. Numerous ovarian genes are positively regulated by FSH during the period of follicular maturation, with their expression levels peaking in preovulatory follicles on proestrous afternoon. These include the genes encoding the FSH receptor (FSH-R) *(10,11)*, LH receptor (LH-R) *(10,11)*, cytochrome P450 aromatase *(62)*, PKA regulatory subunit RIIβ *(19)*, and the inhibin and activin α and β subunits *(63,64)*. Following the preovulatory LH surge, expression of these genes is

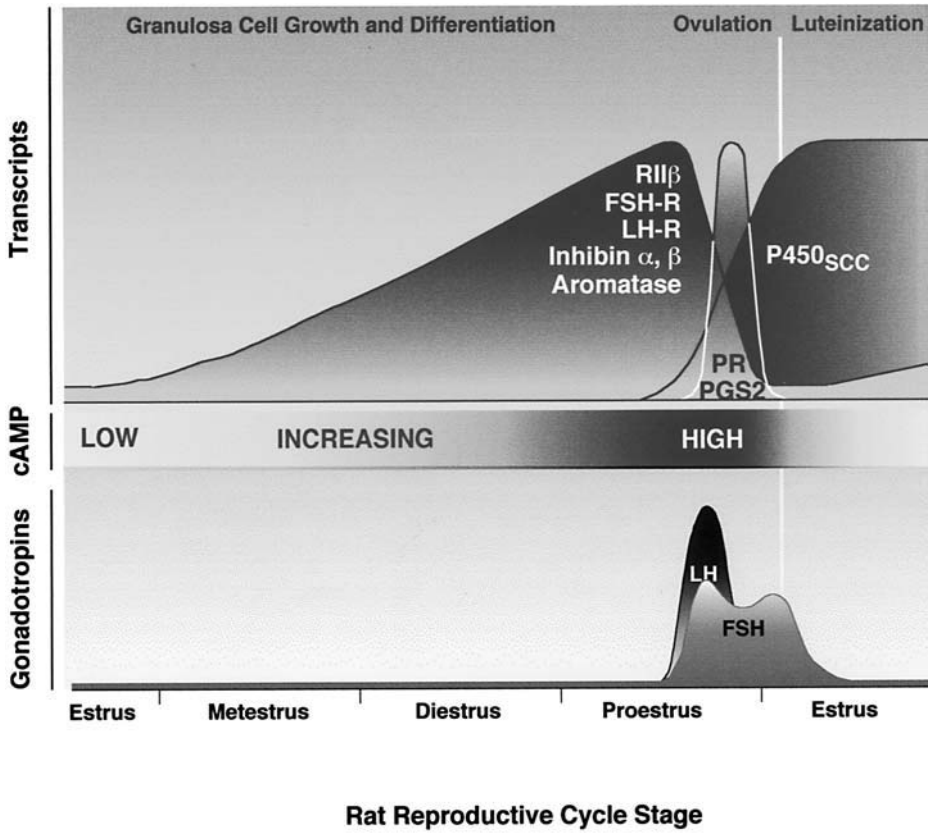

Fig. 4. Gene regulation in the ovary during the estrous cycle. This diagram depicts the relationship among estrous cycle stage, circulating gonadotropin levels, granulosa cell cAMP levels, and mRNA levels for several genes of interest. Three basic patterns of gonadotropin-regulated gene expression are shown and are discussed further in the text.

dramatically downregulated during the periovulatory period, and they are not expressed in luteal cells *(63–66)*.

A second pattern of gene expression shown in Fig. 4 is exemplified by the progesterone receptor (PR) and prostaglandin synthase-2 (PGS-2) *(67–70)*. Both PR and PGS-2 are implicated in ovulation, and consistent with this implication, these genes are transiently expressed at high levels during the periovulatory period in response to the LH surge and then downregulated in the corpus luteum of the rat. Finally, steroidogenic enzymes expressed predominantly in luteal cells, such as P450 side-chain cleavage (P450scc), are induced in response to the LH surge and continue to be expressed following ovulation and luteinization *(71,72)*.

As can be seen in Fig. 4, the genes encoding the subunits of the inhibins and activins are highly regulated during the rat estrous cycle and are induced during follicular maturation and repressed following the preovulatory LH surge. In the following sections, we review the basic biology of the ovarian inhibins and activins, and then consider the potential mechanisms by which the pituitary gonadotropins regulate inhibin gene expression in ovarian cells.

INHIBIN AND ACTIVIN SUBUNITS AND GENES

Inhibins and activins are dimeric polypeptide hormones that belong to the transforming growth factor (TGF-β) superfamily of growth and differentiation factors *(73–76)*. Inhibins suppress FSH secretion from anterior pituitary gonadotroph cells, whereas activins stimulate FSH synthesis and secretion. Inhibins are heterodimers of a unique α subunit and one of two highly related β subunits (inhibin A = $\alpha\beta_A$ and inhibin B = $\alpha\beta_B$). Activins are formed by the heterodimeric combinations of the two β subunits (activin A = $\beta_A\beta_A$, activin AB = $\beta_A\beta_B$, and activin B = $\beta_B\beta_B$).

cDNAs encoding the three inhibin and activin subunits have been cloned from multiple species *(77–83)*. These cDNAs indicate that the α, β_A, and β_B mRNAs encode larger precursor proteins that include the mature subunits at their carboxy terminus, a characteristic of all proteins within the TGF-β superfamily. The genomic structures of the inhibin and activin subunit genes have also been determined in several different species. Figure 5 shows the structures of the rat α, β_A, and β_B subunit genes. The structures of the inhibin α *(81,84,85)* and β_B *(83,86)* genes are fairly similar, with two exons separated by a single intron of 1.7 or 2.3 kb, respectively. Both exons 1 and 2 encode the precursor protein, but the mature hormone coding sequences are localized to exon 2. The structure of the β_A subunit gene is similar, but there is a small additional 5′ exon containing only nontranslated sequences, and the two coding exons are separated by a larger intron of approx 9 kb *(87)*. The α and β_B subunit genes are linked on human chromosome 2 and mouse chromosome 1, whereas the β_A gene is located on human chromosome 7 and mouse chromosome 13 *(88)*.

Analysis of the putative promoter regions of these genes, summarized in Fig. 5, indicates that the α and β_A subunit genes have "CAAT"- and "TATA"-like sequences and unique transcriptional start sites, whereas the β_B subunit gene promoter lacks these elements, has multiple transcriptional start sites, and is extremely GC-rich, features more commonly associated with unregulated "housekeeping" genes. Functional analyses of the promoter regions of each of the three inhibin and activin subunit genes have been carried out using transfection of gonadal cell lines *(84,86,89)*, but the inhibin α subunit promoter has been the most extensively studied, as discussed in the following sections.

INHIBIN SUBUNIT GENE EXPRESSION IN THE OVARY

Inhibin subunit gene expression in the rat ovary and in ovarian granulosa cells has been studied using a variety of in vivo and in vitro model systems that provide unique and complementary types of information. Studies in normally cycling adult animals provide important information on the normal physiological regulation of these genes, but are difficult to perform and require correlations to be made between the observed regulation and changes in endogenous gonadotropin levels. An often used alternative is to treat immature rats with exogenous gonadotropins. PMSG is most commonly used to mimic the activity of FSH in stimulating follicular maturation. PMSG also has weak LH-like activity *(90)*, and the recently available recombinant human FSH *(27)* is an alternative treatment that avoids this potential problem. PMSG- or FSH-primed animals can subsequently be treated with hCG to mimic the preovulatory LH surge and induce ovulation and luteinization. Finally, primary cultures of granulosa cells can be readily prepared from the rat ovary *(84,91)*, and these provide an important in vitro system

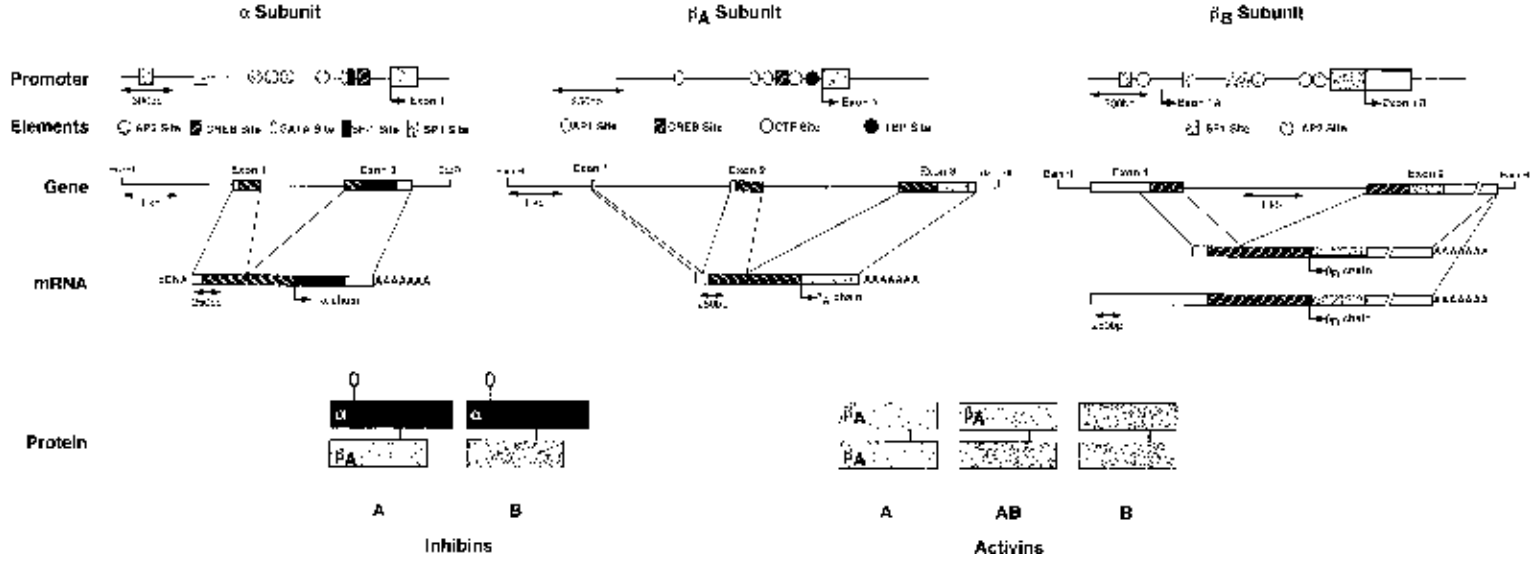

Fig. 5. Structural features of the rat inhibin and activin α, β_A, and β_B subunit genes. The structures of the three subunit genes and the corresponding mRNAs are shown. A schematic of each promoter region showing key transcription factor binding sites discussed in the text is also shown. **(Bottom)** The combinatorial assembly of the mature α, β_A, and β_B subunits to generate the multiple forms of inhibin and activin.

for studying and manipulating the molecular events involved in inhibin subunit gene regulation.

All three of the inhibin and activin subunit genes are expressed in the rat ovary. However, the α subunit mRNA is much more abundant than the β subunit mRNAs, consistent with the observation that inhibin rather than activin is the predominant secreted product from the ovary *(63,64)*. *In situ* hybridization reveals that the inhibin subunit mRNAs are localized to the granulosa cells of healthy ovarian follicles *(64,92)*. Lower levels of α subunit mRNA are also observed in thecal and interstitial cells and in the newly formed corpus luteum *(93)*, and the stromal expression of the α subunit mRNA is reported to increase in the aged rat *(94,95)*.

To exemplify the pattern of expression and regulation of the inhibin subunit genes, Fig. 6 shows *in situ* hybridization to localize the inhibin α and β_A subunit mRNAs during the rat estrous cycle. In general, the α and β_A subunit mRNAs are colocalized and coordinately regulated, and although the β_B subunit mRNA is not shown in Fig. 6, it is expressed in a quite similar fashion. The subunit genes are expressed in small follicles during the early part of the cycle, and peak expression is observed in the granulosa cells of large preovulatory follicles during proestrous morning. Following the preovulatory LH surge, expression is downregulated and lost in ovulatory follicles and the corpus luteum. The downregulation of the β_A subunit mRNA is somewhat more rapid and complete than that of the α subunit, and significant α subunit expression is observed in stromal cells during this period (e.g., see 2100-h proestrus in Fig. 6). When the LH surge is pharmacologically blocked using a gonadotropin-releasing hormone (GnRH) antagonist in adult cycling rats, inhibin subunit gene expression is no longer downregulated on proestrous evening and remains elevated into the following morning *(96)*. Inhibin subunit expression has also been followed during pregnancy, and similar localization of the mRNAs to the granulosa cells of healthy follicles is observed *(97)*.

The relative increase in inhibin subunit gene expression seen during follicular maturation in the normal estrous cycle is likely a direct reflection of the actions of FSH on the granulosa cell. In the immature rat model discussed previously, *in situ* hybridization experiments demonstrate that the mRNAs for all three subunits are induced in granulosa cells in response to PMSG stimulation *(93,98)*. Figure 7 shows an example of this. A more quantitative measure of the effects of FSH can be obtained using cultured rat granulosa cells. In this system, treatment with recombinant human FSH results in a three-to sixfold increase in the steady-state levels of all three subunit mRNAs, suggesting direct positive regulation of these genes by FSH (*see* Fig. 7 for an example). The effects of FSH in this system can be mimicked with pharmacological reagents that increase intracellular cAMP levels such as dibutyryl cAMP and forskolin, and these agents stimulate inhibin subunit gene expression as well as levels of immunoreactive and bioactive inhibin protein in cultured granulosa cells *(73,99–103)*.

The negative regulation imposed by the preovulatory LH surge during a normal estrous cycle can also be mimicked in the immature rat model with exogenous hCG. Figure 8 shows localization of inhibin α subunit and LH receptor (as an index of LH responsiveness) mRNAs in the gonadotropin-treated immature rat ovary. Twelve hours after hCG treatment, there is a dramatic downregulation in both mRNAs, consistent with the estrous cycle studies. Interestingly, negative regulation of inhibin gene expression by LH is not observed in primary granulosa cells, and, in general, LH has a stimulatory effect on inhibin gene expression in cultured cells *(73)*. This suggests that cellular or

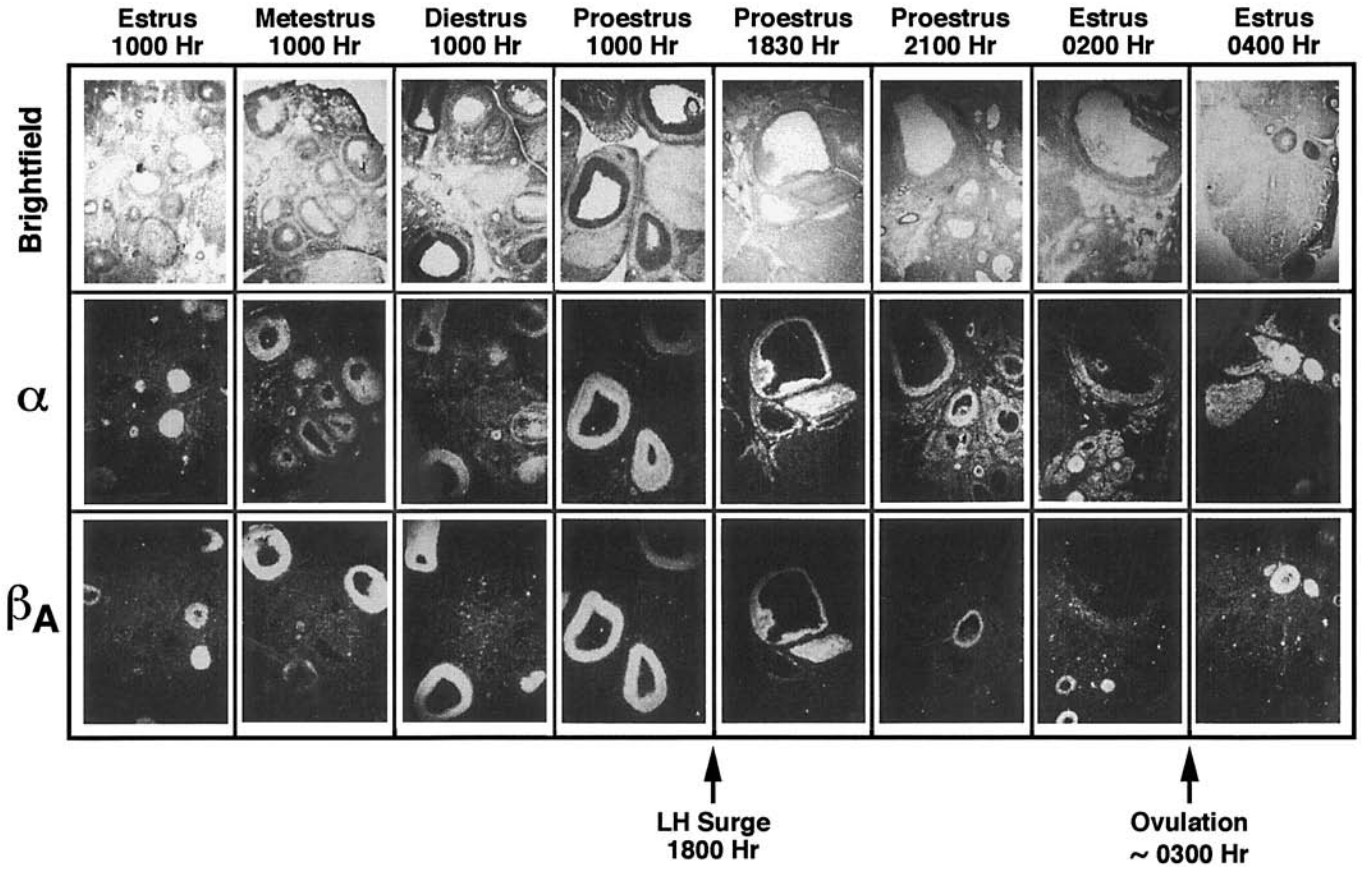

Fig. 6. Regulation of inhibin gene expression during the rat estrous cycle. The panels are bright field or dark field photomicrographs of *in situ* hybridization studies to localize inhibin α and β_A subunit mRNAs in the cycling rat ovary. **(Top)** Cycle times; **(bottom)** for reference the approximate times of the LH surge and ovulation.

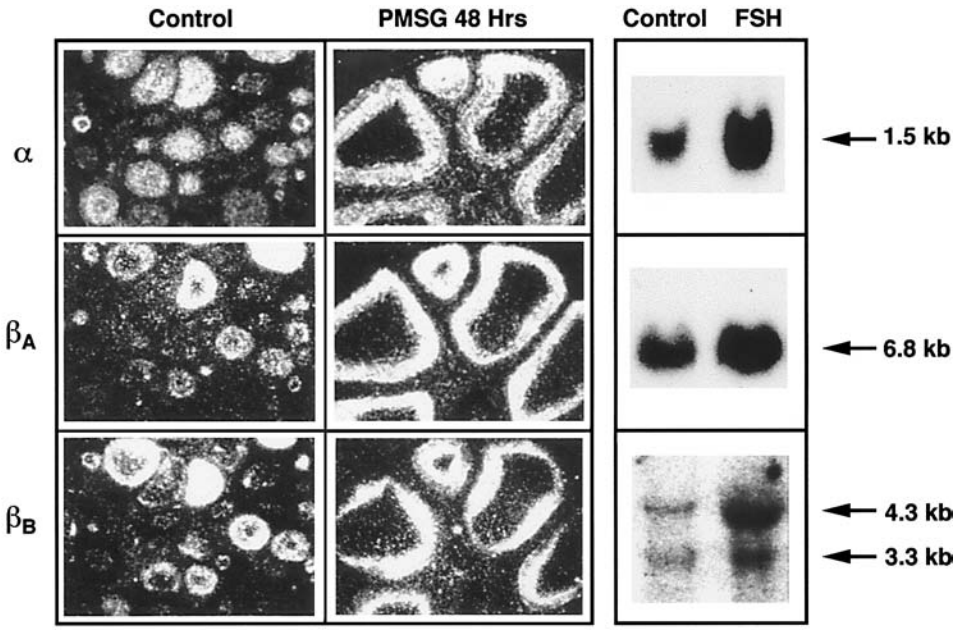

Fig. 7. Regulation of inhibin subunit gene expression by FSH. **(Left)** An *in situ* hybridization analysis of ovaries from immature rats that were either untreated or treated with PMSG for 48 h. Localization of the inhibin α, β_A, and β_B subunit mRNAs is shown. **(Right)** RNA blot analyses of inhibin α, β_A, and β_B subunit mRNAs from cultured granulosa cells that were either untreated or treated with recombinant human FSH for 48 h.

paracrine interactions found within the intact follicle are likely to be an important component of this negative regulation.

INHIBIN SUBUNIT GENE PROMOTER ANALYSIS

To try to understand the mechanisms responsible for tissue- and cell-specific expression and hormonal regulation of the inhibin subunit genes, gene promoter activity has been investigated in transfected gonadal cells or cell lines and in transgenic mice. Although studies of all three subunit genes have been performed, the most extensive characterization is of the α subunit promoter, which is the primary focus of this and the following sections.

With respect to tissue specificity, Hsu and collaborators *(104)* have shown that a 6-kb fragment 5′ to the mouse α subunit gene can direct many aspects of the normal expression pattern of the gene in transgenic mice. In particular, this construct is expressed in the gonads and ovarian granulosa cells, although it appears to also be expressed in thecal and interstitial cells at levels higher than typically observed for the endogenous gene. This same 6-kb construct also targets expression to the adrenal, a secondary site of inhibin α subunit gene expression. A 2.5-kb construct gave a less complete expression pattern in which adrenal expression and some aspects of gonadal cell–type specific expression were lost *(104)*.

Inhibin α subunit promoter regulation has been studied in many laboratories using cell transfection approaches *(86,105,106)*. Consistent with the finding that FSH and

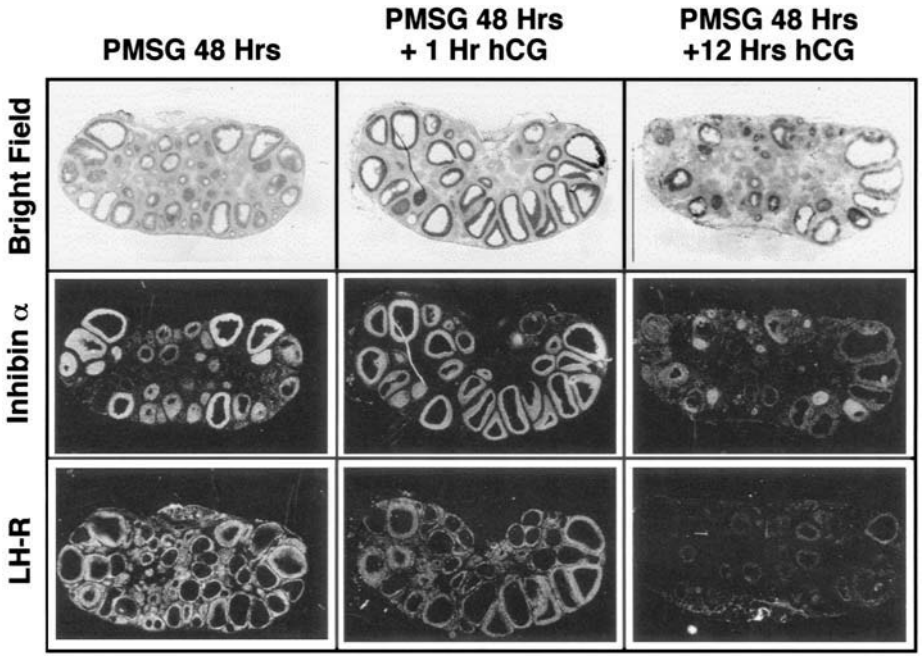

Fig. 8. Suppression of inhibin gene expression in the rat ovary by LH. An *in situ* hybridization analysis of ovaries from hormonally treated immature rats is shown. Rats were untreated or treated with exogenous gonadotropins as indicated. Localization of the inhibin α subunit mRNA, as well as the LH receptor mRNA, is shown.

cAMP stimulate expression of the endogenous gene, these same agents increase α subunit promoter activity in cultured granulosa cells. An example of this is shown in Fig. 9, in which a rat inhibin α subunit promoter fragment was fused to a luciferase reporter gene, and luciferase activity in transfected primary granulosa cells that were untreated or forskolin treated was measured. Forskolin results in a fourfold increase in luciferase reporter-gene activity. If the promoter is sequentially deleted from the 5′ end, it retains forskolin or cAMP responsiveness until the region between −163 and −96 bp is deleted, implicating this region in the cAMP response *(84,106)*. A similar study using the mouse inhibin α subunit gene promoter indicated that cAMP responsiveness localized to a proximal promoter region extending to −165 bp *(105)*.

The region required for FSH or cAMP regulation of the rat inhibin α subunit gene includes an atypical CRE centered at −122 bp *(84)*. To test the importance of this site, the experiments summarized in Fig. 10 were carried out. To determine whether CREB could interact with this potential CRE, in vitro DNA footprinting was performed, which demonstrated that CREB protects a region of the promoter between −117 and −132 bp. When a two-nucleotide mutation was made in the core of the CRE in the context of the normal inhibin α subunit promoter, CREB was abolished. More important, this CRE mutant construct exhibits a dramatic reduction in both basal and forskolin-induced activity in transfected granulosa cells (Fig. 10). Electrophoretic mobility shift assays indicate that granulosa cell extracts include proteins capable of binding to this same

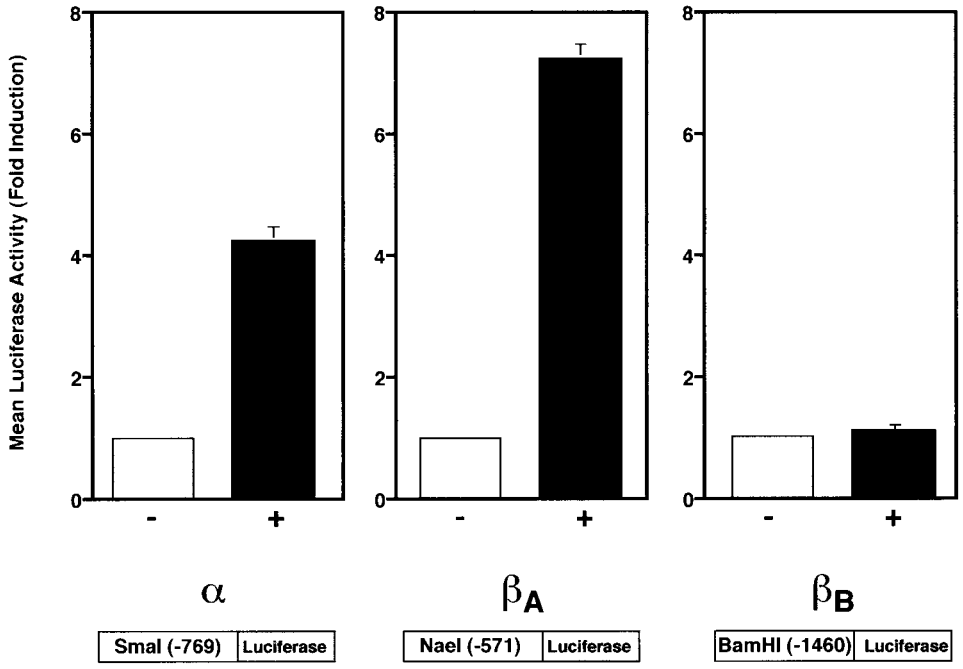

Fig. 9. Regulation of the inhibin subunit gene promoters by forskolin in rat granulosa cells. Inhibin α, β_A, and β_B subunit promoter fragments of the indicated sizes were linked to a luciferase reporter gene and transfected into primary cultures of granulosa cells. +, treatment with 10^{-5} *M* forskolin for 24 h. Luciferase activity is shown as a fold induction in response to forskolin.

CRE site, and a CREB antibody supershifts one of these complexes, indicating that endogenous CREB interacts with the inhibin α subunit CRE *(84)*.

For comparative purposes, Fig. 9 also shows the responses of the β_A and β_B subunit gene promoters to forskolin in transfected granulosa cells. The β_A subunit promoter gives a robust response to forskolin similar to that observed for the α subunit. By contrast, the β_B subunit gene is not stimulated by forskolin, despite the observations that the endogenous β_B mRNAs are increased by FSH in granulosa cells (*see* Fig. 7) and that the β_B subunit gene is regulated at the transcriptional levels by cAMP in Sertoli cells or cell lines *(107)*. Deletion analysis of the rat β_A subunit gene promoter localized cAMP inducibility to the proximal 300 bp upstream of the transcription start site, and this region contains a variant CRE *(108,109)*. This variant CRE has been implicated in both cAMP and phorbol ester regulation of the β_A subunit gene in GRMO2 cells, a granulosa cell line *(110,111)*. Gel mobility shift assays using phorbol ester and/or forskolin-stimulated GRMO2 cell nuclear extracts indicate that this variant CRE preferentially binds AP-1 family proteins, although it is capable of binding CREB at low affinity *(109)*. Thus, two signaling pathways converge on this key element to regulate transcription of the inhibin and activin common β_A subunit gene in granulosa cells. It can be concluded that although the three inhibin and activin subunit genes are coordinately regulated by FSH stimulation of cAMP-dependent pathways in vivo and in cell lines, the molecular mechanisms of this regulation are distinct for each of the three genes.

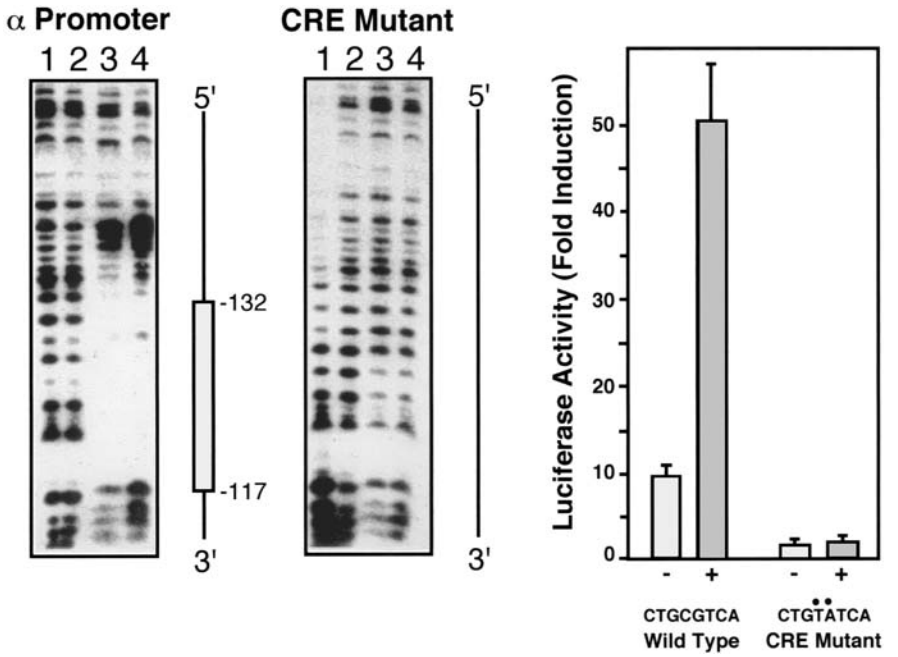

Fig. 10. Interaction of CREB with the inhibin α subunit gene promoter. **(Left** and **middle)** DNA footprinting of the inhibin α subunit promoter with recombinant CREB protein; **(right)** promoter activity in transfected rat granulosa cells. The constructs are either the wild-type inhibin α subunit promoter or a mutant in which two nucleotides of the CRE element were replaced as shown in the right panel. The footprinting lanes are (1) no protein, (2) control extract, and (3/4) CREB extract. (Adapted from ref. *84*.)

CREB PHOSPHORYLATION AND INHIBIN α GENE EXPRESSION

The data discussed in the previous sections indicate that the ovarian inhibin α subunit gene is regulated by FSH in vivo, that this regulation can be mimicked by agents that stimulate intracellular cAMP levels in vitro, and that the cAMP-responsive transcription factor CREB is a central component of this regulation. In this section, we consider further the involvement of CREB in FSH-stimulated inhibin gene expression and the regulation of CREB activity in granulosa cells.

The simplest explanation for gonadotropin-mediated induction and subsequent down-regulation of the inhibin α subunit might be a stoichiometric control of the levels of the cAMP-responsive transcriptional activator CREB. In this model, FSH would induce CREB expression, leading to increased inhibin α subunit gene transcription, whereas LH might suppress CREB expression, resulting in attenuated transcription following the LH surge. Indeed, cyclical control of CREB isoform expression is observed in rat testicular cells *(33)*. However, quantitative mRNA measurements and *in situ* hybridization performed in the immature rat ovary indicate that CREB mRNA is ubiquitously expressed at low levels and is not regulated by either FSH or LH stimulation *(112)*. Another potential mechanism of CREB regulation is through alternative RNA processing to generate isoforms with differing transcriptional activities. In the testis, the CREB transcript can be alternatively processed in a regulated fashion to include an exon, termed W, that contains multiple in-frame stop codons as well as a cryptic initiation

site (*see* Fig. 3). This results in the translation of CREB proteins that either lack the DBD or contain only the DBD. Thus, activating forms of CREB are not generated from this alternative mRNA *(33)*. However, reverse transcriptase polymerase chain reaction (RT-PCR) experiments using oligonucleotides designed to differentiate CREB transcripts that include the W exon indicate that this transcript is not expressed at appreciable levels in the rat ovary, and that it is not regulated by the pituitary gonadotropins *(112),* suggesting that this posttranscriptional mode of regulation is not operative in the rat ovary. Finally, gonadotropins do not alter levels or localization of the CREB protein in the rat ovary, as assessed using Western protein blotting and tissue immunohistochemistry approaches *(112)*.

As discussed in some detail in a previous section, the transcriptional activity of CREB is controlled in part by a posttranslational modification—protein phosphorylation. Since CREB levels in the ovary did not appear to change in response to the gonadotropins, the status of CREB phosphorylation was investigated to determine whether this was a target for gonadotropin regulation. Figure 11 summarizes these data. Using an antibody specific for CREB phosphorylated at serine residue 133 by PKA, phospho-CREB levels were seen to be very low in granulosa cells maintained in primary culture. However, following a brief (20-min) exposure of the cells to FSH, most of the cells stained with the phospho-CREB antibody, and the epitope was completely localized to the cell nucleus. This phosphorylation of CREB is transient in the cultured cells, and there is a return to the basal state by 3 h after FSH stimulation. Other agents that increase intracellular cAMP levels, including hCG and forskolin, cause a similar increase in CREB phosphorylation in cultured granulosa cells *(112)*.

CREB phosphorylation in granulosa cells has also been observed by Carlone and Richards *(113)* and correlated with changes in aromatase gene expression. In their study, chronic treatment with hormone led to the reappearance of phospho-CREB 48 h after the initial transient increase in phospho-CREB *(113),* a time more closely associated with the induction of potential target genes such as the inhibin α subunit and aromatase. In our study, CREB phosphorylation was examined at longer times using an in vivo model in which immature rats were subjected to a standard gonadotropin treatment regimen, granulosa cells were rapidly isolated, and the status of CREB phosphorylation was determined by Western protein blotting using the phospho-CREB antibody. The bottom panel of Fig. 11 indicates that in our study, levels of phospho-CREB remained elevated for at least 18 h following PMSG stimulation, a time when inhibin α subunit gene expression in this same animal model is maximal.

There is increasing evidence that CREB-mediated gene transcription can be impacted by other transcriptional regulatory factors. These proteins may compete with CREB for binding to CRE-like sites *(51),* form heterodimers with CREB having altered transcriptional activity *(114),* and synergize with CREB, either directly *(115)* or through adaptor proteins such as the CREB coactivator CBP *(69,113)*. Gonadotropins, particularly, LH, regulate some of these transcription factors, including steroidogenic factor-1 (SF-1) *(69,113)* and CCAAT/enhancer-binding protein β (C/EBPβ) *(116,117)*. SF-1 does not interact with CREB directly, but it appears to synergize with CREB in the transcriptional control of aromatase gene expression. It is postulated that SF-1 interacts, either directly or via another coactivator protein, with CBP *(118),* and that this interaction leads to increased transcription from the aromatase promoter *(69,113)*. LH leads to a small suppression of SF-1 expression in granulosa cells, and this may contribute to the

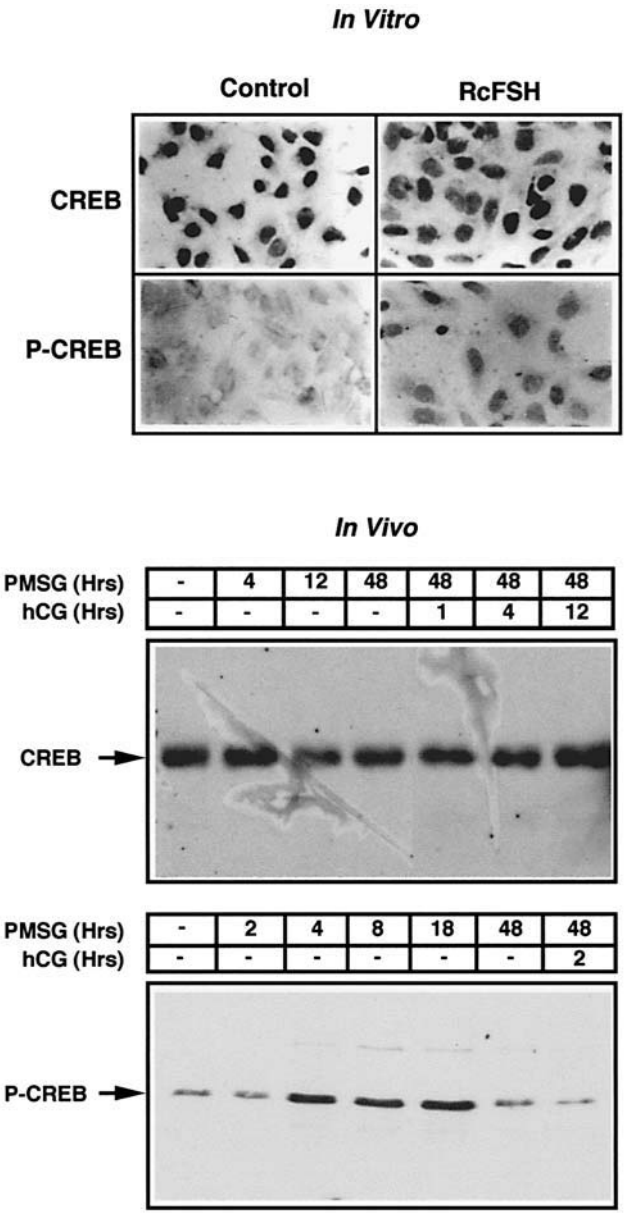

Fig. 11. CREB phosphorylation in FSH-stimulated granulosa cells. **(Top)** Immunocytochemistry of cultured granulosa cells with antiserum to CREB or phospho-CREB. The cells were untreated or treated with recombinant human FSH. **(Bottom)** Western protein blots of extracts from granulosa cells isolated from the ovaries of rats treated with gonadotropins in vivo as indicated. The blots were probed with antiserum to CREB or phospho-CREB. (Adapted from ref. *112*.)

ability of LH to downregulate aromatase gene expression *(113)*. C/EBPβ may also play a role in modulating CREB-mediated transcription. This transcription factor is known to be rapidly induced in granulosa cells following LH stimulation. Recently, C/EBPβ null mice were generated *(117)*. The female mice have severe reproductive disorders and are infertile. There is an ovulatory failure, and the ovary lacks corpora lutea and

exhibits follicular cysts. At a molecular level, several ovarian genes, including aromatase, fail to downregulate in response to an exogenous LH surge, suggesting that C/EBPβ is normally required for this downregulation. There is evidence that CREB can interact with the C/EBP family of transcription factors, and in one example a heterodimer of C/EBP and CREB appears to bind preferentially to a CRE rather than a C/EBP consensus site *(115).* C/EBPβ is induced by a cAMP-PKA-CREB-dependent pathway in HEPG2 cells *(119),* providing a potential mechanism for the induction of C/EBPβ observed in ovarian cells following the LH surge. Thus, changing interactions with other LH-regulated transcription factors such as SF-1 and C/EBPβ may be one mechanism by which CREB activity is altered at the time of the preovulatory LH surge. In the following section, we consider an additional pathway involving the transcriptional repressor ICER, a product of the CREB-related CREM gene.

ICER EXPRESSION AND REGULATION OF THE INHIBIN α SUBUNIT GENE

Because some isoforms of the CREB and CREM family of bZip proteins can function as transcriptional repressors, an attractive model to explain the ability of LH- and cAMP-dependent pathways to downregulate inhibin gene expression would be to postulate the induction of such a repressor at the time of the preovulatory LH surge. As mentioned previously, the identified repressor isoforms of CREB do not appear to be expressed in the rat ovary. We therefore examined the expression of the CREM gene in the ovary, focusing on the potent cAMP-inducible transcriptional repressor ICER *(47,120).*

Using *in situ* hybridization and RT-PCR approaches, ICER was found to be rapidly induced in ovarian granulosa cells following LH or hCG stimulation *(121).* The left panel of Fig. 12 shows *in situ* hybridization to the immature rat ovary 1 h after hCG stimulation of PMSG-primed animals, and a robust induction of ICER mRNA is observed in both granulosa and thecal cells. Colocalization of the LH receptor indicates that ICER is induced only in LH-responsive follicles *(121).* The right panel of Fig. 12 illustrates an experiment in which granulosa cells were isolated from the ovaries of gonadotropin-stimulated immature rats, and RNAs from these cells were used for RT-PCR studies of ICER gene expression. Four distinct isoforms of ICER (I, Iγ, II, IIγ) are observed; these have been previously identified and their structures are shown in Fig. 3. ICER transcripts in these granulosa cells are not induced by PMSG stimulation, but they are strongly induced following hCG stimulation, at a time when the inhibin α subunit mRNA begins to decline. In this same immature rat model, ICER protein is also induced by hCG stimulation, and, as might be expected, it lags behind the mRNA induction and is maximal 4 h following hCG treatment *(121).*

In primary cultures of rat granulosa cells, ICER is transiently induced by both FSH and LH, and this induction can be mimicked with forskolin. ICER induction in the granulosa cell line GRMO2 can be inhibited with the PKA inhibitor H89, suggesting that ICER is induced through a cAMP-PKA-dependent pathway in granulosa cells *(121).* Expression of ICER mRNA was also studied in the normal rat estrous cycle *(121).* ICER expression in the ovary is observed only following the preovulatory LH surge on late proestrous afternoon, and this expression is extinguished within about 4 h. When the LH surge is blocked using either pentobarbitol or a GnRH antagonist, ICER mRNA is no longer induced in the ovary of these cycling animals, suggesting

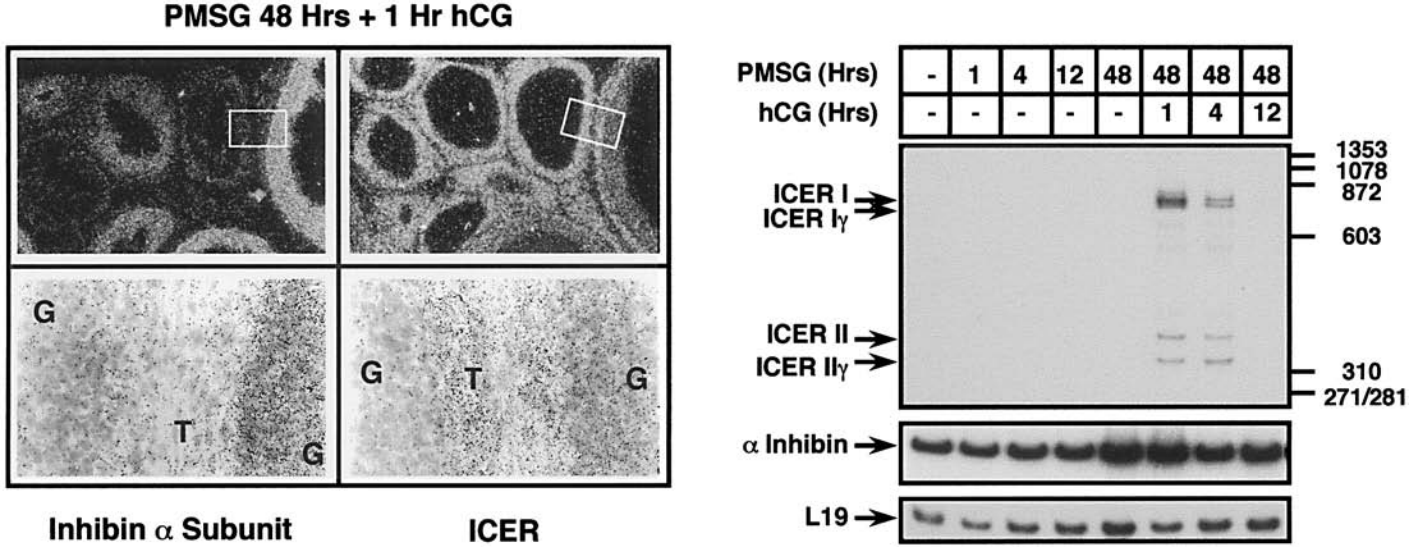

Fig. 12. ICER mRNA expression in the rat ovary. **(Left)** An *in situ* hybridization analysis of ICER and inhibin α subunit mRNA expression in ovaries from immature PMSG-primed rats treated with hCG for 1 h. The higher magnification views **(bottom)** show silver grain localization to the granulosa (G) or thecal (T) cell compartments. **(Right)** An RT-PCR experiment showing ICER expression in granulosa cells isolated from immature rats treated in vivo with gonadotropins as indicated. For comparative purposes, inhibin α subunit and ribosomal protein L19 mRNAs in these granulosa cells are also shown. (Adapted from ref. *121*.)

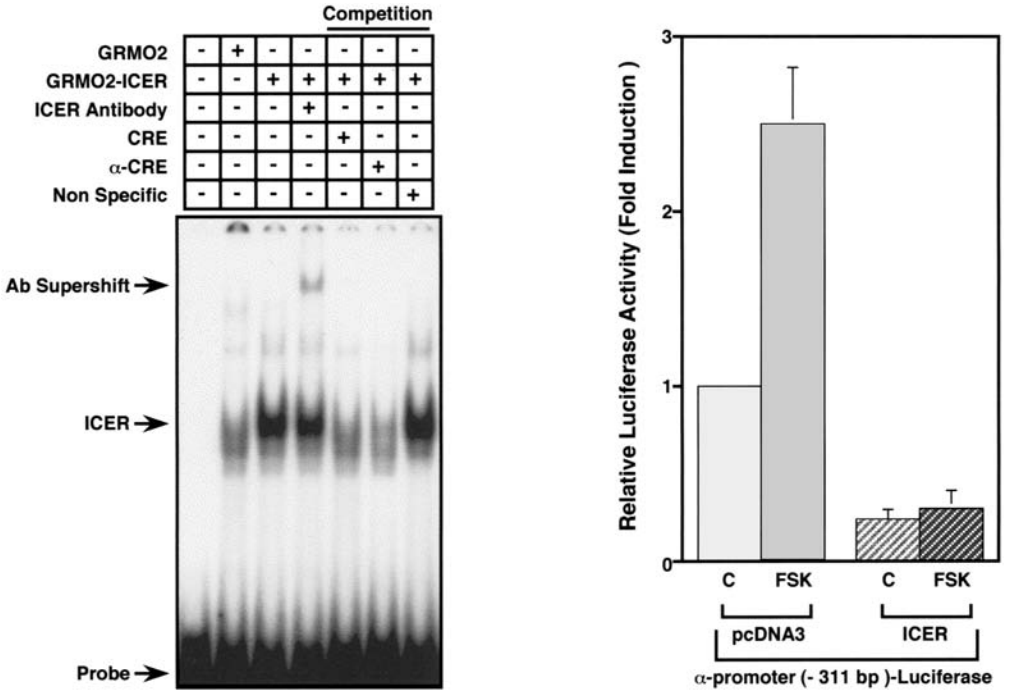

Fig. 13. Regulation of the inhibin α subunit promoter by ICER. **(Left)** An electrophoretic mobility shift assay demonstrating specific interaction of ICER with the inhibin α subunit CRE. Extracts used were from control GRMO2 cells, or cells expressing ICER-I. Competition and antibody addition was analyzed in the various lanes as indicated in the grid at the top. The positions of the specific ICER complex and an antibody supershifted complex are shown. **(Right)** A transfection assay in GRMO2 cells, showing the ability of an ICER expression construct to suppress basal and forskolin-stimulated expression of the inhibin α subunit gene promoter. pcDNA3 is the vector used to express ICER-I. Activities are shown as fold inductions relative to the control. (Adapted from ref. *121*.)

that the induction of ICER mRNA is LH dependent in the adult cycling rat, as it is in the immature rat.

Taken together, these expression data indicate that ICER is specifically and transiently induced by LH in the granulosa cells of preovulatory follicles. Thus, the pattern of ICER expression is consistent with its potential role in the repression of inhibin α subunit gene expression following the LH surge. To determine whether ICER can interact with the inhibin α subunit CRE to regulate transcription of this gene, the studies summarized in Fig. 13 were performed. Electrophoretic mobility shift assays using extracts from GRMO2 granulosa cells expressing recombinant ICER-I protein demonstrated that ICER-I can specifically bind the inhibin α subunit CRE, as shown in the left panel of Fig. 13. To test directly whether ICER can affect inhibin α subunit gene expression, an ICER-I expression construct was cotransfected with an inhibin α subunit promoter-luciferase reporter-gene construct into GRMO2 cells. As shown in the right panel of Fig. 13, both basal and forskolin-stimulated reporter-gene activity was substantially attenuated, consistent with an ability of ICER to repress CREB-stimulated expression of the inhibin α subunit gene *(121)*.

The mechanism by which ICER antagonizes CREB-dependent transcription is

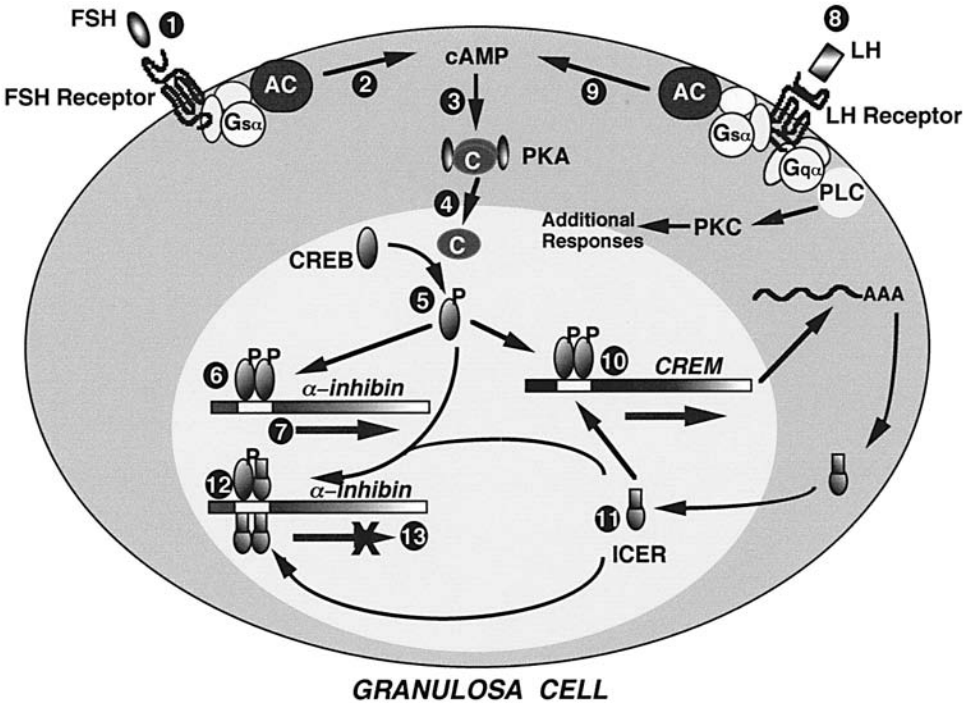

Fig. 14. A model for gonadotropin regulation of inhibin α subunit gene expression. FSH interacts with FSH receptors on granulosa cells (1), leading to adenylate cyclase (AC) activation and accumulation of moderate levels of cAMP (2). cAMP binds (3) to the regulatory subunits of protein kinase A (PKA) and releases the catalytic subunit (C) of PKA (4). PKA then activates nuclear CREB proteins by phosphorylation (P) at serine 133 (5). Binding of phosphorylated CREB (6) to the inhibin α subunit promoter induces inhibin α subunit gene expression (7). When preovulatory surge levels of LH bind LH receptors (8), higher levels of cAMP are accumulated (9) in the granulosa cells of preovulatory follicles. cAMP again activates PKA, which in turn activates CREB by phosphorylation. Fully phosphorylated and activated CREB interacts with the CREM gene CAREs (10), leading to production of the ICER proteins (11). ICER can then bind the inhibin α subunit CRE (12) as a homodimer or as a heterodimer with CREB, attenuating inhibin α subunit gene expression (13).

unknown, but it has been shown that CREB activity can be modulated by heterodimerization with related proteins *(56,114,122)*. Preliminary studies suggest that ICER homodimers have a higher affinity for the inhibin α subunit CRE than CREB homodimers, indicating that a likely mechanism of transcriptional repression is occupancy of the CRE by transcriptionally inactive ICER dimers immediately following the LH surge.

SUMMARY

Based on the data reviewed in this chapter, we can propose a model for how FSH and LH might exert their opposing actions on inhibin α subunit gene expression in ovarian granulosa cells. Figure 14 schematically shows this model. During the early phase of the estrous cycle, basal FSH stimulates the growth and maturation of ovarian follicles, and this is accompanied by increasing expression of the inhibin α subunit gene in granulosa cells. FSH, by stimulating intracellular cAMP production and activating PKA (steps 1–4 in Fig. 14), leads to the phosphorylation of CREB (step 5), making

it competent to bind to and transactivate CRE-containing target genes such as the inhibin α subunit gene (steps 6 and 7). Once ovarian follicles mature and acquire functional LH receptors, the preovulatory LH surge triggers the morphological and biochemical changes associated with ovulation and luteinization. These actions of LH are thought to be mediated largely by cAMP-dependent pathways (step 8 and 9), although additional signaling pathways, such as the PKC and tyrosine kinase signaling pathways are likely to be important. In this model, the LH-stimulated cAMP signal leads to the rapid transcriptional activation (via CREB) of the CREM gene intronic promoter and to the production of ICER mRNA (steps 10 and 11). The ICER protein then acts to attenuate rapidly the transcription of CRE-containing target genes such as the inhibin α subunit gene (steps 12 and 13), thus completing a cycle of cAMP-dependent activation and repression of gene expression.

A question of primary importance with respect to this model is, Why do FSH and LH, both acting predominantly through cAMP-dependent mechanisms, have such divergent actions on inhibin gene expression? One answer likely relates to the observation that although LH clearly represses inhibin gene expression in the ovary, this action is not observed in cultured granulosa cells. Thus, paracrine factors or cell-cell interactions in the ovary are likely to impact LH action, and it will be important to establish what these factors or interactions might be. A second likely answer comes from studies on ICER activation by cAMP signaling. The CREM gene intronic P2 includes four clustered CRE-like elements called cAMP autoregulatory elements (CAREs). *(47).* These CARE elements mediate cAMP induction, probably through activating factors such as CREB, and subsequently bind ICER, resulting in an autorepression of ICER expression and a resetting of the system. Because the CAREs are fairly poor CRE elements, they are likely to be occupied by activators only when the cAMP signaling pathway is fully activated. The magnitude of the intracellular cAMP signal induced in preovulatory granulosa cells by LH is known to be much larger than that induced by FSH in small antral follicles, providing a likely mechanism for the selective activation of ICER by LH in vivo.

The CREM/ICER gene has been disrupted in mice *(123,124),* and although the mutant male mice are infertile owing to defects in spermatogenesis, the mutant female mice are reported to be fertile *(89,90).* Although this might seem to be inconsistent with a critical role for ICER in inhibin gene expression, there has to date been no study of ovarian morphology or ovarian-specific gene expression in these mice, and thus it is not clear whether they are normal. In addition, we have overexpressed the inhibin α subunit in transgenic mice from a metallothionein promoter, and although these animals have repressed serum FSH and are subfertile as assessed by litter size, they are nonetheless fertile (manuscript in preparation). Thus, a subtler misregulation of inhibin expression might result in only minor effects on fertility. It is also possible that other repressor protein such as inhibitory CREB forms are upregulated in these mutant mice, compensating for the absence of ICER. Finally, while these data suggest an involvement of ICER in inhibin subunit gene expression, there are certain to be many other signaling pathways and transcriptional regulatory molecules that are modulated in response to the LH surge, some of which are likely to be important regulators of inhibin gene expression.

Recent studies in a pituitary cell line have addressed the important concept of the dynamics of cAMP-induced transcriptional responses *(125).* These experiments define

a refractory period following the initial stimulation of cAMP-dependent genes that is determined in part by the duration of the initial stimulus. These findings may have strong parallels to the ovary that would explain the observation that whereas the primary gonadotropin surges on proestrous evening strongly induce ICER expression, the secondary FSH surge early on the morning of estrus does not induce ICER expression *(121)*. The preovulatory LH surge might, in addition to causing the initial induction of ICER, make granulosa cells refractory to further ICER induction, thus allowing the secondary FSH surge to stimulate the gene expression and cell proliferation events that are critical to the recruitment and maturation of a new cohort of ovarian follicles. It seems reasonable to speculate that cycles of gonadotropin-induced and cAMP-mediated induction and attenuation of transcriptional responses may be a key mechanistic component for maintaining the cycles of ovarian follicular development characteristic of reproduction in many mammals.

In summary, it is clear that gene expression in the ovary is regulated in a highly dynamic fashion through the complex cycle of follicular recruitment, maturation, ovulation, and luteinization. The pituitary gonadotropins FSH and LH are perhaps the best studied regulators of ovarian gene expression, but ovarian steroids and paracrine-acting growth and differentiation factors also are important regulators of gene expression. The inhibin α subunit gene is tightly regulated during the ovarian cycle, and the gonadotropins, acting through cAMP-dependent pathways, seem to be critical determinants of this regulation. Our studies suggest that the cAMP-regulated transcription factors CREB and ICER are key targets of gonadotropin action, and they in turn are necessary for the appropriate regulation of inhibin gene expression. Clearly much more remains to be learned about the mechanisms of cAMP-regulated gene expression in this system, about how the transcriptional activities of multiple ovarian genes are coordinately regulated, and about how these changes in gene expression drive the cell proliferation and differentiation events that are key to ovarian function.

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14 Placental Trophoblast Cells

Transcriptional Regulation and Differentiation[1]

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CONTENTS

INTRODUCTION

The placenta is essential for implantation of the mammalian embryo into the uterus, protection of the developing fetus from the maternal immune system, establishment of a nutrient/waste exchange system between the maternal and fetal compartments, and production of pregnancy-specific hormones that regulate maternal and fetal physiology. Given the many roles of the placenta in mammalian reproduction and development, considerable effort has been made to understand the extraembryonic development of this organ. The first steps in the process of placental development include the formation of the trophectoderm in the blastocyst and the subsequent differentiation of trophectodermal cells into the trophoblasts, the major cell type of the placenta. The placenta is actually composed of more than one type of trophoblast, and another key event is the terminal differentiation of proliferative trophoblasts into large, nonproliferative cells (giant cells in rodents and syncytial trophoblasts, or syncytiotrophoblasts, in humans).

Based on results obtained in other developmental systems such as the pituitary and skeletal muscle, it seems likely that the key factors that drive trophoblast differentiation will prove to be transcriptional regulatory proteins that directly activate trophoblast-specific genes. Thus, one approach to find the factors involved in placental development

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has been to work upstream from genes expressed in the placenta to characterize the corresponding *trans*-acting factors. A second approach is to analyze transcription factors expressed in the placenta for their effects on trophoblast differentiation; such factors have emerged from searches for trophoblast members of transcription and differentiation regulatory protein families, and from targeted gene disruption experiments in mice that revealed placental defects. In this chapter, we focus on the recent progress that has been made in analyzing the transcriptional regulation of trophoblast gene expression and identifying the potential regulatory components of trophoblast differentiation.

TRANSCRIPTION OF PLACENTA-SPECIFIC GENES

The approach of identifying placenta-specific transcriptional enhancers and then characterizing the factors that act through these elements first required the isolation of genes expressed solely in the placenta, or genes that display a restricted pattern of tissue expression that includes the placenta. Many of these genes encode secreted proteins, including hormones, growth factors, cell-surface receptors, and proteases that are important for trophoblast attachment and invasion of the uterus, as well as the subsequent growth of the placenta. Since these genes appear to encode many of the key regulators of placental function, their expression is likely to be closely tied to placental development. Therefore, these genes are expected to prove valuable as markers for trophoblast differentiation and as systems for identifying transcription factors that may participate in the broader control of placental gene expression and placental development. Studies on several of these genes have now reached the point where the regulatory elements and factors involved in placental expression have been at least partially defined. In this chapter, additional genes are also described that are in the early stages of characterization; these genes may prove to be important systems for identifying trophoblast-specific transcriptional regulatory components.

Hormones Related to Prolactin and Growth Hormone

The placenta in rodents and humans is the source of numerous pregnancy-specific hormones, the most numerous and abundant of which are members of the prolactin (PRL) and growth hormone (GH) family. In the mouse, at least nine placental hormones in this family are encoded by genes closely linked to the PRL gene *(1–9);* the transcriptional regulation of three of these hormones—placental lactogen I (PL-I), placental lactogen II (PL-II), and proliferin (PLF)—has been studied most extensively. All three of these hormones are produced exclusively by the trophoblast giant cells *(10–13),* the outermost cells of the embryonic compartment that establish connections to the maternal uterus. PL-I and PLF synthesis initiates early in gestation, whereas the onset of PL-II gene transcription is delayed until midpregnancy, coincident with a decrease in PL-I and PLF expression *(1,3,4,10).*

Transcription of the PL-I and PLF genes appears to be coordinately regulated. Both gene promoters have similar architectures, with closely spaced binding sites for Activator Protein-1 (AP-1) and GATA factors *(14).* These sites represent functional elements for the PL-I promoter in transfected Rcho-1 cells, a cell line derived from a rat choriocarcinoma that is able to differentiate in culture into giant cells *(15,16).* The precise form of AP-1 responsible for gene transcription in trophoblast giant cells is not known, but

the PLF promoter in transfected fibroblasts has been shown to respond primarily to an AP-1 heterodimer containing fra-1 and junB *(17)*.

The GATA factors that activate the PL-I gene promoter in Rcho-1 cells have been shown to be GATA-2 and GATA-3 *(16)*. Furthermore, the introduction of either GATA-2 or GATA-3 into fibroblasts converts these cells into a permissive environment for transcription from the PL-I gene promoter, thus suggesting that these two factors are able to direct a nontrophoblast cell to become a giant trophoblast-like cell *(16)*. The importance of GATA-2 and GATA-3 in trophoblast gene expression is not restricted to cell culture systems, since mice unable to produce either of these factors are unable to express wild-type levels of the PL-I and PLF mRNAs *(14)*. In the absence of either GATA-2 or GATA-3, though, giant cells are able to form in vivo; the definitive experiment of eliminating both factors and analyzing placental development has been hampered by the surprising lethality of the GATA-2 +/–, GATA-3 +/– double heterozygote *(14)*.

Although sequences within the first 300 bp upstream of the transcription start site are sufficient for trophoblast-specific promoter activity of the PL-I gene in transfected Rcho-1 cells *(15)*, this region is not sufficient for expression in vivo in transgenic mice (G. Ma, V. Soloveva, and D. Linzer, unpublished results). By contrast, a region of the PL-II gene has been identified that is active in transgenic mice *(18)*, and two functional elements in this region from −1471 to −1340 have been mapped by transfection into Rcho-1 cells *(19)*. Factors that bind to these elements are found in trophoblast but not fibroblast extracts, and the levels of these factors increase on giant cell differentiation *(19)*. Since PL-II gene expression initiates at midgestation in the same cells that had been producing PL-I and PLF *(13)*, the onset of PL-II gene transcription provides a molecular marker for a later stage of placental development. The regulatory factors responsible for activation of the PL-II gene may not only promote the switch from PL-I to PL-II gene expression, but may also participate in the further differentiation of trophoblast giant cells at midpregnancy.

The transcriptional regulation of PRL family genes in the placenta is also actively investigated in other rodents, ruminants, and primates. In humans the PL (or chorionic somatomammotropin [CS]) genes and a placentally expressed GH variant gene arose from the duplication and divergence of the closely linked GH (rather than PRL) gene *(20)*. Two human CS (hCS) genes are expressed exclusively in placental syncytiotrophoblasts and encode the same 22-kDa hormone *(21)*. The regulatory regions of one of these genes (alternatively designated as hPL-3, hCS-2, or hCS-B) has been analyzed by transfection into human choriocarcinoma cell lines and primary trophoblast cultures, leading to the identification of a placenta-specific enhancer approx 2 kb downstream of the gene *(22,23)*. The minimal enhancer has been localized to a region of 240 bp that is able to stimulate transcription in human BeWo and JEG-3 choriocarcinoma cells (but also in monkey kidney COS-1 cells), but not in HeLa or pituitary cells *(24,25)*. Within this enhancer is a site recognized by transcription enhancer factor-1 (TEF-1) and an uncharacterized factor named CS enhancer factor-1 (CSEF-1) with high affinity, and several other sites are recognized by these factors with lower affinity *(23–26)*. A separate regulatory region within the enhancer comprises adjacent repression and derepression elements and is required for maximal activity *(27)*. Positive enhancer activity correlates with the binding of CSEF-1 *(25)*, whereas TEF-1 appears to inhibit transcription through the titration of the TATA-binding protein *(28)*.

Chorionic Gonadotropin

Chorionic gonadotropin (CG), luteinizing hormone (LH), follicle-stimulating hormone (FSH), and thyroid-stimulating hormone (TSH) are heterodimers consisting of a hormone-specific β-subunit complexed to a common α-subunit. FSH, TSH, and LH are synthesized in the pituitary gland of all mammals, whereas CG is expressed in the primate and equine placenta *(29)*. Because of its pattern of expression, the hCG α-subunit has provided an excellent model for both placental and pituitary gene expression.

Placenta-specific expression of the α-subunit gene depends on the combined action of five closely spaced regulatory elements in the 5′ flanking region of the gene: tandem cAMP response elements (CREs), an α-activator element (α-ACT), a trophoblast-specific element (TSE), the junctional regulatory element (JRE), and a CCAAT box. All five elements are required for full promoter activity in transfected choriocarcinoma cells, but two of the elements (TSE and JRE) appear to be the primary contributors to trophoblast specificity *(30)*. The human α-subunit gene promoter is also able to direct transcription in the transgenic mouse placenta *(31)*. Thus, even though the mouse does not express a CG protein, the elements and factors responsible for placenta-specific expression appear to be conserved between humans and mice, strengthening the idea that the regulation of placental gene expression and placental development in an individual species may rely on general strategies shared among mammals.

One element that appears to be required for hCG α promoter activity in transgenic mice is the tandem CRE, since the bovine α-subunit gene promoter, which has only one functional CRE, is inactive in the transgenic mouse placenta *(31)*. A single nucleotide substitution in one of the CREs in the α-subunit gene promoter also results in only one functional CRE in rodents, and this CRE has a 200-fold lower affinity for the CRE-binding protein (CREB) and displays a marked reduction in placental expression *(31)*. In addition to CREB, the α-subunit gene promoter CRE can also interact with the activating transcription factor ATF1, ATF2, and AP-1 *(32,33),* which can lead to transcriptional repression *(33);* hence, this element may integrate information from several signaling pathways.

The α-ACT element is an important contributor to the activity of the promoter, but by itself this element is not sufficient to drive placenta-specific transcription. The α-ACT in combination with the TSE has been designated as the upstream regulatory element (URE) *(34,35)*. The URE and tandem CREs can act together as a placenta-specific enhancer in transfected choriocarcinoma cells *(31,36)*. The two transcription factors GATA2 and GATA3 bind to the α-ACT and stimulate transcription *(35,37)*. Thus, the PL-I, PLF, and hCG α-subunit gene promoters each have functional and closely spaced binding sites for GATA factors and AP-1, suggesting that this combination of elements is an important motif for placenta-specific transcriptional regulation.

The TSE was initially defined as an element recognized by a trophoblast-specific factor *(34,35),* but a recent finding indicates that this factor is the widely expressed transcription factor AP-2 *(38)*. Another, but as yet undefined, factor is able to bind the entire URE, spanning both the α-ACT and TSE elements *(35)*. It is therefore still not clear how the TSE contributes to trophoblast-specific transcription. Similar to the TSE, the JRE also contributes to trophoblast specificity despite being bound in an electrophoretic mobility shift assay by a factor that is found in multiple cell types *(39)*. However, by a Southwestern assay, binding of a 40-kDa protein from choriocarcinoma cells was detected, and this protein was not seen in extracts from pituitary or mammary

cells *(30)*. The fifth element, the CCAAT box, appears to interact with a protein that is distinct from the many already characterized CCAAT factors *(40),* but the molecular characterization of the factor that binds to the hCG α-subunit element has not yet been reported. This element also may contribute to cell type–specific transcription, since a mutation in the CCAAT box decreases promoter activity in transfected choriocarcinoma cells, but not pituitary cells *(30)*.

Unlike hCG α-subunit expression, the regulation of expression of the hCG β-subunit in the placenta is complicated by the presence and transcription of multiple hCG β-subunit genes *(41–43)*. Transgenic mice bearing a 36-kb segment of the human genome containing 6 hCG β-subunit genes accumulate hCGβ mRNAs from three of these genes at low levels in the placenta *(44)*. Thus, the elements necessary for placental transcription of these human genes are present in this region and are conserved well enough to be recognized by mouse transcription factors. However, the timing of expression relative to gestational age is not the same in mice and humans; transgene expression was detected only during the last third of gestation in the mouse, whereas in humans CGβ expression is elevated early in pregnancy *(44)*. The precise mapping of the elements driving placental expression has not yet been achieved, but similar to the hCG α promoter, the hCG β promoter is responsive to cAMP; surprisingly, though, the cAMP-responsive region does not contain consensus binding sites for CREB *(45)*. The TSE region from the hCG α promoter can compete for protein binding to sequences that coincide with the cAMP-responsive region of the hCG β promoter, so the TSE binding protein may provide a mechanism for the coordinate transcription of the α- and β-subunits in the placenta *(46)*.

Transcription of the hCG genes is also under negative regulation. The AP-1 component c-*jun* is able to repress the activity of both the hCG α and β promoters in cotransfected choriocarcinoma cells *(33),* and the transcription factor Oct-3/4 inhibits the activity of the hCG β promoter in choriocarcinoma cells *(48)*. The binding site for Oct-3/4 was localized by footprinting to the same region involved in cAMP responsiveness and TSE binding protein recognition *(47)*. The single Oct-3/4 binding site is functional, since mutation of this site results in the loss of Oct-3/4 repression, but it has been proposed that Oct-3/4 acts to repress transcription by a mechanism distinct from blocking the binding of positive regulatory factors *(47)*. The negative regulatory effect of Oct-3/4 has also been detected for hCG α-subunit promoter activity, thereby coordinately regulating the synthesis of both components of hCG *(48)*. However, repression of the α-subunit promoter is not mediated by Oct-3/4 DNA binding, but is probably attributable to protein-protein interactions through which Oct-3/4 neutralizes the activity of a transcription factor that is essential for promoter activation *(48)*.

To date, the focus of the studies on hCG gene expression has been on understanding the regulation of expression of this hormone. Thus, the ability of any of the positive or negative regulators of hCG α or β gene expression to influence trophoblast differentiation has not yet been evaluated.

Adenosine Deaminase

Adenosine deaminase (ADA) is the enzyme that converts adenosine and deoxyadenosine to inosine and deoxyinosine *(49)*. Mice lacking ADA die soon after implantation with a severe deficiency in purine metabolism, demonstrating that this enzyme is essential *(50,51)*. ADA is expressed in a variety of tissues, but it is expressed at

particularly high levels in the placenta *(49)*. Expression of ADA in trophoblast cells is detected at d 7.5 of mouse gestation and increases in all trophoblast lineages during placental development. Expression of ADA has been reconstituted in the placenta by the introduction of a transgene containing 6.4 kb of 5′ flanking region *(52,53),* and the expression of ADA in trophoblast cells was sufficient to restore viability to ADA-deficient fetuses *(54)*.

The study of ADA expression may reveal general features of trophoblast gene expression, since ADA is expressed in all trophoblast cell types. Within the 6.4-kb 5′ flanking region, the essential elements for placental expression map to a 770-bp region and include at least two binding sites for proteins that are present in both the placenta and the liver, and one site that binds a factor (designated FP1) detected only in placental nuclear extracts *(53)*. The binding site for FP1 is similar to that of the hCG α TSE, so the binding factor may coordinately stimulate expression of both of these genes. In addition, two GATA elements are required for placental ADA enhancer activity *(53),* and a sequence near the 5′ end of the mouse ADA enhancer is similar to binding sites DF3 and DF4 in the human PL enhancer *(55)*. Thus, placental expression of the mouse PL-I and PLF genes, the human PL gene, the hCG α gene, and the ADA gene may share several common regulatory components. The ADA enhancer also includes binding sites for helix-loop-helix (HLH) proteins, two of which are discussed as follows.

4311

Initially isolated as a placenta-specific cDNA clone encoding a protein of unknown function, 4311 is expressed in the ectoplacental cone and the spongiotrophoblasts in the mouse *(56)*. Since ectoplacental cone trophoblasts are proliferative diploid cells that give rise to differentiated trophoblasts, the regulation of 4311 expression may provide insights into the early stages of trophoblast cell determination before terminal differentiation into nonproliferative cells occurs. To identify the regulatory region that confers trophoblast-specific expression of 4311, 5′ flanking genomic sequences linked to a *LacZ* reporter gene were introduced into transgenic mice *(57)*. This approach enabled the spongiotrophoblast enhancer to be localized to a region of only a few hundred base pairs, but the functional elements within the enhancer have not yet been defined. A potential binding site for HLH proteins occurs within this region, but sequences resembling TSE, CRE, GATA, and AP-1 elements were not seen. Thus, spongiotrophoblast-specific and giant cell–specific transcription may utilize distinct regulatory components.

Pregnancy-Specific Glycoproteins

The placenta is a source of a group of gene products known as the pregnancy-specific glycoproteins (PSGs), proteins that are closely related to the carcinoembryonic antigens and that belong to the immunoglobulin superfamily *(58)*. These abundant PSGs are encoded by multiple genes, and additional variants arise from alternative RNA splicing; however, the function of these proteins remains obscure *(58)*. Proximal promoter protein binding sites required for transcriptional activity have been identified in several human PSG genes *(59,60),* but these elements apparently do not act in a cell type–specific manner *(60)*. Recently, a zinc finger transcription factor, designated the core promoter-binding protein (CPBP), was identified that interacts with the human PSG5 promoter and stimulates transcription *(61)*. CPBP is enriched in the placenta,

but it is also expressed at lower levels in many other organs and tissues *(61)*. Two promoter elements have also been mapped in a rodent PSG gene that bind to placental proteins, and one of these factors has been found to be the widely expressed leucine zipper protein C/EBP-β *(62,63)*.

Cytochrome P450 Enzymes

Cytochrome P450 17α-hydroxylase plays a central role in steroidogenesis. This enzyme is expressed in the placenta *(64)* and differentiated Rcho-1 choriocarcinoma cells *(65)*. A 500-bp promoter region is active in both transfected Rcho-1 cells and Leydig MA-10 cells. Curiously, promoter activity is inducible by cAMP in the MA-10 cells, but not in the Rcho-1 cells, suggesting that this promoter can be differentially regulated in the testis and placenta *(65)*.

Transcription of a second cytochrome P450 enzyme, aromatase, which is involved in estrogen synthesis, relies on the utilization of alternative, tissue-specific promoters *(66)*. A region 300 bp upstream of the placenta-specific transcription start site is sufficient to direct trophoblast-specific expression in transfected human choriocarcinoma JEG-3 and BeWo cells *(67,68)*. Two binding sites in this promoter form a TSE that can compete for factor binding to the TSE within the hCG α-subunit gene promoter *(68),* and two other positive regulatory elements have been mapped approx 2 kb upstream of the transcription start site by transfection into BeWo cells *(69)*.

A third cytochrome P450, the side-chain cleavage enzyme P450scc, localizes to the mitochondria, where it initiates steroid hormone biosynthesis *(70)*. P450scc is expressed in syncytiotrophoblasts in humans and trophoblast giant cells in rodents, as well as in other steroidogenic tissues *(71,72)*. Transfection analysis of the P450scc promoter into JEG-3 cells has revealed several regions that contribute to placenta-specific transcription *(73)*. In addition to the basal promoter region, two proximal activator regions and one negative element have been identified *(73)*. Expression of P450scc mRNA is induced on differentiation of Rcho-1 cells *(72),* and an upstream promoter region has been mapped that is responsible for this differentiation-dependent expression *(74);* however, the functional elements and corresponding factors remain to be identified.

HLA-G

HLA-G is a nonclassical member of the major histocompatibility complex that is expressed in placental trophoblasts and is predicted to participate in protecting the genetically and antigenically distinct embryonic compartment from the maternal immune system *(75)*. A transgene containing 1.4 kb of 5′ flanking sequence is able to direct transcription in the mouse placenta, whereas a shorter region of 1.2 kb is much less effective *(76)*. Transgene expression was detected in the spongiotrophoblasts *(77,78)*. The approx 250-bp region 1.4–1.2 kb upstream of the transcription start site that is important for expression in trophoblasts contains a consensus AP-1 site *(78);* however, the role of that site has not yet been determined. Proteins present in placental cell extracts form specific complexes with this regulatory region *(79),* but the identities of the factors that contribute to placental HLA-G expression remain to be determined.

GH-Releasing Hormone

GH-releasing hormone (GHRH), a hypothalamic factor that stimulates pituitary GH secretion, is also expressed in the placenta (and several other tissues). A construct that

includes 7.5 kb upstream of the transcription start site utilized in the placenta is transcriptionally inactive in the transgenic mouse placenta *(80)*. However, the addition of sequences extending downstream from the first placental exon in the GHRH gene results in placental expression in transgenic mice *(80)*. The critical regulatory elements within this downstream region have not yet been mapped.

Interferon-τ

Interferon-τ (IFN-τ) is produced by bovine and ovine trophoblast cells and suppresses the production of prostaglandin $F_{2\alpha}$, thereby blocking prostaglandin-mediated luteolysis *(81)*. IFN-τ expression is first detected very early in gestation and its expression decreases as implantation begins *(81)*. Genes for IFN-τ have been cloned from several ruminant species, including sheep, cattle, goat, musk ox, and giraffe, and the corresponding gene promoters among these species display a high degree of sequence conservation *(81)*. Two regions of the IFN-τ gene are required for expression in transfected human choriocarcinoma cells *(82)*. Furthermore, proteins present in nuclear extracts from early stage ruminant conceptuses bind to both the proximal and distal regions *(82)*. The exact sequence requirements for these elements to function and bind factors, as well as the identity of the factors, remain to be elucidated.

TROPHOBLAST-SPECIFIC TRANSCRIPTION/ DIFFERENTIATION FACTORS

The second approach to characterize the regulators of trophoblast gene expression and trophoblast differentiation is to identify placental transcription factors and test their actions. One possible shortcut is to assume that the key factors will resemble transcription/differentiation regulators in other systems, especially HLH proteins.

HLH Proteins

Cross et al. *(83)* screened a trophoblast library for novel HLH proteins and identified a clone that they called Hxt. Expression of high levels of Hxt in Rcho-1 cells enhances their differentiation into giant cells *(83),* and disruption of the Hxt (now designated Hand1) gene results in abnormal giant cell development, placental insufficiency, and embryonic death *(84)*. Thus, Hand1 appears to be a key component in trophoblast differentiation. However, this same factor was simultaneously discovered in other cell types *(85,86),* indicating that either Hand1 by itself is insufficient to induce a trophoblast cell fate or that Hand1 in combination with other factors induces a distinct differentiation program. Gene targets for Hand1 have not yet been identified; Hand1 has been reported to stimulate transcription from a cotransfected PL-I promoter *(83),* but this effect may be indirect since binding sites for Hand1 in this PL-I promoter region have not been detected (J. Cross and D. Linzer, unpublished results). Since active HLH transcription factors are heterodimers, target gene specificity will also depend on a positively acting cofactor associated with Hand1, which may be E47 *(83)*.

Whereas Hand1 can induce giant cell differentiation, another HLH protein, Mash-2, may inhibit this transformation. Consistent with this model, Mash-2 is strongly expressed in the spongiotrophoblasts but not in the trophoblast giant cells *(87)*. The most compelling evidence for an inhibition of giant cell differentiation by Mash-2 comes from the targeted disruption of the *Mash-2* gene *(88)*. In these mice, a profound

loss of the spongiotrophoblasts and an abundance of giant cells are observed, with the resultant placental failure responsible for the inability of the embryo to develop beyond midpregnancy *(88)*. Hand1 and Mash-2 may therefore have antagonistic activities in regulating trophoblast differentiation. It will be of interest to determine whether these two factors have opposing effects on common trophoblast gene targets, or whether they act on different sets of genes. One attractive possibility would be if Hand1 and Mash-2 form a regulatory loop by regulating each other's synthesis.

In contrast to Hand1 and Mash-2, a second class of HLH proteins lacks a functional DNA-binding domain (DBD) and acts through heterodimerization as a dominant negative transcription factor. Such factors, including Id-1, are also detected in trophoblasts and are able to repress PL-I promoter activity in transfected Rcho-1 cells *(83)*. In addition to the Id class of repressor proteins, a non-HLH protein, designated I-mfa, can also interact with and block the action of HLH proteins that induce muscle cell differentiation *(89)*. Recently, the targeted disruption of the *I-mfa* gene revealed that this repressor protein is also important in placental development; in the absence of I-mfa, the number of giant cells is reduced, whereas no effect is seen on the spongiotrophoblasts *(90)*. Since I-mfa can apparently interact with both Mash-2 and Hand1 *(90),* the effects of I-mfa on placental development may involve its ability to titrate out the activities of these two HLH transcription factors. Thus, the activities of Hand1 and Mash-2 may be subject to regulation both at the level of gene expression (since these two proteins are synthesized in distinct trophoblast populations) and by association with various positive and negative cofactors.

POU-Homeobox Domain Proteins

Pit-1 was identified as a pituitary-specific inducer of PRL and GH transcription *(91,92)* and, based on sequence relatedness, was grouped with the Oct-1 and Unc-86 proteins as the POU transcription factor family *(93)*. The ability of Pit-1 to act not only as a transcription factor but also as a regulator of pituitary development *(94,95),* and the discovery of an even larger family of POU factors *(96),* suggest that POU proteins may also participate in trophoblast gene expression and placental development. Indeed, Pit-1 itself is also expressed in the placenta in both humans and rodents *(97,98),* and a Pit-1 binding site in the human renin gene promoter is necessary for transcriptional activity in placental cell primary cultures *(99)*. No placenta-specific POU factors have been reported to date, but factors expressed more broadly (including Pit-1) may still represent key factors in trophoblast differentiation.

Pit-1 also contains a conserved homeobox domain, a motif commonly found in proteins that specify cell fate and tissue patterning. Other homeodomain proteins are also found to be expressed in the placenta, including Cdx-2, the mouse homolog for the *caudal* gene in *Drosophila,* which is expressed in the trophectoderm and later in development in the spongiotrophoblasts *(100)*. Homozygous deletion of the *Cdx-2* gene results in the failure of the blastocysts to implant in the uterus, presumably owing to abnormal trophoblast differentiation *(101)*. Pem is another putative transcription factor that contains a homeobox DBD that can be detected in extraembryonic tissues, including the chorion, ectoplacental cone, and giant cells *(102)*. In a screen for homeobox genes that are expressed in the human placenta, a novel gene that is closely related to the *distal-less* gene in *Drosophila* was identified along with three known factors, Hb24, GAX, and MSX2 *(103)*. Target genes for any of these placental homeobox factors

have not yet been identified, but these factors are strong candidates for regulators of the specific patterns of gene expression that are required for trophoblast differentiation.

Nuclear Receptors

The estrogen receptor–related receptor β (ERR-β) is an orphan member of the nuclear hormone receptor superfamily. It is expressed in a subset of cells in the extraembryonic ectoderm that subsequently form the dome of the chorion *(104)*. Homozygous ERR-β mutant mice die at d 10.5 of gestation (midgestation) and display a severe deficiency in placental development *(104)*. These mutant mice lack a chorion and therefore fail to fuse the chorion and allantois. Also, multiple layers of giant cells form, but the spongiotrophoblasts and labyrinthine trophoblasts are severely reduced in number. Embryonic lethality in these mutants can be rescued by aggregating ERR-β mutant blastocysts with tetraploid wild-type cells (which will form the extraembryonic structures), demonstrating that lethality is owing to an extraembryonic, not a fetal, defect. Thus, ERR-β is required for early development of the placenta. This factor may be directly involved in both chorion formation and trophoblast differentiation; alternatively, an ERR-β-dependent signal may be produced by the chorion required for normal trophoblast proliferation and differentiation.

Factors Expressed in Many Tissues

In addition to the transcription factors displaying restricted patterns of expression, several widely expressed factors also participate in the regulation of placenta-specific transcription. For example, AP-1, which is composed of jun-fos or jun-jun dimers, is required for maximal PL-I promoter activity *(15)* and apparently plays a role in the regulation of the hCG gene promoter in trophoblasts *(33)*. The *c-fos* gene is expressed in trophoblasts *(105),* and in mice lacking c-fos, placental development is affected, as evidenced by a reduction in placental weight *(106)*. Similarly, C/EBP-β is expressed at high levels in the placenta and other tissues, and this factor contributes to the activity of multiple genes in trophoblasts, including the rat pregnancy-specific glycoprotein promoter *(63)*. Several ets family transcription factors are detected in the human placenta, including c-ets1 *(107),* myeloid elf-1-like factor *(108),* and ERM-ets-related molecule *(109)*. The promoter activity of the human placental folate-binding protein gene is regulated by tandemly repeated ets binding sites that are able to bind GABP, another ets-related factor *(110)*. Although it seems unlikely that an ubiquitously expressed factor alone is able to confer tissue specificity of gene expression, a combination of these factors, as well as a synergistic action with tissue-specific regulators, could dictate a restricted pattern of transcription in certain cell types at distinct developmental stages.

SUMMARY

The two approaches being taken to characterize the transcription factors that regulate trophoblast-specific gene expression and trophoblast cell differentiation—working upstream from trophoblast-specific genes to identify the factors and working downstream from transcription factors found to be expressed in the placenta to identify their targets and effects—have uncovered a large number of experimental systems and potential regulatory components. The upstream direction has implicated transcription factors such as GATA-2, GATA-3, and AP-1 as essential for the expression of multiple

trophoblast-specific genes; however, the roles of these factors in trophoblast differentiation have not yet been established. By contrast, the evidence for direct roles of several HLH transcription factors (Mash-2 and Hand1) and proteins that inhibit HLH-dependent transcription (Id and I-mfa) in trophoblast differentiation and placental development in the mouse is particularly striking. However, the key targets of these transcriptional regulatory factors in the genesis and maintenance of spongiotrophoblasts and giant cells remain to be defined.

A tremendous boost in identifying additional regulatory factors for placental development has come from the rapid application of gene-targeting technologies in the mouse. If a factor is required for placental development, the disruption of the gene encoding that factor will lead to a placental defect that may well be the first (and therefore only significant) phenotype seen. The mutations of some genes originally identified as important in nonplacental tissues (such as I-mfa) can unexpectedly reveal placental phenotypes and therefore implicate these factors in placental development. As the combination of the upstream and downstream approaches to the study of trophoblast gene expression leads to exciting convergences, these gene-targeting studies could continue to add novel components to the mix of factors that need to be accounted for in the overall regulation of placental development.

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15 Alternative Splicing of mRNAs for cAMP-Responsive Transcription Factors and Modulation of Gene Transcription in the Testis

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Contents

INTRODUCTION

Sperm and eggs are derived from what arguably may be the only true totipotential stem (germ) cells of living organisms. The existence of germ cells is critical for the continued propagation of all animal species. In the female, the full complement of mature germ cells in the form of oocytes is attained at birth. In the male, germ cells continue to develop after birth, in contrast to oogenesis in the female, in a process of continued spermatogenesis, which progresses at the time of puberty and continues throughout adult life. Spermatogenesis occurs during adulthood in repeated "waves" or cycles within the seminiferous tubules of 12.5-d duration, which in the rat encompass

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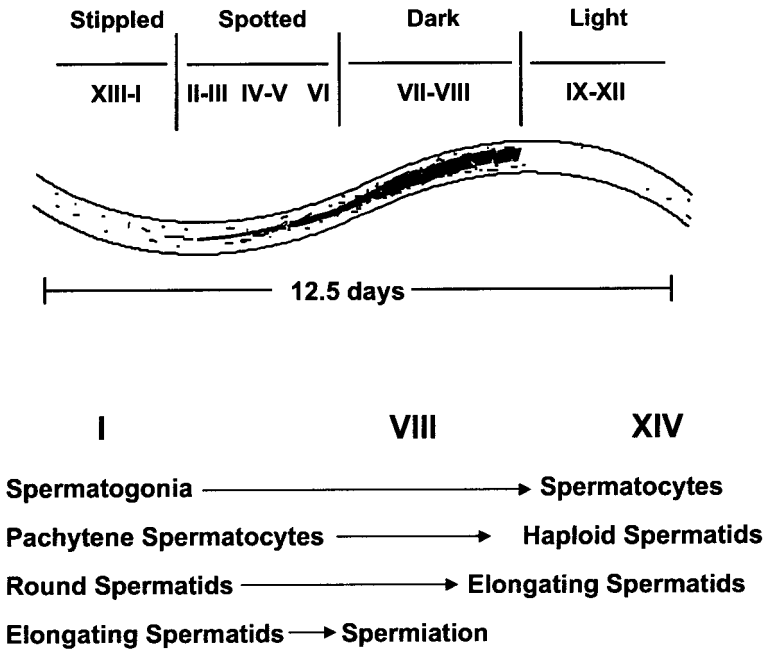

Fig. 1. Summary of spermatogenesis and cellular association stages in rat seminiferous tubules.

approx 45 d (3.5 cycles) for the progressive differentiation of stem spermatogonia to mature spermatozoa. The germ cells are stacked in four layers within the seminiferous tubule in the order of spermatogonia, spermatocytes, spermatids, and spermatozoa oriented from the basal to luminal border of the tubule, respectively (Fig. 1). By virtue of this unique anatomic arrangement of germ cell associations, cohorts of the four stages of germ cell experience the same local environment at the same time during the cycles of spermatogenesis.

cAMP SIGNALING IN THE TESTIS

The cAMP Signaling Pathway Is Critical in Spermatogenesis

Signaling by cAMP plays a major role in driving the cycles of spermatogenesis. Levels of cAMP in the rat seminiferous tubules wax and wane rhythmically during the 12.5 d cycles. The source of cAMP appears to be the somatic Sertoli cells that reside along the basal border of the tubules interspersed among the spermatogonia. The Sertoli cells produce many substances—sterols, heavy metal binding proteins, cAMP, and so forth—that "nurse" the germ cells and are essential for their differentiation to mature spermatozoa.

The production of cAMP by Sertoli cells is enhanced by receptors coupled to the stimulatory GTP-binding proteins (Gs) in turn coupled to adenylyl cyclase that converts adenosine triphosphate (ATP) to cAMP. There are at least two relevant Gs-protein-coupled receptors located on Sertoli cells: those for the pituitary gonadotropic hormone, follicule-stimulating hormone (FSH) and pituitary adenylyl cyclase-activating peptide (PACAP). Notably, PACAP is produced in high amounts by the germ cells themselves *(1)* and serves as an endogenous generator of cAMP within the testis by actions on

Sertoli cells *(2),* independent of FSH secreted by the pituitary. The high levels of production of PACAP within the testis *(3,4)* may explain why in the complete absence of FSH male mice and humans remain fertile, whereas females, in which the ovary may not have such a source of high levels of endogenous cAMP, are completely infertile *(5,6)*. However, PACAP is expressed at low levels in the ovary and has been implicated in ovulation and luteal steroidogenesis *(7–12)*. The somatic Leydig cells, located in the interstitium between tubules, are the major source of the production of testosterone in response to the stimulation of cAMP-coupled receptors by the other pituitary gonadotropin, luteinizing hormone. Testosterone works in concert with FSH and cAMP signaling in the testis in the regulation of spermatogenesis.

The differentiation of germ cells during spermatogenesis involves a highly complex orchestrated expression of a multitude of genes in both spatial and temporal patterns. The expression of many of these genes is regulated by cAMP signaling pathways that terminate in the activation of cAMP-responsive transcription factors, paramount of which are cAMP response element–binding protein (CREB) and cAMP response element modulator (CREM).

In this chapter, we describe several unique aspects of cAMP signaling and the expression of CREB and CREM in the testis. Distinct isoforms of components of the cAMP signaling pathway are expressed specifically in the testis. In addition, the alternative expression of activator and repressor isoforms of CREB and CREM are a result of alternative RNA splicing as well as alternative translation are unique to the testis.

cAMP Signaling in the Testis

cAMP is an important second messenger that arises intracellularly in response to the activation by hormones of receptors in the plasma membrane *(13)*. One well-studied route of cAMP-mediated signal transmission in cells is via the family of seven-transmembrane receptors, which are activated on engagement of the receptor with peptide or glycoprotein hormones. The binding of hormone induces dissociation of heterotrimeric G-protein subunits attached to the intracellular loops of the receptor. Certain of the G proteins (Gαs) in turn stimulate the adenylyl cyclases to convert ATP to cAMP. All of the nine known adenylyl cyclases are activated by GαS, but subsets are capable of integrating signals from calcium, protein kinase C, and other G-protein subunits (reviewed in refs. *14* and *15)*.

cAMP Binds to Regulatory Subunits, Resulting in Liberation of Catalytic Subunits

Protein kinase A (PKA), a heterotetrameric protein consisting of two regulatory and two catalytic subunits, is activated in the presence of cAMP. cAMP binds to the regulatory subunits, resulting in the liberation of catalytic subunits. The catalytic subunits then phosphorylate target proteins in the cytoplasm and the nucleus on serine or threonine residues defined by the conserved amino acid motif RRX (S/T) *(16)*.

In some tissues, small proteins called protein kinase inhibitors (PKIs) play a role in modulating PKA activity. These proteins interact with the catalytic subunit of PKA through a pseudosubstrate motif and inhibit phosphorylation activity and nuclear accumulation. PKIs are the products of at least three genes, each showing distinctive tissue distribution. All three forms (PKIα, β, and γ) are expressed in the testis *(17,18)*. PKIα, also prevalent in skeletal muscle, is expressed solely in the Sertoli cells. PKIβ is highly

expressed in the germ cells, starting around 25 d of age coincident with round spermatid development *(19)*. Two smaller isoforms of PKI (70 and 78) are the products of alternative translation initiation *(20)*. However, several larger isoforms (X, Y, and Z) have not been fully characterized. Unlike the shorter PKIβ isoforms, X and Y also inhibit cGMP-dependent protein kinase. The Y and X isoforms emerge later than the 70 and 78 forms, at about d 40 or later (elongating spermatid stage). The larger isoforms dominate in mature spermatozoa. It is uncertain whether these larger isoforms arise from alternative splicing, alternative translation, or posttranslational modification.

Also specific to the testis is an isoform of the PKA catalytic subunit called Cγ *(21)*. The product of a unique gene *(22)*, Cγ differs markedly from other C-subunit isoforms in that it is not inhibited by a peptide based on the pseudosubstrate motif of PKIα *(23)*.

CREB

A determination of the causal link between cAMP signaling and gene transcription began with the identification of a consensus cAMP response element (CRE) (TGACGTCA) by analysis of several cAMP-inducible promoters *(24,25)*. Thereafter followed the identification and isolation of CREB, a protein capable of binding this DNA element *(26,27)*. CREB was further characterized as a factor that promotes gene expression in a cAMP-responsive manner *(26,27)*. CREB comprises four distinct domains (Fig. 2A). These domains include two glutamine-rich transactivation domains flanking a phosphorylation box (P-box), otherwise known as a kinase-inducible domain (KID) *(27)*, which contains the PKA phosphorylation site. The carboxy-terminal region contains a basic region involved in DNA contact, and an adjacent leucine zipper region required for dimerization. Together they form the bZIP domain critical for DNA recognition and binding to CREs.

CREB is expressed ubiquitously in all tissues examined to date. The dominant forms of CREB are the larger activator proteins. A small exon D is alternatively spliced in CREB mRNA in all tissues, and changes the overall length of CREB from 327 to 341 amino acids. The function of exon D is uncertain. One report suggests that it may alter the transactivation potential of CREB *(28)*. The transcription of the CREB gene is autoregulated via the interactions of CREB and/or CREB-like proteins with two adjacent CREs located in the promoter of the gene. As discussed subsequently, the cyclical changes in the levels of cAMP in the seminiferous tubules during the 12-d cycles of spermatogenesis appear to control the expression of the CREB gene.

In the testis, several additional exons (Ψ, W, Y, or γ, and Z) are alternatively spliced into CREB mRNAs *(29–32)*. Exons W, Y, and, in humans, Z, are incorporated into a substantial (~50%) proportion of CREB transcripts, and their splicing is regulated during the course of spermatogenesis. The significance of these exons is discussed further in "Temporal Regulation." Other splice variants of mRNAs transcribed from the *creb* gene also exist (reviewed in ref. *33*), including a thymus-specific form lacking the second glutamine-rich transactivation domain *(34)*.

Targeted disruption of the *creb* gene has been accomplished in mice. The *creb* null mice have a perinatal lethal phenotype, owing to a deficient production of respiratory surfactant–associated protein and resultant atelectasis. They also have developmental defects including brain and T-cell lymphocyte development *(35)*. Heterozygous (*creb* +/–) mice are fertile but the testes have not been examined in detail. Interestingly, an

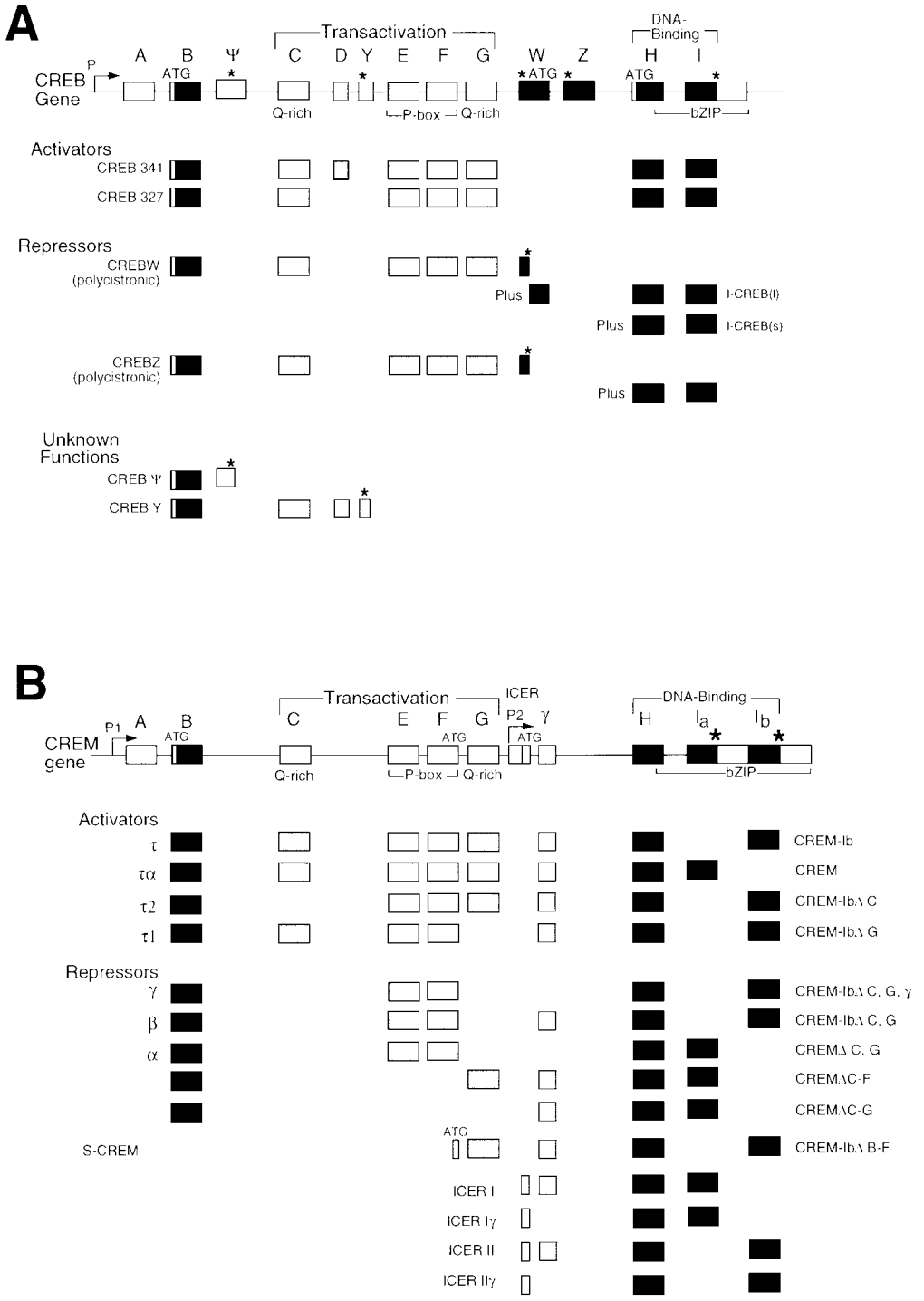

Fig. 2. (A) Isoforms derived from the CREB gene by alternative splicing and use of alternative translation initiation codons. **(B)** Isoforms derived from the CREM gene by alternative splicing, use of alternative translation initiation codons, and an internal promoter. (Modified from ref. *33*.)

earlier attempt to knock out the *creb* gene by targeting the first coding exon (exon B) was only partially successful owing to the upregulation of a previously unrecognized CREB isoform, called CREBβ, and present at low levels in tissues of normal animals *(36)*. The isoform retained activity by splicing the untranslated exon A to the second coding exon (exon C). Translation then initiated from a cryptic AUG codon in exon C, resulting in a partial deletion of the first transactivation domain. Consequently, the mice survived gestation and appeared normal, although subtle defects in long-term memory consolidation, opiate withdrawal, and gliosis were found *(36)*.

CREM

CREM is closely related in its structure to CREB. The full-length activator isoform (CREMτ) is almost identical in structure to CREB (Fig. 2B). The pattern of alternative exon splicing of CREM is even more complex than it is for CREB. In the majority of tissues examined, the dominant CREM isoforms lack the activator domains required for transactivation of gene transcription *(37)*. CREMτ was originally discovered in germ cells of the testis. The protein first appears during stages VII–VIII of spermiogenesis. Its regulation and role in spermatogenesis are discussed in "Expression of CREM Activator and Repressor Isoforms." An important feature of the *crem* gene that distinguishes it from the *creb* gene is the existence of two alternative DNA-binding domains (DBDs), selected by alternative RNA splicing. The upstream of the two domains (DBD I) is more homologous to the DBD of CREB, whereas the second domain (DBD II) is more divergent, and therefore may have a DNA-binding selectivity different from that of DBD I. CREM may also have a role in compensating for the partial knockout of the *creb* gene, because CREM mRNA levels were greatly elevated in these mice *(38)*. Although CREMτ is most strongly expressed in testis, it is also expressed diffusely in brain and at lower levels in other tissues *(39)*.

In addition to the formation of multiple CREM isoforms that arise from the translation of alternatively spliced RNAs, the *crem* gene has a second internal promoter (P2) that drives expression of a truncated protein containing either DBD I or DBD II, but contains no amino-proximal transactivation domains. These truncated CREM proteins bind to CREs and repress cAMP-induced transactivation by competing for the binding of transactivator isoforms of CREM and CREB. These CREM proteins are produced by the P2 promoter and are called inducible cAMP early repressors (ICERs) *(40)*. Interestingly, the internal P2 promoter is strongly induced by cAMP because it contains four closely spaced cAMP-activated response elements and is particularly abundant in neuroendocrine cell types *(41–43)*.

Mice with a targeted disruption of the *crem* gene exhibit an interesting reproductive phenotype. Male *crem* null mice are sterile (female mice are fertile). Spermatid development is arrested at stage VII, the time of maximum CREMτ expression in the seminiferous tubule *(44,45)* (*see* "Expression of CREM Activator and Repressor Isoforms."). The *crem* null mice also have elevated levels of FSH and androgens. Because disruption of the *crem* gene eliminates both DBD I and II, the ICER CREM isoform is also absent in these mice. Consequently, *crem*-deficient mice overexpress serotonin *N*-acetyltransferase, a rate-limiting enzyme in melatonin synthesis and a regulator of circadian rhythms *(46)*.

cAMP signaling activates CREB and CREM by way of PKA-mediated phosphoryla-

tion on a specific serine residue within the KID domain of CREB and CREM. Because the catalytic subunits of PKA rapidly translocate to the nucleus following cAMP-induced dissociation from the regulatory subunits *(47),* the phosphorylation of CREB and CREM occurs within the nucleus. Phosphorylation of CREB and CREM by PKA is required for them to associate with the transcriptional coactivator, CREB-binding protein (CBP) *(48,49).* CBP is proposed to be a multifunctional coactivator protein. The KID domain alone appears to mediate the CBP interaction *(50),* although phosphopeptides based on this sequence have a lower affinity for CBP in comparison to longer CREB-derived proteins *(49,51).* For transactivation, CREB and CREM also require one or both glutamine-rich transactivation domains *(52).* Thus, the CREM isoforms α and β act as inhibitors at CREs, although they may still be able to recruit active CREB to promoter sites through heterodimerization *(53)* and thereby act as pseudoactivators.

ROLE OF cAMP SIGNALING IN THE CONTROL OF SPERMATOGENESIS

The developmental maturation of the germ cells in the seminiferous tubules of the testes is a cyclical process. Each cycle takes place in approx 12.5 d in the rat. Approximately 3.5 cycles are required for the stem spermatogonia cells to become mature spermatozoa (*see* Fig. 1). In the rat, tubule segments of roughly 2.5–3.0 cm encompass a single spermatogenic cycle, which can be visualized by transillumination as a gradual darkening of the tubule interior. This darkening of the transilluminated tubule is caused by a condensation and a thickening of the density of germ cells, and abruptly changes back to translucency at the time of the release of the spermatozoa from the lumen of the tubule, which is called spermiation. In experimental practice it is possible to dissect and isolate 1- to 3-mm segments of the tubules at precisely identified and defined temporal and anatomical stages of germ cell development. Those small segments of the seminiferous tubules can be analyzed for the expression of specific genes (by reverse transcriptase synthesis of cDNA and amplification of the cDNAs by the polymerase chain reaction [PCR] procedure). The cycle is arbitrarily divided into 14 distinct cell association stages *(54)* (Fig. 3).

Stem germ cells that are committed to develop into spermatozoa pass through approx 3.5 cycles (Fig. 3). During the first cycle, spermatogonia cells reach the stage of spermatocytes, commencing the early stages of meiosis, and become pachytene spermatocytes by the start of the second cycle. Meiosis is completed at the end of the second cycle (stage XIV). In the third cycle, haploid round spermatids develop acrosomes and begin elongating. In stages I–VIII of the fourth cycle, the spermatids mature, shed the residual body, and finally release into the tubule lumen. The stages therefore define a series of cellular association states, and at any given point in a cycle, three to four generations of developing germ cells will be present, in addition to the population of originating stem cells/spermatogonia (reviewed in ref. *55*). Each cycle takes approx 12.5 d, making a total of approx 42 d from the initiation to the completion of spermatogenesis.

A defining feature of the 14-stage cycle is the marked elevation in cAMP levels between stages XII and V. This cyclic modulation occurs in part as a consequence of changes in sensitivity to pituitary FSH by Sertoli cells in the tubule *(56).* As previously discussed, an endogenous cAMP generator also exists in the form of PACAP, which is produced stage specifically by germ cells *(1).* PACAP acts on cAMP-coupled receptors

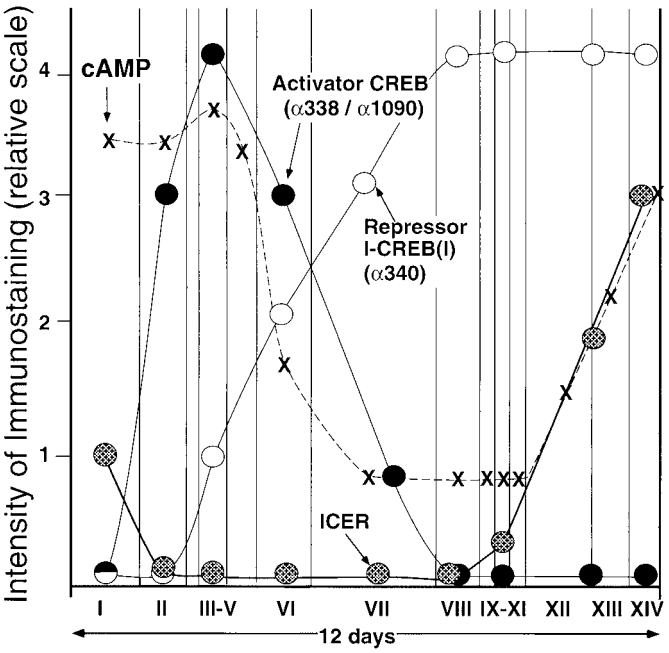

Fig. 3. Proposed cycles of expression of cAMP, activator CREB, repressor CREB (I-CREB), and repressor ICER in the seminiferous tubule during spermatogenesis. Shown are the relative levels of expression of the transcription factors during the repetitive 12-d cycles of germ cell development (germ cell association stages I–XIV) in the seminiferous epithelium. The cyclical fluctuations in cAMP levels is as reported by Parvinen et al.). The relative amounts of the transcription factors were determined by semiquantitative immunostaining of tubules, using the antisera (designated α) specific for the detection of the CREB isoproteins. Vertical lines delineate the successive cell association stages during germ cell development; spacing between the vertical lines denotes the approximate fraction of time spent in each stage during the 12-d cycle.

located on Sertoli cells *(2)* and possibly germ cells *(57)*. It is conjectured that PACAP actions on Sertoli cell receptors is a major source of the cyclical fluctuations in levels of cAMP in the seminiferous tubules during the stages of spermatogenesis (*see* Fig. 3).

TEMPORAL REGULATION OF THE ALTERNATIVE SPLICING OF EXONS IN THE CREB mRNA DURING CYCLES OF SPERMATOGENESIS INTERCONVERT TRANSCRIPTIONAL ACTIVATORS TO REPRESSORS

In the testis of mouse, rat, and human, several additional exons are spliced in and out of the CREB transcripts in a stage-dependent fashion. These additional exons (W, Y, and Z) contain multiple translational stop codons in all three reading frames and thereby result in a premature termination of translation. Exon W is perhaps the best studied of these translational terminating exons. A potential function for exon W, and by extension to other exons that terminate translation, has been demonstrated; namely, to activate internal translation from a cryptic AUG translation codon resulting in the synthesis of truncated CREB repressor isoforms. Exon W (Fig. 4) consists of 63 nucleotides in rat and 62 nucleotides in human. The rat and human exon Ws are highly

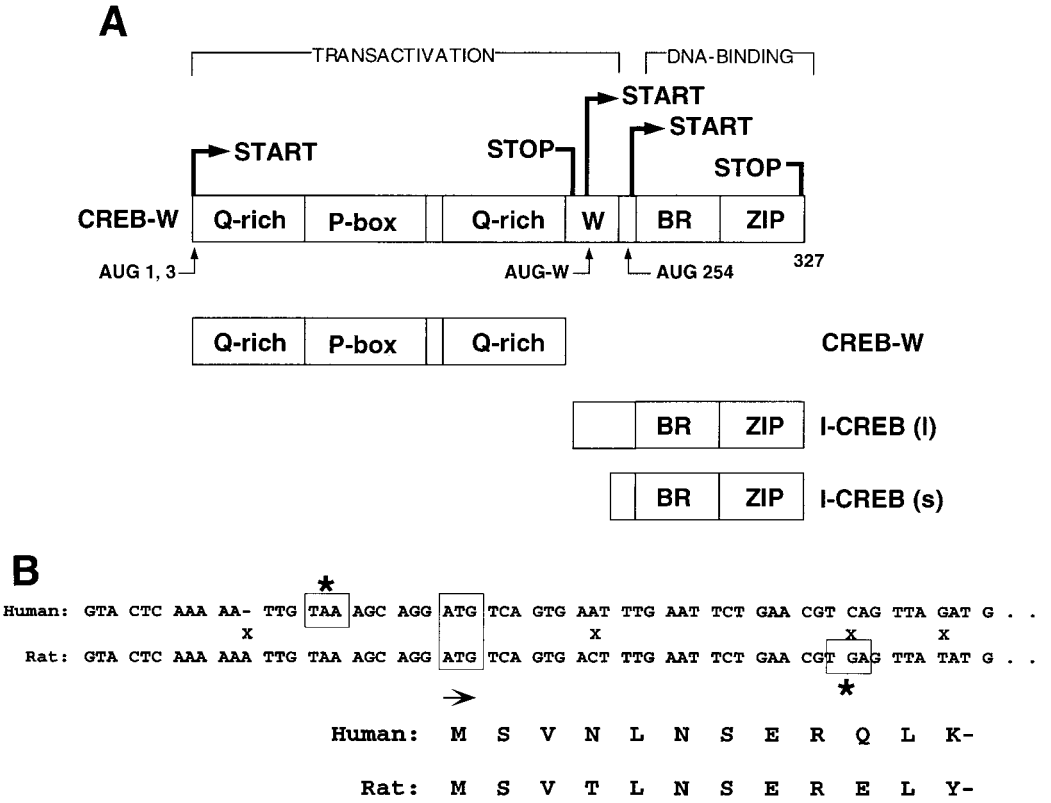

Fig. 4. **(A)** CREB-W encodes small repressor CREB isoforms. Diagrams are of the CREB-W functional domains encoded by the alternatively spliced CREB-W mRNA. Methionine initiator codons in-frame with CREB are shown. Translation of CREB initiates at either AUG1 or -3 and continues to the stop codon encountered after codon 327. Insertion of exon W terminates translation at a stop codon within exon W, resulting in the formation of the amino-proximal protein CREB-W. Insertion of exon W also allows reinitiation of translation at codon 8 of exon W (AUG-W, 7 nucleotides downstream of the stop codon in exon W) and at Met-254 to produce I-CREB(l) and 1-CREB(s) proteins, respectively. Sequences flanking AUG-W and AUG254 are consistent with the consensus translation initiation motif. **(B)** CREB exon W, showing conservation of nucleotide sequence between rat and human. Termination codons are marked with an asterisk. Translation from the marked ATG (arrow) produces I-CREB long, featuring the bZIP region of CREB prefaced with a unique 12 amino acids.

conserved; they differ in only four nucleotide changes. Exon W resides between exons G and H. The reading frame of CREB mRNA normally used in the synthesis of CREB is terminated within the sequence of exon W. The resultant truncated amino-terminal product is not predicted to have any DNA binding or nuclear localization domains *(58),* and its function, if any, is unknown. However, exon W contains a cryptic AUG codon that reinitiates translation in frame with the carboxy-terminal domain of CREB. The novel protein so formed by internal reinitiation of translation consists of the DNA-binding bZIP domain of CREB devoid of the transactivation domain, designated (inhibitor-CREB) (I-CREB). I-CREB is analogous to the CREM product ICER, because it binds to the same DNA control elements as the full-length protein but is unable to

transactivate and thereby function as repressors of CREB (and CREM). An antiserum raised against the unique 14 amino acids encoded by the RNA sequence in exon W peptide does, in fact, detect products in the germ cells of seminiferous tubule sections encompassing stages V–XIV *(59)*.

In addition to the I-CREB produced by translational reinitiation within exon W, a second I-CREB is produced by reinitiation of translation within exon H (Fig. 4). Thus, two internally translated I-CREBs are formed, called I-CREB long (l) and I-CREB short (s), of 16 and 8 kDa, respectively. Both potential start codons feature surrounding nucleotide sequences consistent with the "Kozak" consensus sequence for translation initiation *(59)*. The mechanism of the internal translation originating with exon H was examined by the insertion of a stable stem-loop RNA structure between the exon W and exon H start AUG codons, a circumstance recognized to abolish further scanning of the mRNA by the 40S ribosome. The presence of the stem-loop structure enhanced I-CREB(s) production in in vitro translation assays, supporting the existence of an internal ribosomal entry site in the CREB mRNA between exons W and H *(59)*.

In human testis, an additional exon Z is cospliced along with exon W into the CREB mRNA *(32)*. A conserved sequence homologous to exon Z resides in the intron between exons W and H in the genomes of rats and mice, but is not spliced owing to naturally occurring mutations in the RNA splice sites. The inclusion of exon Z adjacent to exon W results in a termination of translational reading frame of I-CREB(l) and increases the expression of I-CREB(s).

The Y exon is also highly conserved between rats and humans, and terminates the translation of CREB when it is included in the CREB mRNA. However, exon Y does not feature any AUG translation start codons suitable for the reinitiation of translation within the CREB reading frame. By using reverse transcriptase-PCR to examine the expression of exons W and Y during the course of the spermatogenic cycle, it was determined that the splicing of the two exons is highly regulated *(60)*. The levels of CREB mRNA rise and fall during the spermatogenic cycle, reaching their peak along with cAMP levels, a transcriptional response of the *creb* gene attributable to the CREs that reside in the CREB promoter. The proportion of transcripts from the *creb* gene containing exons W or Y also undergoes significant variation. The proportion of *creb* transcripts that contain exon W closely correlates with the increase in *creb* mRNA levels. Exon Y, however, reaches a peak in relative abundance later than exon W in the spermatogenic cycle and remains high even as overall CREB levels decrease *(60)*.

Although the precise identification of the cells in the testis that express the W and Y forms of CREB mRNA is uncertain, mid-to-late pachytene spermatocytes have the strongest immunostaining for exon W using an antiserum specific for the unique epitope in I-CREB(l) *(59)*. A significant amount of mRNA containing both the W and Y exons is also detectable in segments of tubules that repressed from stages II–VIII of spermatogenesis, showing that the expression of transcripts containing exons W and Y overlaps in at least one cell type. The functional effects of exon Y on I-CREB expression remain uncertain. The high degree of conservation between rat and human exon W (and exon Y) suggests that I-CREBs have a functional role in spermatogenesis. A likely function of the I-CREBs is to downregulate cAMP-induced gene transcription by acting as repressors to inhibit the binding of activator forms of CREB and CREM to CRE sites in the promoters of cAMP-responsive genes. The persistence of high levels of the I-CREBs throughout stages V–XIV of spermatogenesis *(59)* may contribute

to the unresponsiveness of the cells to cAMP-induced gene transcription at these stages, thus reinforcing the importance of cAMP levels and the expression of the *creb* and *crem* genes at different stages of spermatogenesis.

EXPRESSION OF CREM ACTIVATOR AND REPRESSOR ISOFORMS DURING SPERMATOGENESIS

CREMτ is believed to be a critical regulator of genes involved in the maturation of postmeiotic germ cells. As discussed previously, a predominant phenotype of mice in which both CREM DBDs have been deleted by targeted disruption of the *crem* gene is male sterility, owing to a developmental arrest and apoptosis of germ cells at the stage of the development of round spermatids *(44,45)*. Heterozygous animals are fertile but have a reduced sperm count and also have increased numbers of spermatozoa that are developmentally deformed.

CREMτ binds to CRE control sequences located within promoters for several spermatid-specific proteins, including transition protein 1, and protamines 1 and 2 *(61)*. The mRNAs encoding these proteins are undetectable in the *crem* null mice, as is mRNA for calspermin, mitochondrial capsule selenoprotein, *krox 20,* and *krox 24*. A direct effect of CREMτ has been demonstrated on the activation of the promoters of the calspermin *(62)* and testicular angiotensin-converting enzyme genes *(63)*. CREMτ activates testis-specific promoters within the genes for calmodulin IV and somatic ACE, resulting in the production of truncated isoforms of the proteins that exert novel functions important for the maturation of spermatids.

The regulation of gene transcription by CREMτ has several unusual features. First, the change from repressor to activator isoforms occurs as a result of splicing in of the τ1 and 2 exons during spermatocyte development to convert the repressor CREMα mRNA to the activator CREMτ mRNA *(64)*. The accumulation of CREMτ mRNA is further augmented by a change in polyadenylation site selection that deletes RNA destabilizing sequences located within the 3′ untranslated region of the mRNA and thereby stabilizes the half-life of the CREMτ mRNA *(65)*. Second, the synthesis of CREMτ mRNA is enhanced in pachytene spermatocytes, but translation of the mRNA to the CREMτ protein is delayed until the stage of round spermatid development *(61)*. Sequestration of mRNA for later translation is an important generalized feature of germ cell development (reviewed in ref. *66)*. Such delayed translation of masked mRNAs is believed to be mediated by RNA-binding proteins such as TB-RBP *(67),* which binds to specific translational control sequences located in the 3′ untranslated regions of mRNAs *(68)*. The *deleted in azoospermia* gene product (DAZ) may also perform this function *(69)*. Another form of CREM apparently unique to germ cells is CREMΔC-G, a putative negative regulator expressed at significant levels in haploid germ cells, especially elongating spermatids *(70)*.

SUMMARY

An important shift in splicing events takes place in spermatocytes following the emergence of the pachytene spermatocyte, resulting in the conversion of activator to repressor CREB isoforms and, conversely, conversion of repressor to activator CREM isoforms. Notably, the germ cell line GC2spd, believed to be representative of the midspermatocyte stage of development, expresses several splice variants of CREB

mRNA, including transcripts containing exons Y and W, and CREM ΔC-G. The developmental switches in the alternative splicing of exons in the CREB and CREM transcripts must be driven by the emergence of factors capable of altering the specificity of splice-site recognition. The eventual identification and characterization of the spliceosome components expressed in the testis at specific stages of spermatogenesis will help elucidate the precise mechanisms involved in the alternative splicing of CREB and CREM mRNAs. Likewise, the change in the stability of the mRNA encoding CREMτ is also brought about by modifications in RNA processing.

The identification and elucidation of the functional aspects of the RNA-binding proteins that enhance either mRNA stability or translational efficiencies will provide novel insights into the control of gene expression in the testis. Based on the encouraging but incomplete information obtained so far regarding the role of the cAMP-responsive transcription factors CREB and CREM in the control of spermatogenesis, it seems reasonable to anticipate that continued investigations will shed new light on the functions of these transcription factors. It seems clear that both the CREB and CREM genes can be expressed in the form of either activators or repressors of gene transcription in the testis. Furthermore, the interconversion of transactivator to repressor, and vice versa, is regulated during the spermatogenic cycle by mechanisms of alternative exon splicing, alternative promoter usage, and changes in RNA stability and translational efficiencies of mRNA. Inasmuch as the whole process of spermatogenesis, encompassing the progression of stem spermatogonia cells to the development of mature spermatozoa is highly complex, it stands to reason that the target genes responsive to the CREB and CREM gene regulators will also be regulated in a complex manner.

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16 The Androgen Receptor, Androgen Insensitivity, and Prostate Cancer

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CONTENTS

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INTRODUCTION

Androgens control a wide range of processes in vertebrates, from important developmental events in embryogenesis, to functions occurring as a part of normal adult physiology *(1)*. In mammals, two steroids, testosterone and its 5α-reduced metabolite 5α-dihydrotestosterone (5α-DHT), serve as the principal circulating androgens. Individually, each of these hormones exerts specific functions relative to the events modulated by androgen. Both hormones, however, are required to account for the entire spectrum of androgen-regulated phenomena. How some processes are preferentially dependent on one or the other of the two hormones remains the subject of active study *(2)*.

Regardless of the mechanisms by which specific genes and processes are modulated preferentially by testosterone or 5α-dihydrotestosterone, it appears that androgen-mediated events are exerted via a single androgen receptor (AR) protein that is encoded on the X chromosome. Because normal 46,XY males are hemizygous for genes encoded on the X-chromosome, only a single AR gene is present in normal men. This, and the fact that androgens do not appear to be essential for life, likely account for the relatively frequent occurrence of clinically apparent defects of virilization that constitute the spectrum of androgen resistance caused by defects of the AR *(1,3)*.

THE AR: STRUCTURE AND ORGANIZATION

The AR is a member of a large gene family that includes all of the classic steroid receptors, as well as a host of related proteins such as the thyroid hormone receptor (TR), vitamin D receptor, and retinoid receptors *(4)*. Each of these proteins shares certain structural characteristics, including a centrally located DNA-binding domain (DBD), a carboxy-terminal hormone-binding domain, and an amino terminus of variable length (Fig. 1). In addition to receptors for classically defined hormones such as the steroid hormones, thyroid hormone, and the retinoids, a large number of proteins have been characterized that share the same overall organization, but for which ligands have not been identified (the "orphan" receptors) *(4)*.

The functional attributes of each of these domains have been carefully examined

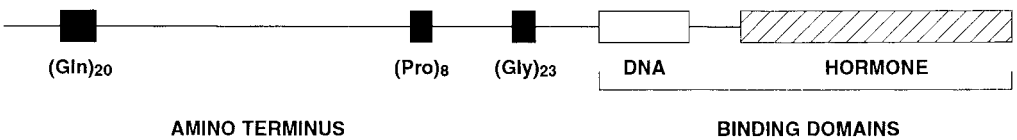

Fig. 1. A schematic of the structure of the human AR is presented based on the predicted amino acid sequence of the receptor protein. The relative positions of the DBD and hormone-binding domain are indicated. The amino terminus comprises more than half of the predicted amino acid sequence and contains a number of polymorphic repeat elements, which are indicated. As the size of the glutamine repeats varies among the different AR cDNAs that have been isolated, the numbering coordinates employed by the individual groups are different. (The numbering systems used in many publications differ, owing to the differences in amino acid numbering resulting from different sizes of the polymorphic glutamine repeat in the cDNAs isolated by the different groups. The Androgen Receptor Mutation Database [available at http://www.mcgill.ca/androgendb] has adopted the numbering protocol of Lubahn et al. *[5]* and this numbering system has been employed in references to nucleotide and amino acid coordinates in this review as well.)

for a number of different receptors. It has been established that the central DBD is responsible for mediating the contact of the receptor protein with its specific DNA targets. This region, approx 80 amino acids in length, contains a series of eight conserved cysteine residues that serve to coordinate two zinc atoms as a component of each DBD (zinc fingers). Classic studies performed using site-directed mutagenesis demonstrated that specific residues within and adjacent to the two zinc fingers that comprise the DBD were responsible for the DNA binding specificity and dimerization of receptor molecules. These regions were referred to as the P- and D-boxes (reviewed in ref. *6*). The details of these structures and the interactions that they mediate have been defined for several receptors at the molecular level using nuclear magnetic resonance (NMR) spectroscopy and crystallographic methods (*see* below).

The carboxy-terminal hormone-binding domain is responsible for recognition of the specific ligand bound by the receptor protein. Although this segment does not display the same remarkable level of conservation between the different family members that is seen for the DBD, considerable sequence conservation is still evident when comparing even widely disparate members of this family. Deletion and linker-scanning mutagenesis demonstrated that the final ~250 amino acids of the receptor proteins are necessary for the high affinity binding of ligand *(7–9)*. Deletion mapping of the human AR indicates that the boundaries of the hormone-binding domain (HBD) of the human AR are quite similar *(10)*.

The function of the amino terminal domain, however, remains somewhat unclear. In transfection studies, this portion of the receptor protein is clearly required for full functional activity. Studies conducted to determine the exact mechanism by which this portion of the receptor molecule is required have not been completely fruitful. Attempts to define discrete segments of the amino terminus that are required for mediating full receptor function have not identified individual critical residues within this region, as was possible for studies of the DBDs and hormone-binding domains. Instead, these studies have identified larger regions of the receptor that must be present within the amino terminus that are required for full receptor function to be achieved (*[11–13]*, and references within). Whether this requirement is owing to the contribution of residues that contact ancillary proteins, or because the conformation of the amino terminus influences the capacity of the remainder of the receptor to bind hormone or to bind DNA properly, has not been established.

In addition to these functional domains, the human AR is somewhat unusual in that it contains three segments within the amino terminus that are composed of direct repeats of single amino acid residues (Fig. 1). Although unusual as to their size and number, these homopolymeric repeats are not unique to the AR and are encountered in a number of other transcription factors. Recent studies have suggested that the present structures of these elements have only recently evolved in primates *(14)*.

The importance of these repeated domains varies considerably. The proline homopolymeric repeat appears to be relatively constant in size and instances in which variations in the length of this segment exist in the normal population have not been reported. Likewise, the glycine homopolymeric repeat is also relatively invariant in size, although length polymorphisms have been identified in some analyses *(15)*. In contrast to the glycine and proline repeats, the glutamine homopolymeric domain displays considerable variation, even in the normal population *(16–18)*. These polymorphisms have even been employed in family studies to track the inheritance of

specific AR alleles. In addition to their importance as markers, it appears that variations in the length of the glutamine homopolymeric segment of the human AR have pathological implications as well. Expansion of the glutamine repeat is implicated in the pathogenesis of spinal bulbar muscular atrophy (SBMA) *(19),* and a shortening of the glutamine homopolymeric domain has been suggested by recent studies to identify individuals having an inherent increased incidence of developing aggressive forms of prostate cancer (*[20–23], see* "AR Polymorphisms and Prostatic Cancer").

METHODS TO MEASURE AR ABUNDANCE

The identification of steroid receptors was the result of the synthesis of tritiated steroid hormones of sufficient specific activity to permit the detection of specific binding moieties in tissues and cells *(24).* In like fashion, the AR was originally characterized on the basis of its capacity to bind tritiated androgens, such as 5α-DHT, and assays to detect the AR using these reagents were initially limited to binding assays *(25–27),* exchange assays, or autoradiography using the tritiated ligands *(28–30).* Although ligand-binding assays permit the detection and quantitation of functional, unoccupied receptor molecules, the sensitivity of such assay methods is limited by the specific activity of the tritiated ligand that is employed, and such methods cannot measure altered forms of the receptor that do not bind hormone normally. Furthermore, although exchange assays have been employed to measure AR abundance, it is not clear how accurate such exchange methods are in measuring the AR, because such techniques have only a limited capacity to measure the levels of AR to which hormone is bound. Finally, with the exception of autoradiography, these methods employ broken cell preparations, and any heterogeneity within the individual tissues will go undetected.

Subsequent to the determination of the predicted primary sequence of the AR, a number of investigators developed specific antibodies that permitted the detection of the AR using immunoblot and histochemical assays *(31–37).* Antibody-based assays of the AR possess several distinct advantages. First, they require only that the epitopes recognized by the individual antibodies are intact. As such, the results are not usually affected by the occupation of the receptor by agonist or antagonist ligands. Second, such assays do not require that the hormone-binding domain be capable of binding ligand and can thus detect even forms of the receptor that are unable to bind hormone. Finally, the use of such antibodies in histochemical assays permits the facile identification of individual cells or cell populations expressing even low levels of the receptor protein.

Despite these distinct advantages, however, such assays also possess inherent limitations. First, the antibodies are unable to distinguish functional molecules from denatured forms of the receptor. Second, many of the most widely used antibodies have been raised to a limited number of epitopes within the amino terminus. As such, receptor proteins that do not contain the epitope recognized by the antibody used will not be detected.

METHODS TO MEASURE AR FUNCTION

Studies of the effects of mutations of the AR have demonstrated that the degree to which androgen action is deranged is reflected to varying extents, depending on the assays that are employed. Measurements of the levels of binding of ligand or the levels

of immunoreactive AR may not reflect the degree to which the activity of a mutant receptor has been altered. For this reason, it has been necessary to develop methods to assess the functional activity of normal and mutant ARs. These tests have centered principally on measuring the capacity of the AR to modulate the transcription of model-responsive genes. Several different reporter genes have been employed in such measurements, including elements derived from the mouse mammary tumor virus (MMTV) promoter *(38,39),* the prostate-specific antigen (PSA) promoter *(40),* and the probasin promoter *(41),* as well as artificial constructions based on the thymidine kinase promoter (e.g., the PRE_2-TK promoter construct). In experiments employing these plasmids, cDNAs encoding the normal or mutant receptor are introduced into recipient cells, incubations are performed with or without the ligand being tested, and the activities of the reporter gene (most commonly chloramphenicol acetyltransferase or luciferase) are measured. By comparing the activity of mutant receptors in such assays to those in which aliquots of the normal AR cDNA are introduced, it is possible to assess the functional activities of individual mutant receptors.

THE AR AND MODELS OF STEROID RECEPTOR FUNCTION

A great deal of progress has been made in unraveling the mechanisms by which steroid hormones alter the activity of responsive genes. Initial work centered on the use of biochemical assays to characterize the AR using the binding and fractionation techniques that were available. These studies established that the AR, like many other steroid hormone receptors, exists in cells as a complex with a number of ancillary proteins, including chaperones such as HSP70 and -90, when examined under conditions of low ionic strength and in the absence of ligand *(42,43).* Incubation with ligand results in the dissociation of the ancillary proteins and the acquisition of the capacity to bind to DNA. Although some differences may be evident under selected circumstances, it appears that for the most part, the majority of the immunoreactive AR is localized to the nucleus. Studies conducted in transfected cells demonstrated that this localization is the result of sequences contained within and adjacent to the DBD *(44,45).*

The nature of the conformational changes that take place following the binding of ligand have been explored using several different techniques. Studies using limited proteolysis demonstrated that the patterns of protease cleavage products change after the incubation of receptor preparations with ligand. Such studies, conducted first for the glucocorticoid receptor (GR) and progesterone receptors (PR), established that the binding of steroid receptor agonists and antagonists resulted in discernibly different conformations of the ligand-binding domain (LBD) *(46,47).* Such studies have been extended to an assessment of the conformational changes occurring with the binding of the agonists or antagonists by the human AR *(48,49).* As with the PR and GR, distinctive conformations result after the binding of agonist or antagonist ligands.

In recent years, conclusions regarding the structure and conformation of the domains of the nuclear receptors that have been derived from such indirect studies have been extended to the molecular level for several members of the nuclear receptor family. Crystal and solution NMR structures have now been solved for important domains of the GR, PR, and ER, as well as retinoid X receptor and peroxisome proliferator–activated receptor (PPAR). Although the primary amino acid sequences of the corresponding segments differ, considerable conservation of form has been observed at the molecular

level that parallels the homologies of sequences that are evident among the different proteins.

The structures of the DBD of the steroid receptors were first determined for the GR and ER DBDs using solution NMR spectroscopy *(50,51)*. More detailed information has been obtained from the crystal structures of these and other DBDs that have been solved complexed to target DNA sequences *(52–59)*. These studies established that the organization that had been postulated on the basis of similarities to the structure of TFIIIA was correct and consisted of two zinc atoms coordinated by the conserved cysteine residues. α-Helical segments follow each of the zinc fingers and are separated by a more extended segment (Fig. 2). The two helical segments are oriented perpendicular to each other.

The solution of the crystal structures of steroid receptor DBDs complexed to target DNA sequences has revealed the molecular basis of the binding specificity and spacing of the monomeric units on model hormone response elements. Binding of the individual units to a palindromic response element is accomplished by the binding of a monomer to each half-site of the response element in a head-to-head fashion. In this configuration, the first α-helix presents itself to bases in the major groove of each half-site, and protein-protein contacts between the residues that constitute the two D-boxes contribute to the dimer interfaces. Direct contacts are made between residues at the tip of the amino-terminal zinc finger and the phosphate backbone at the inner aspect of the dyad spacer. Additional contacts are made between residues at the tip of the carboxyl-terminal zinc finger and the phosphate backbone. Residues within the residues comprising the P-box make direct contact with bases within the half-site target sequence, and such interactions contribute a large part of the specificity inferred from mutagenesis studies *(60–62)*. Structures solved for complexes between the DBDs and "noncognate" DNA targets suggest that the intercalation of additional water molecules results in the formation of complexes that are structurally similar, but that are presumably less entropically favorable *(56)*. Although similar structures have not been solved for the AR DBD, the high degree of sequence conservation has permitted modeling of these structures to be performed *(63,64)*.

Increasingly detailed information is becoming available regarding the structures of the HBDs of several members of the steroid and nuclear receptor family *(65–73)*. Although the structures available represent a divergent group of proteins within this family, in each instance the overall organization of the LBD is highly similar and consists of a three-layered collection of α-helices. The central core is composed of the helices H5/6, H9, and H10, whereas the outer layers are composed of the helices 1–4 and H7, H9, and H11. This helical framework surrounds a hydrophobic core that comprises the ligand-binding pocket. The LBD of the hPR is among the most highly related to that of the hAR and is thus deserving of particular note. The PR LBD structure—although highly conserved compared to other members of the nuclear receptor family—exhibits several distinct structural features *(73)*. Although displaying the same "helical sandwich" *(67)* framework that has been observed previously in other nuclear receptors, it contains no helix 2, and helix 10 and 11 are continuous. In addition, the PR has a longer helix 12 and a 12 amino acid residue carboxy-terminal extension that is important for hormone binding. The bound hormone molecule contacts residues from helices 3, 5, 7, 11, and 12, as well as the beta turn. The determinants of ligand specificity that are observed in the PR LBD (Fig. 3C) are likely to be important

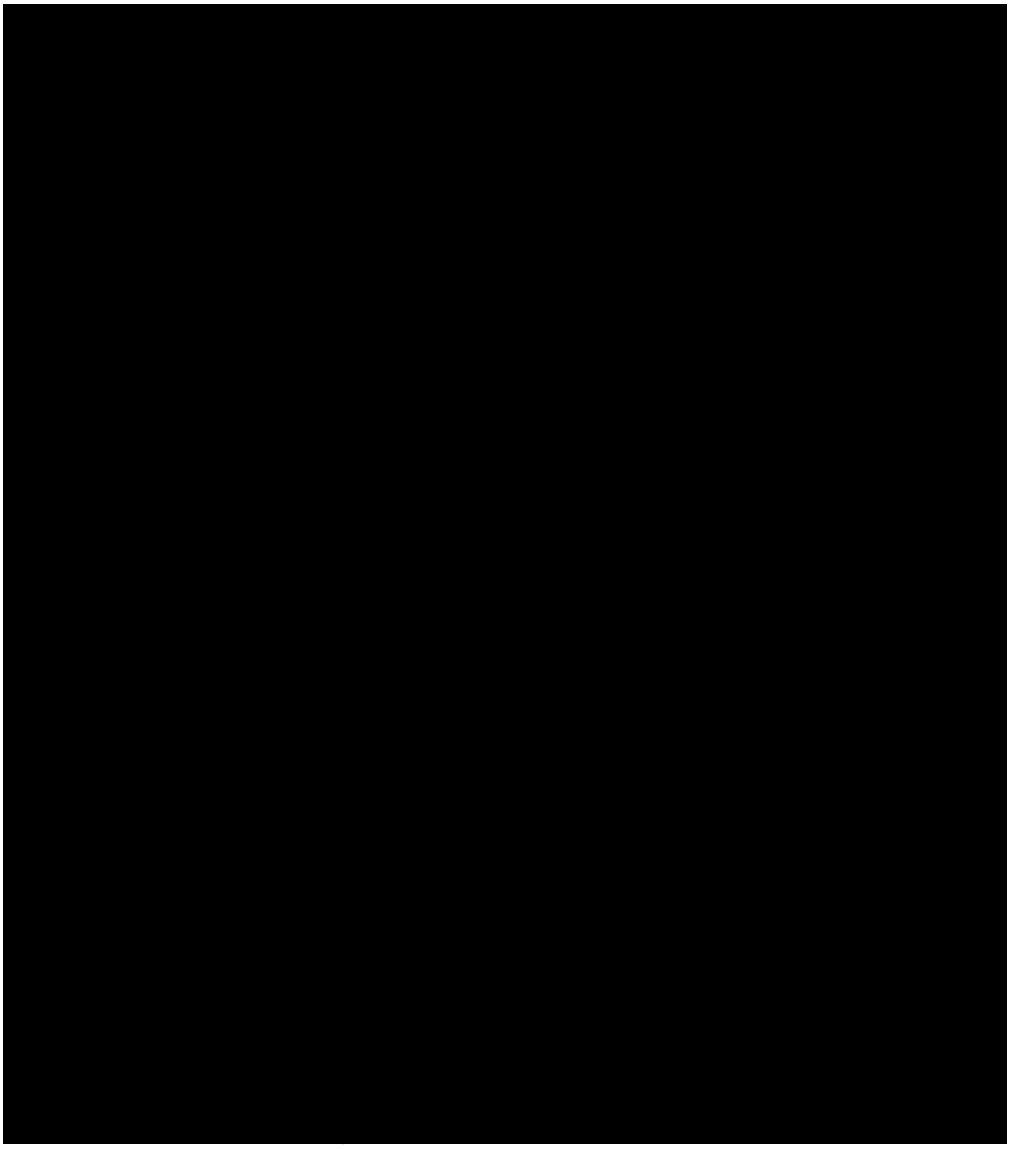

Fig. 2. The binding of the DBD of the rat GR binding to its palindromic response element. A ribbon diagram of the interaction of the GR DBD interacting with a target DNA sequence is shown. Each monomer contains two α-helical elements. The amino-terminal helix lies in the major groove of the DNA helix and makes base-specific contacts. The carboxy-terminal helix is oriented at approximately a 90° angle relative to the amino-terminal helix, and residues within this segment make contacts with phosphate groups of the target DNA backbone. The sites at which the two monomers interact (centered on amino acid residue number 480) are visible in the center of the diagram. The coordinated zinc atoms are represented as black dots. Although such interactions have not been observed directly for AR, the sequence similarity between the AR and GR DBDs and their target DNA sequences predicts that the AR DBD-DNA interactions are similar (*see* text). (Reprinted from ref. *52* with permission. © 1991 Macmillan Magazines Ltd.)

contributors of ligand binding in the AR as well. This inference is based on the facts that the structures of the A-, B-, and C-rings of testosterone and progesterone are identical and that the residues identified as important ligand-receptor contacts in the liganded PR LBD are conserved in the AR LBD.

In the limited number of structures in which antagonist- and agonist-bound structures have been solved, distinctive changes exist between the agonist- and antagonist-bound LBDs. In the estradiol-bound LBD, H 12 is positioned over the ligand-binding pocket and is "packed" against H3, H5/6, and H11. Similar positioning of H12 is present in all of the liganded LBDs that have been analyzed and has been postulated to be required for the formation of a functional activation function-2 surface capable of interacting with coactivators. By contrast, in the antagonist-bound ER LBD, the orientation of the H12 helix is markedly different and interacts with portions of H5 and H3 *(71)*. Although further studies will be required to assess the generality of these observations, it seems likely that the formation of such distinctive structures represents the physical basis by which coactivators and corepressors are differentially recruited by ligand-bound steroid receptors.

MEDIATORS OF AR FUNCTION: COACTIVATORS AND COREPRESSORS

Current models suggest that following the recognition of DNA sequence elements within responsive promoters, activated steroid receptors facilitate the formation and stabilization of active transcription complexes. These complexes contain both basal transcription factors and other peptides that are characteristic of steroid-responsive transcription complexes. These interactions result in an increased synthesis of RNA from the responsive gene *(74)*.

The preceding discussion implies the existence of contacts between the activated steroid receptor and the basal transcription apparatus. These views have become considerably more complex with the identification of families of proteins that act as links between the steroid receptor family member and the core components of the transcription apparatus. Two general classes of molecules have been identified: corepressors and coactivators.

The discovery of corepressor proteins derives, in large part, from observations made in the process of characterizing the activities of cDNAs encoding the TRs. In these experiments, it was noted that the transfections of cDNAs encoding the TR inhibited the activity of model thyroid hormone-responsive genes in the absence of ligand *(75,76)*. Careful experiments traced the sequences within the open reading frame (ORF) to specific elements of the carboxy terminus of the receptor (within the LBD). Subsequent experiments demonstrated that transfer of this segment to heterologous fusion proteins conferred similar inhibitor properties on the resulting fusions. Biochemical and genetic approaches ultimately identified the two related proteins, nuclear receptor corepressor (N-CoR) and SMRT, that interacted with this segment of the thyroid and retinoid receptors and that were responsible for mediating the inhibition of target genes in the absence of ligand *(77,78)*. Additional studies established that N-CoR and SMRT (silencing mediator of retinoid and thyroid hormone receptors) were also important to the inhibitory effects exerted by ligands capable of acting as steroid receptor antagonists as well (*[79,80]; see* "Antiandrogens and AR Function").

Fig. 3. (A) Alignment of the primary amino acid sequences of members of the steroid receptor family and a summary of observed or predicted secondary structural features. The sequences of the LBDs of the human PR, estrogen receptor-α (ERα), AR, and mineralocorticoid receptor are aligned at the level of their primary amino acid sequences. Elements of the secondary structure of the PR that were observed in the PR crystal structure are shown above ("secondary structure"), and the shaded areas below represent regions in which conservation of such secondary structure elements has been observed or is predicted in the LBDs of other nuclear receptors. α-Helices are shown as light gray shading and β-sheets are shown as dark gray shading. (Reprinted by permission of ref. *73*. Copyright 1998, Macmillan Magazines Ltd.)

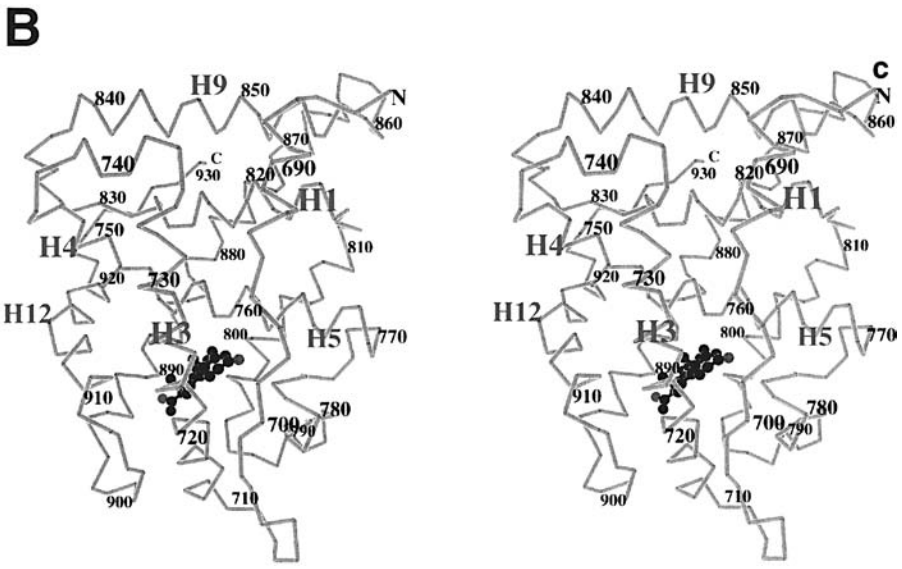

Fig. 3. (B) A stereo view of the α-carbon tracing of the structure of the human PR LBD. The position of the ligand within the layered α-helices that comprise the LBD is indicated as a red and black ball-and-stick model. The high degree of sequence similarity between the predicted amino acid sequences of the different nuclear receptor family members (above) and the conservation of the amino acid residues that correspond to the contacts between PR-progesterone suggest that the structure of the liganded AR LBD is similar to that observed for PR.

Investigations focused on characterizing the proteins that act as links between activated steroid receptors and the transcription apparatus to mediate the activation of responsive genes proceeded in parallel with studies of the corepressor proteins. The first of this class of molecules, termed steroid receptor coactivator-1 (SRC-1), was identified by Oñate and coworkers *(81)* as a protein that interacted with the carboxy terminus of the PR in a ligand-dependent fashion. Subsequent studies have identified a number of other proteins, several of which are related to SRC-1, that appear to serve similar properties roles in the activation of responsive genes *(82–92)*. Note that many of these proteins are capable of interacting with a number of different members of the nuclear receptor family. Recent studies of the structure of a ternary complex of PPAR-γ, its ligand, and segments of SRC-1 suggest that highly conserved residues in the LBD bind to backbone atoms of SRC-1 via "charge clamp" mechanism *(93,94)*. It remains to be determined whether such a mechanism will be found to underlie the recruitment of coactivators by other, more distantly related members of the nuclear receptor family, such as the AR.

As noted, many of the proteins that have been identified as coactivators of nuclear receptor function have been shown to be capable of interacting with multiple members of the nuclear receptor family. A much smaller number of proteins have been suggested to exhibit restricted specificity in terms of the receptor proteins with which they will interact. For this reason, Yeh and Chang's *(95)* report is of particular interest. In this initial report, these investigators identified a protein, ARA70, that interacted with the human AR in a ligand-independent fashion. This protein, also known as RFG (RET-fused gene) or ELE1, was previously identified as a fusion protein in a case of thyroid

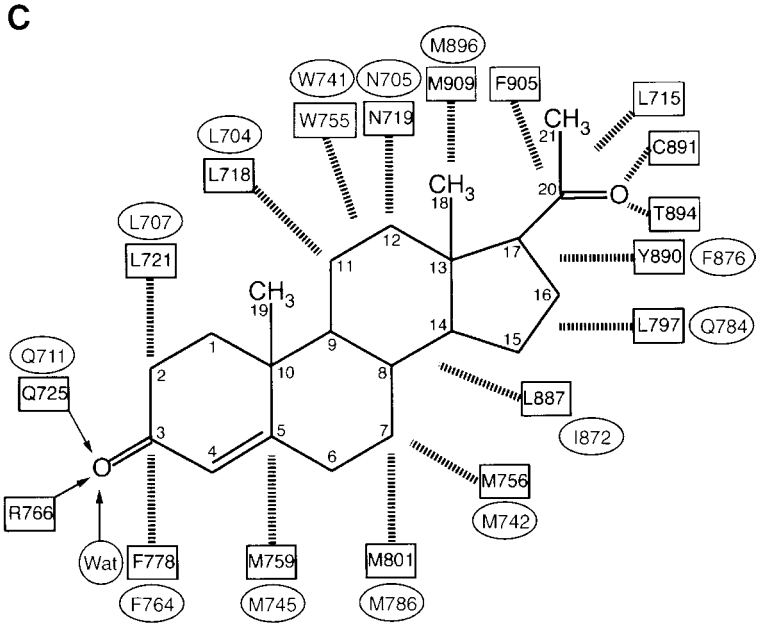

Fig. 3. (C) A summary of the interactions between the PR and AR LBDs and ligand. The amino acid residues indicated in squares are those contacts that were identified from the PR-progesterone crystal structure. Those shown in ovals are the corresponding residues of the human AR. Inspection of the PR-progesterone contacts and comparison of these residues to the AR primary amino acid sequence demonstrates that identical residues are present within the corresponding segments of the LBDs in both the PRs and ARs in those regions that are predicted to contact the ligand (at positions in which the structures of progesterone and testosterone are identical). This suggests that the ARs and PRs interact with their ligands in a similar fashion in the regions that their ligands possess identical structures (i.e., the A, B, and C rings of the steroid nucleus). (Modified from ref. *73* with permission. © 1998 Macmillan Magazines Ltd.)

carcinoma *(96,97)*. Interestingly, Yeh and Chang *(95)* reported that this interaction was restricted to the liganded AR, and was not observed with related members of this nuclear receptor family. Further, these investigators found that the expression of this protein enhanced the activity of the human AR receptor in a dramatic fashion in cotransfection assays *(95),* and that the expression of this protein even altered the ligand responsiveness of the AR *(98)*.

Although intriguing, these results have been the subject of considerable debate. Several reports have now appeared that complicate the interpretation of the suggestion that ARA70 (RFG, ELE1) acts as a specific coactivator of AR function. Gao and coworkers *(99)* found that although ELE1 (ARA70) was capable of interacting with the AR, it also interacted with the liganded PR *(99)*. Furthermore, these investigators found that the coexpression of ELE1 and the AR resulted in only small changes of receptor function, and no alteration in terms of the response to physiological levels of androgen. Such results have been mirrored by the findings of Alen et al. *(100),* who found little effect on the function of the human AR in the presence of the expression of the RFG cDNA. More recent studies have suggested that the RFG/EEE1/ARA70 protein is, in fact, localized to the cytoplasm *(101)*. At present, it is difficult to reconcile such divergent observations.

HIGHER-ORDER INFLUENCES: COINTEGRATORS, HISTONE MODIFICATION, AND CHROMATIN

Experiments conducted in several laboratories have identified additional "higher-order" components that participate in effecting or modifying nuclear receptor function. CREB-binding protein (CBP), originally identified as a modifier/modulator of CREB function, has been found to interact with a number of coactivators, such as SRC-1, and to play an important role in the "integration" of signals from a variety of sources *(102–106)*. Thus, it appears that the effects of corepressors and coactivators of steroid receptor function, including the AR *(107),* are mediated or modified by cointegrators, such as CBP and related proteins.

Much of the information summarized in the preceding discussion was derived from experiments performed in defined systems or in transfection assays that minimize or neglect the contribution of the chromatin structure in which genes exist in the nuclei of cells. Recent studies have proceeded on two fronts to attempt to assess the contribution of such influences. One line of experimentation has attempted to define the factors that regulate the assembly of chromatin and the use of such systems to reconstitute native chromatin structures in vitro *(108,109)*. When such studies have been applied to hormonally responsive systems, it has been found that the level of chromatin structure exerts profound effects on the responsiveness of target genes *(110)*. This type of influence has been even more directly implicated by the recognition that many of the cofactors involved in the transmission of signals between the nuclear receptor and the basal transcription apparatus possess the capacity to enzymatically modify chromatin structure. These activities, particularly histone acetyltransferase, are intrinsic properties of several coactivators or activities that are recruited by proteins that interact with these molecules (111–115). These results have complemented studies suggesting that an important aspect of nuclear receptor function is to recruit molecules that act to remodel the structure of chromatin to permit the accessibility of the transcription apparatus *(116,117)*.

Although parallel studies to examine each of these influences have not been adapted to the study of the AR, it is likely that the broad outlines of the pathway defined for related family members is applicable to the AR as well.

ANTIANDROGENS AND AR FUNCTION

Compounds that act to interfere with the normal actions of androgen have been in clinical use for many years. In most instances, such compounds have been defined on the basis of their ability to interfere with the binding of labeled androgen and the capacity to block the effects of androgen in a variety of bioassays *(118–120)*. Although a number of different agents have been described that are active both in vitro and in vivo, flutamide and related compounds are among the best studied.

Flutamide is a hydrophobic nonsteroidal molecule that has been widely studied for its activity as an antiandrogen. It has long been recognized that although flutamide itself is not particularly potent, it is rapidly converted by the liver to a hydroxylated derivative, 2-hydroxyflutamide, which is considerably more active as an antiandrogen. Cytochrome P450 1A2 has recently been identified as the enzyme responsible for catalyzing this reaction *(121)*. Several compounds have been synthesized using the 2-

hydroxyflutamide nucleus as a starting point for additional derivatives. Some, such as Casodex, have been approved for clinical use *(120)*. Interestingly many of the molecules that possess the 2-hydroxyflutamide nucleus as a component of their structure retain some degree of agonist activity *(122)*. Recently, a new class of compound—structurally unrelated to the flutamide class of molecules—has been described *(123)*. The properties of these agents are only now being explored.

Detailed studies exploring the corepressors and coactivators that mediate the agonist and antagonist properties of AR antagonists have not been described. It is likely, however, that much of what has been learned regarding the factors that modulate the agonist or antagonist activities of steroid receptor antagonists can be extrapolated to an understanding of AR antagonists as well.

As noted, the binding of agonist or antagonist ligands by the AR results in distinct conformations (e.g., that can be distinguished by limited proteolysis). These distinct conformations lead to the recruitment of coactivator or corepressor molecules that recruit components that result in the formation of an activated or a repressive complex. It has been suggested that molecules that display mixed activities (i.e., agonists/antagonists) display surfaces capable of permitting the formation of both types of complexes. In this context, it seems likely that several influences could act to alter the degree of agonism or antagonism that is exhibited by a compound in any given cell type or tissue. In some instances, the presence or relative abundance of corepressor or coactivator molecules could affect the activity that predominates, as has been suggested for selected nuclear receptor antagonists *(124–128)*.

While the preceding discussion takes into account information that has been learned relating to the activities of nuclear receptor coactivators and corepressors, other factors may also contribute the mixed agonism/antagonism that is characteristic of some agents. Hedden et al. have proposed that the existence and selective occupancy of distinct binding sites on the estrogen receptor α (ERα) might contribute to the different agonist or antagonist activities that are observed *(129)*. In the same way, changes in the metabolism or in the intracellular concentration of a compound that display a mixed agonist/antagonist profile (or its metabolites) could also act to influence the behavior exhibited by the parental compound *(121)*.

ALTERNATE MODES BY WHICH ANDROGENS MAY ACT TO REGULATE GENES

A large proportion of studies directed at understanding the mechanisms by which steroid receptors modulate the activities of responsive genes have focused on how steroid receptors regulate transcriptional activity by binding to the palindromic steroid response element (SRE) within or adjacent to a target gene. Despite this focus, there is substantial literature suggesting that steroids also act to regulate genes by more indirect mechanisms involving posttranscriptional effects or alterations in the stability of messenger RNAs. The repression of target genes by steroid receptors has also been described and has been suggested to involve negative response elements or transcriptional interference *(130–132)*. With this backdrop, the recent studies of Reichardt et al. *(133)* are particularly intriguing. These studies employed animals in which GRs were expressed that were defective in dimerization and thus incapable of inducing

genes regulated via canonical SREs. In contrast to mice in which GR function had been completely abolished, such GR_{dim} mutant mice were viable. Such results demonstrated that mechanisms other than those mediated by palindromic SREs may play an important—and in some instances unsuspected—role in mediating the responses to other steroid receptors as well.

MODIFIERS OF THE AR FUNCTION: LIGAND-INDEPENDENT AR ACTIVATION

Seminal experiments by Power et al. *(134)* demonstrated that under some conditions, steroid hormone receptors—normally dependent on the presence of ligand to modulate their activities—could be activated in the absence of ligand. These observations stimulated many investigations that examined the conditions under which the different members of the steroid hormone receptor family, including the AR, could be regulated in a ligand-independent fashion *(135–137)*. Although the physiological importance of these different pathways is, in many instances, still under active investigation, it appears that, at least in certain circumstances, such pathways are biologically significant *(138)*.

A consideration of different states in which androgen regulation of genes is perturbed makes the AR a logical potential target of ligand-independent pathways of AR activation. In particular, such pathways might provide a possible mechanism by which some prostatic tumors might progress to a state in which androgens are no longer required for growth (androgen-independent prostate cancer growth). Despite considerable interest by several groups, a substantial contribution of such pathways to the androgen-independent growth of prostatic carcinomas has been difficult to discern. Apart from a few observations, evidence supporting the concept that such pathways are important has not been forthcoming *(139–141)*. Note, however, that the demonstration that such mechanisms are operative might require specific physiological contexts not reproduced by many of the model cell systems available for study.

ANDROGEN ACTION AND THE DEVELOPMENT OF THE MALE PHENOTYPE

An understanding of the physiology of the genetic defects of the AR that have been described requires that the processes by which sexual development occurs be considered. In mammals, sexual development is a process that is controlled by the types of hormones produced by the gonads during specific periods of embryogenesis. The peptide and steroid hormones that are produced are dictated in turn by the differentiation events that occur as a result of the fetal chromosomal composition. In male embryos, at approx 9 wk of development, the testes begin to secrete testosterone. This androgen, in combination with its 5α-reduced metabolite, 5α-DHT, acts to induce the virilization of the internal and external genitalia. In this process, the external genitalia respond with the enlargement of the phallus and fusion of the genital ridges to form the scrotum. At this same time, the structures of the Wolffian ducts grow to form the pelvic portion of the urogenital sinus and give rise to the seminal vesicles and the epididymis. In addition to the events that are mediated by the action of the androgens, Müllerian-inhibiting substance, a polypeptide hormone produced by the Sertoli cells of the testes, acts to induce a regression of the Müllian duct–derived structures, including the uterus

and fallopian tubes. A number of reviews touching on the endocrine and molecular aspects of sexual development have been published *(1,142)*.

ANDROGEN INSENSITIVITY: A SERIES OF OVERLAPPING PHENOTYPES

Two aspects of androgen resistance caused by mutations in the AR gene are particularly remarkable: the number of patients who have clinically apparent defects of androgen action, and the range of subjects with different phenotypes that are available for study *(1,3)*. The relatively high frequency of these disorders has been attributed to at least three factors. First, because the AR gene is located on the X-chromosome, the effects of androgen are mediated by the product of a locus that is present in only a single copy in a normal 46,XY genotypic male. Thus, any functional defect of this single structural gene will be manifested (and not compensated for by an additional allele). Second, although defects of androgen action can result in abnormalities of sexual development, it appears that such individuals—even those severely defective in AR function—are normally viable. As a result, individuals affected by such defects are able to live and are therefore available for ascertainment. Third, abnormalities of sexual development are often discernible at birth, and their recognition stimulates studies designed to evaluate and diagnose such disorders.

The genesis of this spectrum is most easily understood as reflecting the degree to which the androgen-mediated steps of male sexual development have been disturbed. In instances in which the function of the AR is completely defective, none of the internal or external male structures develop. This clinical phenotype has been referred to as complete testicular feminization or complete androgen insensitivity (AIS). Owing to the inability to respond to androgen, these individuals show no sign of virilization and have normally developed external female genitalia. Although unable to respond to androgens, the high circulating levels of androgen can be aromatized to estrogen, which can act via the normal ERs present to mediate feminization. As such, affected individuals appear as normally developed females with normal breast development. Examination of such subjects may detect testes either within the labia majora or within the abdominal cavity. Because the testes are normal in such patients and produce both androgen and MIS, the Müllerian-derived structures—the uterus and fallopian tubes—are absent and the vagina is blind ending.

Individuals in which the function of the AR is less completely impaired may display a range of intermediate phenotypes *(1,3)*. These clinical syndromes have been referred to using a variety of terms, including Reifenstein syndrome, partial AIS, and incomplete testicular feminization. The phenotypes of affected individuals are characterized by differing degrees of virilization. Such individuals may exhibit a phenotype that is predominantly female (incomplete testicular feminization) or may display a predominantly male phenotype with severe urogenital abnormalities, such as perineal hypospadias (the Reifenstein phenotype). In recent years, some authors have defined a more detailed system to categorize patients with partial AIS *(3)*.

At the other end of the spectrum from patients with complete forms of androgen resistance are individuals in whom male sexual development is normal or near normal, but in whom processes that are mediated by androgen are not normal. In some such patients, subtle but distinct signs of undervirilization, such as gynecomastia, may be

present. In others, infertility or oligospermia appear to be the only manifestations of defective AR function *(1)*.

TYPES OF AR MUTATION

Mutations in the AR gene that cause androgen resistance can be categorized in a number of different ways. Owing to the manner in which the AR was discovered and the nature of the tools that were subsequently developed to study it, much information has been accumulated with respect to the effect(s) that such mutations have on the binding of ligand *(143,144)*. At this point, however, the numbers of mutations that have been identified permit substantially different characterization schemes to be employed. (A listing of mutations in the AR can be found on the Internet at the Androgen Receptor Mutations Database Web site at http://www.mcgill.ca/androgendb.)

Interruption of the AR Open Reading Frame

Several different mechanisms have been identified that result in an interruption of the primary sequence of the human AR. These mechanisms, although not unique to defects of the human AR, result in a reproducible spectrum of biochemical and phenotypic abnormalities. These mechanisms include large- and small-scale deletions, insertions, and alterations in AR structure caused by changes in mRNA splicing. Although the specific mechanisms causing each of these types of mutations are mechanistically different, the end result is a protein product that differs in primary amino acid sequence from that of the normal human AR protein sequence. This difference may be caused by a premature truncation or by the addition or removal of one or more amino acids from the receptor sequence. Note that although mutations that result in the premature termination of the receptor can have a dramatic effect on receptor function when introduced at virtually any position within the primary amino acid sequence, mutations that serve to insert or remove single or multiple amino acids—while maintaining the receptor reading frame—have been shown to result in syndromes of androgen resistance only when occurring within the DNA- or hormone-binding segments of the receptor protein.

MUTATIONS IN THE DNA-BINDING DOMAIN OF THE AR

A substantial proportion of patients with endocrine studies or family histories consistent with a defect of the AR do not display discernible abnormalities of ligand binding *(1)*. This group includes patients with diverse phenotypes such as individuals with complete testicular feminization, incomplete testicular feminization, and less severe effects. These defects were postulated either to be the result of subtle defects of AR function or to represent defects in genes other than the AR required for the normal AR function.

The analyses of the AR genes from affected individuals in a number of such families have now demonstrated that in pedigrees in which the family history suggests the inheritance of an X-linked trait, mutations within the conserved DBD of the receptor are frequently detected. In one such study, the AR genes of four unrelated subjects with complete or near complete forms of androgen resistance were analyzed, and in each instance, amino acid substitutions were localized to the DNA-binding region of the receptor protein. When analyzed in detail, these mutant ARs were found to bind

androgen with normal or near normal kinetics, as was predicted on the basis of studies performed using fibroblasts established from the individual patients. These same receptor proteins were found to be markedly impaired when assayed using a model androgen-responsive reporter gene. Studies performed in vitro using fusion proteins containing the normal or mutant AR DBDs indicated that in each instance the receptors displayed an impaired capacity to bind to target DNA sequences (androgen response elements) *(145)*. Similar findings have been obtained in studies conducted on patients with complete and partial forms of androgen resistance in several different laboratories *(146–150)*.

Furthermore, it is clear that mutations other than amino acid substitutions have the same effect on receptor function when they occur within the DBD and maintain the AR ORF. Such findings are based on the study of patients in whom in-frame deletions have removed one or more amino acid residues. Based on such studies, it appears that regardless of the nature of the causative mutation, mutations that alter the structure of only the DBD of the receptor cause AIS by interfering with a capacity of the receptor to recognize specific target DNA sequences. The studies published to date, although few, suggest that the degree to which DNA binding by the mutant receptors is impaired correlates with the degree of functional receptor impairment and the type of phenotypic abnormality that is observed.

MUTATIONS IN THE HORMONE-BINDING DOMAIN OF THE AR

Amino acid substitutions in the hormone-binding domain of the AR are the most frequent single type of mutation that has been encountered in patients with androgen resistance, accounting for approx 60% of all mutations in the AR genes that result in clinical phenotypes. Such mutations have been implicated as causing the entire spectrum of phenotypes: from complete testicular feminization to those that result in infertility or undervirilization. When carefully analyzed, these mutations fall into two general categories: those resulting in the absence of ligand binding, and those that result in qualitative abnormalities of ligand binding.

Amino Acid Substitutions that Cause Absent Binding

Amino acid substitutions in the hormone-binding domain of the AR that result in undetectable levels of ligand binding (in patient fibroblasts) appear to fall into two groups. The first is infrequent and likely represents the substitution of residues in critical regions of the hormone-binding domain. Such replacements apparently result in such major alterations of the structure of the hormone-binding domain that it is no longer capable of interacting with ligand. One such mutation is that (W739R) described by McPhaul et al. *(151)*. This amino acid substitution replaces a hydrophobic residue at the amino terminus of the helix 5, a residue that is predicted to make important contacts with the C-ring of testosterone, based on the structure of the liganded PR hormone-binding domain *(73)*. In addition, this residue is predicted to be located deep within the hydrophobic core of the hormone-binding domain, and the insertion of a charged residue into this location is likely to have dramatic effects on the overall tertiary structure of the hormone-binding domain.

Far more frequently, although studies performed in patient fibroblasts indicate that the mutant AR lacks the capacity to bind ligand, when such mutant receptors are expressed in heterologous cells, they are frequently found to be capable of interacting

with ligand, although often with reduced stability or affinity. The mutant receptor produced as a result of an amino acid replacement at residue 774 (R774C) provides an illustrative example. When assayed in cultured fibroblasts, the levels of ligand binding are below the assay detection limits. When the mutant receptor (R774C) was expressed in heterologous cells, the mutant receptor was capable of binding ligand, although these studies demonstrated that the binding of ligand was clearly unstable *(152)*. Many mutants of this type exhibit the same general type of behavior as that observed for the R774C mutant, leading to the synthesis of normal, or near normal, levels of immunoreactive receptor, that exhibits decreased ligand binding *(153)*. The discordance evident between the binding assays in fibroblasts and those performed in heterologous cells should not be interpreted to indicate that the mutant receptors behave differently in different cellular environments. Instead, such results appear to reflect differences in the sensitivity of the assays employed and the levels of receptor expressed in the transfected cell models.

Mutations Causing Qualitative Abnormalities of Ligand Binding

In some instances, whereas the number of ARs measured in standard ligand-binding assays may be normal, abnormalities of the AR can be detected using qualitative tests of ligand binding. The qualitative tests that have been employed in such studies have examined the affinity of the receptor for its ligand, the stability of the AR protein expressed (e.g., to thermal denaturation), and the stability of the hormone-receptor complexes that are formed (tests of ligand dissociation). Note, however, that the comparison of studies published by different groups characterizing qualitatively abnormal mutant ARs can be difficult, because the range and methods used often differ from laboratory to laboratory.

Lubahn et al. *(154)* were the first to report the genetic basis of a qualitative abnormality of the AR. The single amino acid substitution (V866M) localized by these investigators to the hormone-binding domain of the AR of affected family members resulted in an increased K_d in ligand-binding studies and was associated with a phenotype of complete testicular feminization. Studies of the function of this mutant AR uncovered a reduced capacity to stimulate a model androgen-responsive reporter gene *(155)*. This decreased capacity of the mutant AR to induce activity of the responsive gene was less evident when the assays were performed at high ligand concentrations. The observation that high doses of androgen could cause discernible different effects on the activity of mutant ARs has been observed in other studies (*see* below).

Many different amino acid substitutions have now been described that cause the mutant ARs to display qualitative abnormalities of ligand binding. Virtually without exception, these mutations are localized to the hormone-binding domain. Interestingly, the distribution of mutations within the AR HBD that cause qualitative abnormalities of ligand binding is quite similar when compared to the distribution of the mutations that result in the lack of detectable ligand binding *(156)*. This observation suggests that the great degree of disruption of the structure of the LBD is related to the abnormality that is identified: those alterations that result in more dramatic changes of structure leading to absent ligand binding, and those that cause less profound alterations of structure causing qualitative defects of the AR. This inference has been reinforced by studies of mutant ARs in which a single residue has been mutated in different pedigrees to different amino acid residues, such as described in the work of Prior et al. *(157)*.

In this study, replacement of an arginine residue by a cysteine residue (R774C) led to absent ligand binding. By contrast, replacement of this same arginine residue by a histidine led to normal levels of androgen binding that displayed marked thermal instability. Other investigations have identified other mutant receptors in which a different substitution mutation at the same residue led to identifiably different effects on ligand binding and receptor fusion *(158–160)*. In those instances in which replacement of a residue with different amino acids has resulted in discernibly different phenotypes, the level of AR function measured for the mutant receptors has varied in concert with the apparent phenotype.

An important conclusion regarding AR function has also been deduced from studies of mutant ARs with different amino acids in the HBD that exhibited different types of qualitative abnormalities. When assayed in cells capable of metabolizing the androgens testosterone and 5α-DHT, it was observed that the presentation and type of androgen used in functional assay experiments had a dramatic effect on the levels of AR function *(161)*. For each mutant AR, testosterone was the least potent, whereas DHT and mibolerone exhibited higher potencies in functional assays. Comparison of the results for this group of mutant ARs permitted three important conclusions. First, these results suggested that mutant receptors capable of binding hormone—however weakly—could be manipulated pharmacologically to exhibit near normal levels of AR function. Second, these experiments demonstrated the importance of the stability of the hormone-AR complex. Conditions that favored the formation and stability of these complexes could be seen to have major effects on the function of the mutant receptors in functional assays. Third, these experiments demonstrated that extreme caution must be used in attempting to correlate the results of functional assays performed using transfected cells with the phenotype observed in vivo. In such studies, minor alterations in the hormonal stimulation protocol used can lead to major differences in the levels of receptor function that are measured.

MUTATIONS IN THE AR THAT RESULT IN DECREASED LEVELS OF LIGAND BINDING

Unlike the preceding categories of mutation, this category is quite heterogeneous. At least two different mechanisms have been defined. The first mutation causing this type of AR abnormality was reported by Zoppi et al. *(162)*. Fibroblast samples from affected individuals within this pedigree were found to express reduced amounts of AR as assayed using monolayer binding assays. When immunoblot analyses of extracts from fibroblast cultures established from affected individuals in this family were performed using antibodies directed at the amino terminus of the AR, no immunoreactive AR protein was detected. The explanation for this apparent paradox was traced to a mutation in the AR ORF that introduced a premature termination codon in place of a glutamine residue at amino acid 60. Subsequent studies demonstrated that the low level of binding detected in the initial screening assays was the result of the downstream initiation at methionine 188. Finally, more recent studies have demonstrated that this receptor protein (which lacks amino acids 1–187) is synthesized in normal cells *(163)* and is precisely analogous to the A-form of the PR *(164)*. Functional studies performed in heterologous cells demonstrated that the phenotype observed in the original pedigree (complete testicular feminization) was owing to a combination of reduced receptor

expression and a reduced function of the receptor protein that is synthesized on selected response elements *(165)*.

More recently, a second mechanism was identified by Choong and co-workers *(166)* in their studies of a patient with partial AIS in whom reduced levels of apparently normal AR were synthesized. Analysis of the AR gene in subjects carrying the mutant allele revealed a single nucleotide substitution that predicted an alteration of the AR ORF at position 2 (a lysine residue in place of the normal aspartate residue). Although it was not possible to examine the effects of this mutation in cultured fibroblasts from affected individuals in this pedigree, the investigators concluded, on the basis of in vitro and cell transfection studies, that the AIS phenotype was primarily the result of the reduced levels of AR that were expressed *(166)*.

THE RELATIONSHIP BETWEEN PHENOTYPE AND MUTATIONS DETECTED IN THE AR GENE

It has been evident for a considerable period of time that the type of AR defect does not have a simple relationship to the phenotype exhibited by affected individuals. This is particularly true when viewed from the perspective of the data derived from the ligand-binding assays that were originally used to classify patients with androgen resistance *(1)*.

The range of AR mutations that has now been identified in patients with various forms of androgen resistance permits two generalizations to be made. First, truncations of the AR protein result, with few exceptions, in a phenotype of complete androgen resistance. This simple fact can be traced to the fact that the critical DNA- and hormone-binding domains are located at the carboxy terminus of the receptor. As such, truncations of the receptor protein, with rare exceptions, remove one or both of these important functional domains. In contrast to alterations that interrupt the primary amino acid sequence of the AR, amino acid substitutions in the receptor protein can cause the complete range of androgen-resistant phenotypes.

The second pertains to the relationship between the AR gene defect and the androgen-resistant phenotype that is observed clinically. In some instances, this relationship is obvious. In instances in which no AR is expressed or the genetic mutation abolishes AR function, the clinical phenotype—complete testicular feminization—invariably agrees with the results of assays of receptor function. In situations in which the receptor is not completely defective, however, quantitation of the degree of deficiency is considerably more difficult, because the results of functional assays may show marked differences depending on the conditions under which they are performed. Although a number of examples of this can be found in the literature, the experiments of Marcelli et al. *(161)* are representative and demonstrate that variations in hormone presentation or the identity of the ligands employed can lead to dramatic differences in the levels of receptor function.

ANDROGENS, THE PROSTATE, AND PROSTATE CANCER

Androgens are important for the normal growth of the prostate gland during the fetal and neonatal periods and to the maintenance of the structure of the adult prostate gland. Just as the development of the prostate is dependent on the normal action of

androgens, particularly 5α-DHT, during embryogenesis, the adult prostate undergoes a dramatic involution when androgens are withdrawn *(167–169)*. These observations have stimulated a variety of investigations, and have led to the use of medical or surgical castration in the management of patients with advanced prostatic cancers *(170,171)*. More recent approaches have employed pharmacological agents to interfere with the production or actions of androgens to accomplish the same effects *(172–175)*.

Despite the widespread use of these endocrine-based interventions, all appear to be subject to the limitations that characterize the response to castration itself. That is, although the vast majority of advanced prostatic malignancies will respond to any form of androgen ablation therapy, in most instances the duration of the observed response is finite. After an initial period of response when tumor size may shrink or symptoms abate, the tumor again begins to grow and cause symptoms in a hormone-independent fashion. This sequence of events has been postulated to represent an outgrowth of tumor cells—present within the initial tumor cell population—that do not require androgens for growth *(176)*.

AR MUTATIONS AND PROSTATE CANCER

Studies characterizing the growth of one well-differentiated prostate epithelial cell line, LNCaP, demonstrated that this cell line displayed unexpected responses when grown in the presence of various androgenic and antiandrogenic compounds. Of particular note was the growth stimulation observed in response to antiandrogens, responses not predicted on the basis of the pharmacology of such agents in normal human or animal tissues *(177)*. This observation led investigators to postulate a genetic abnormality of the AR in this cell line and to determine the primary amino acid sequence of the AR that it expressed. Such investigations led to the discovery that the LNCaP cell line expressed an AR protein that contained a single amino acid substitution residue within the HBD at amino acid residue 877 (threonine to alanine) *(178)*. Transfection experiments established that this amino acid substitution was necessary and sufficient to confer on the AR a broadening of its steroid responsiveness. In functional assays, the mutant AR was activated by hydroxyflutamide to a degree not observed in experiments in which the normal human AR was expressed.

The detection of a mutation in the LBD of the AR suggested one potential mechanism by which prostatic cancers might progress to a state in which the malignancy was no longer dependent on androgens for growth (or, alternatively, that no longer responded to antiandrogens). In this line of reasoning, the appearance of AR mutations in clinical prostate cancers might lead to the expression of mutant ARs that displayed an altered pattern of hormonal responsiveness. Such mutant receptors might be activated by antiandrogens or steroid hormones (e.g., adrenal androgens) that are usually not able to activate the normal, unmutated AR. Such a mechanism might also contribute to the "flutamide withdrawal" phenomenon *(179–182)*.

These concepts led a number of investigators to examine other cell lines and clinical specimens of prostate cancer to determine the frequency with which such mutations could be identified within the AR coding sequence. While many of the commonly employed prostate cancer cell lines are AR negative, the CWR22 cell line expresses

a mutant AR that displays a responsiveness reminiscent of that displayed by the mutant LNCaP AR *(183)*.

The studies examining the occurrence of AR mutations in clinical specimens are somewhat contradictory. A limited number of publications have examined specimens of advanced prostatic malignancies to determine the nature and frequency of AR mutations in such specimens. These studies suggest that in the more advanced stages of prostatic cancer, substantial numbers of AR mutations can be detected *(184–186)*. Furthermore, in keeping with the expectations that were raised by the studies of the mutant LNCaP AR, the mutant receptors that have been detected display aberrant responses to antiandrogens and to different classes of steroid hormones, such as adrenal androgens *(185,187–189)*.

Despite these intriguing results, two issues remain unsettled. First is the frequency that such mutations occur in less advanced lesions. In some studies, the frequency with which androgen mutations can be identified in early lesions is quite high *(186)*. By contrast, other investigators have reported that the frequency of AR mutations in early stage prostate cancer is quite low *(190–192)*. It is possible that the differences between these two different types of investigation reflect either methodological differences or the selection of the patient samples themselves. The second issue pertains to the degree to which the appearance of such mutations actually contributes to progression to the androgen-independent (or antiandrogen-resistant) phenotype that clinical tumors exhibit. It is hoped that studies of the progression of prostate cancer in available animal models may help establish whether the appearance of these mutations is central or peripheral to prostate cancer progression *(193–195)*.

CHANGES IN THE EXPRESSION OF THE AR IN PROSTATE CANCER

At the outset, it was hoped that studies of the expression of the AR in prostate cancer specimens might provide some information useful to predicting the responsiveness of tumors to endocrine manipulation, even though studies using tritiated steroids in ligand-binding assays were not informative *(196)*. Many of the earlier investigations using immunohistocytochemistry did not reveal a clear-cut relationship between the level or pattern of AR expression and patient prognosis (reviewed in ref. *197*). Despite these negative findings, several more recent studies have suggested that a relationship between the level of expression and outcome can in fact be observed. This relationship is most evident when sophisticated video image analyses are performed *(198–200)*. Interestingly, AR expression has been noted to become inconstant and heterogeneous in animals in which prostate cancer progression has been initiated by the targeted expression of large T-antigen *(194)*. Whether this reflects a transcriptional effect or other nontranscriptional events within the tumor cells remains to be determined.

The preceding comments suggest that the level of AR expression changes only in subtle ways early in the progression of prostate cancer. A separate line of evidence suggests that a different pattern may be true in later stages of prostate cancer. First are the studies demonstrating that the AR expression is frequently observed in late stages of prostatic cancer *(201,202)*. Second, in some studies AR gene amplification has been observed as an event that has been suggested to have functional importance in the progression of prostate cancer *(203,204)*.

AR POLYMORPHISMS AND PROSTATIC CANCER

As noted, the structure of the AR contains a number of polymorphic regions. These regions include a series of repeated glutamine residues, a series of repeated glycine residues, and a series of repeated proline residues. Of these, only the polymorphic glutamine repeats appear to show a substantial variation within the general population. In the general population, the length of this glutamine homopolymeric domain is approx 20–25 residues, although individual alleles have been identified that show differing numbers outside this range *(18)*.

Changes in the glutamine repeat length have been suggested to have a range of functional effects. In instances when this region is expanded to include more than 45–52 amino acid residues, the disease known as SBMA (Kennedy's disease) results *(19)*. Studies using transfection of cDNAs encoding ARs that include differing numbers of glutamine residues within the glutamine homopolymeric domain have demonstrated that changes in the size of this segment of the receptor can lead to altered functional activities. These types of studies have suggested that an increase in the length of the glutamine homopolymeric domain leads to decreases of receptor function, whereas a decrease in the length of the homopolymeric domain leads to increases in receptor function *(205,206)*. Note, however, that this finding has not been uniform, and some investigators have noted that a progressive decrease in the length of the glutamine homopolymeric domain is not associated with a progressive increase of AR function *(13)*. Furthermore, it is believed that the SBMA phenotype results from some toxic gain of function, not a loss of function *(207–210)*. Such toxicity may derive from the accumulation of AR fragments in cell lines and tissue samples expressing ARs that harbor such expanded glutamine repeats *(211–214)*.

Decreases in the size of the glutamine repeat have also been associated with disease. The analysis of a prostate cancer specimen from a patient with an advanced prostastic malignancy demonstrated the presence of a short glutamine repeat in the AR of the patient *(215)*. Such observations led several investigators to examine whether such shortened glutamine repeats were associated with an increased risk of developing prostate cancer, or the risk of developing more aggressive forms of prostate cancer. These studies have examined the possibility that differences within the coding sequence of the AR might be related to the risk of developing specific forms of prostate cancer. To date, four different studies have examined the relationship between the length of the homopolymeric domain in individual patient samples and the risk of developing prostate cancer *(20–23)*. Each of the studies has suggested that a decrease in the length of the homopolymeric domain is associated with an increased risk of developing prostate cancer. These investigators have suggested that the increased receptor function (associated with a decrease in the length of the glutamine homopolymeric domain) resulted in increased androgen stimulation to the prostate during much of adult life. It has been suggested that an increased responsiveness to androgen stimulation might account for the increased probability that these patients will develop prostate cancer (or more aggressive forms of prostate cancer).

Finally, several publications have identified polymorphisms within the AR gene *(216–218)*. The positions of these polymorphisms within the AR gene are such that although they would be unlikely to exert an effect on AR function, they might well have an effect on the level of AR expression.

SUMMARY

The information that is now available permits a number of conclusions to be drawn regarding the types of genetic alterations in the ARs that cause the different forms of androgen resistance. First, it is clear—as has been found for many other genes causing human disease—that a variety of types of mutation may cause defects of AR function. These include complete or partial gene deletions, insertions, premature termination codons, and abnormalities of AR mRNA splicing. With the exception of those individuals in whom the AR gene has been deleted, each of these processes, regardless of mechanism or location, results in the synthesis of a receptor protein that is defective by virtue of the interruption of the primary amino acid sequence. Despite this mechanistic heterogeneity, the phenotype that results is that of complete testicular feminization.

Mutations that result in the substitution of single amino acid residues within the AR protein are the most frequent—and interesting—defects that cause androgen resistance. With a single exception, these mutations are all localized to the DBD or hormone-binding domain of the result of the receptor. For the most part, the locations of these mutations within the AR protein do not appear to reflect sites that are subject to increased rates of mutation, but, instead, identify critical segments of the AR ORF that can be disrupted by single amino acid replacements to disturb AR function.

In addition to permitting conclusions from the locations of mutations that have been identified in different forms of androgen resistance, it is interesting to consider the significance of the paucity of mutations that had been identified within the amino terminus of the receptor protein. Those few mutations that have been localized to the amino terminus have been alterations that result—directly or indirectly—in either the premature termination or inefficient synthesis of the receptor protein. When considered in the context of results derived from in vitro mutagenesis studies, which clearly established the importance of the amino terminus for full AR function, the dearth of mutations within the amino terminus suggests that the functions exerted by this segment of the receptor are so diffuse that single amino acid substitutions are unable to affect AR function significantly.

It is clear that androgens control processes that are crucial to the development of the normal prostate and the growth of prostatic cancers. Several different lines of evidence suggest that alterations of AR structure and/or the levels of AR expression may play an important role in the appearance or progression of prostate cancer. Difficulties defining the importance of these changes are owing to the artificial nature of the cell culture or xenograft models that have been required to study human prostate cancer. Investigations of more manipulable, defined systems, such as transgenic mouse models that employ targeted oncogene expression, may lead to a better understanding of the roles that the AR plays in the evolution of prostate cancer.

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17 Genetic Determination of Androgen Responsiveness

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CONTENTS

INTRODUCTION

In the human fetus, the testes begin to secrete testosterone between wk 6 and 8 of gestation. Androgens, testosterone and 5α-dihydrotestosterone (DHT), are required during the fetal period for growth and differentiation of the male reproductive tract, including the Wolffian ducts, urogenital sinus, and external genitalia primordia *(1)*. This developmental phase is completed by 20 wks of gestation, when testosterone synthesis by Leydig cells in the fetal testes ceases. A second unique window of testicular testosterone secretion lasting approx 6 mo occurs during the immediate postnatal period in humans. Androgen actions during this period may imprint the central nervous system and determine the male pattern of gonadotropin secretion. The human testes then remain quiescent until testosterone synthesis and secretion is reinitiated for a third time at puberty. At this time, androgens promote the appearance of secondary male sex characteristics, including growth of the external genitalia, distribution of body hair, deepening of the voice, and increase in muscle mass. These steroid hormones initiate and maintain spermatogenesis and function of the epididymides, seminal vesicles, and prostate. They exert feedback control on the output of gonadotropins by the hypothalamic-pituitary axis. Androgens also act on the liver, kidneys, muscles, bones, and nervous and cardiovascular systems, but it is within the male reproductive tract that the molecular mechanisms of androgen action are best understood.

Testosterone is the primary androgen synthesized and secreted by the testes. Approximately 6 mg of testosterone is produced daily by the adult human testes *(1)*. The concentration of total testosterone circulating in the blood of young adult men is in the range of 4 to 5 ng/mL, or 14–17 n*M*. However, only about 2% of the testosterone is free, with the remainder bound to sex hormone–binding globulin ([SHBG], also

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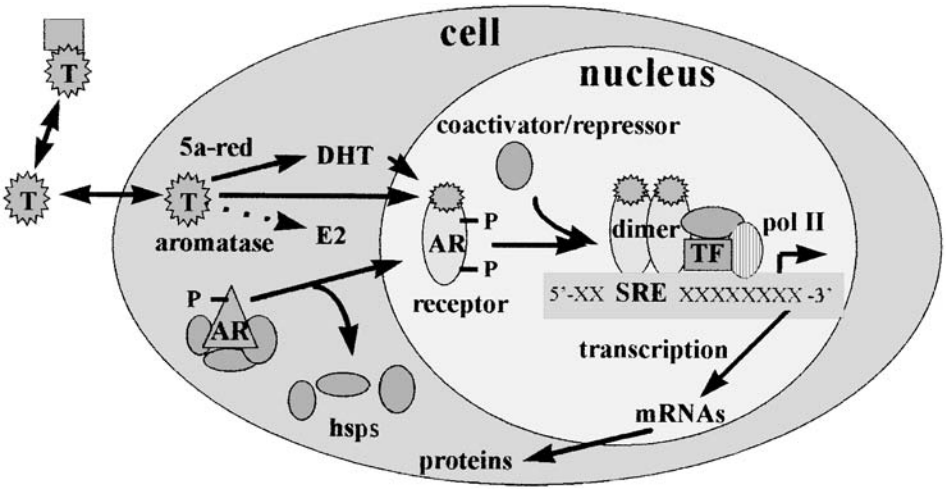

Fig. 1. Mechanism for androgen action in target organs. Testosterone (T) circulates in the blood predominantly bound to carrier proteins, such as sex hormone–binding globulin (SHBG), and the free testosterone enters cells by passive diffusion. Within the target cell, T may act by itself or be converted to its active metabolites, dihydrotestosterone (DHT) by the 5α-reductase enzyme or estradiol (E_2) by the aromatase pathway. E_2 acts through the ER pathway. The cytoplasmic androgen receptor (AR) is a phosphoprotein (—P) that forms a large macromolecular complex with various chaperone proteins, including heat-shock proteins (hsps). On binding of androgen, T or DHT, the receptor undergoes a conformational change and hsps are released. The AR complex undergoes further phosphorylation and acquires increased avidity for binding to DNA. The activated AR-steroid complexes bind as dimers to specific steroid response elements (SREs) defined by nucleotide sequences in regulatory regions of androgen-responsive genes. The chromatin-bound receptors are complexed with other nuclear proteins that function as either coactivators or corepressors of gene transcription that may act to modify the chromatin structure (histone acetylation/deacetylation) and/or interact with the transcriptional initiation complex comprising various transcription factors (TFs) and RNA polymerase II (pol II). This complex acts to facilitate or repress transcription of specific mRNAs, which are subsequently translated into cellular/secretory proteins.

referred to as testosterone-binding globulin) (40–50%) and to albumin (50–60%). Although albumin has a 1000-fold lower binding affinity for testosterone than SHBG, the greater concentration of albumin results in nearly equal amounts of testosterone being bound by the two proteins. The in vivo bioavailable testosterone is often equated with the free steroid, as well as the lower-affinity, readily dissociable albumin-bound steroid, or about half of the total testosterone (Fig. 1). Based on equilibrium kinetics, lipophilic steroids such as testosterone enter cells by passive diffusion across the cell membrane. Adequate levels of circulating testosterone are necessary for normal androgen biological activity; however, testosterone by itself is not sufficient to evoke the full complement of androgenic responses. Tissue-specific expression of the enzyme, steroid 5α-reductase, converts testosterone to its active metabolite, DHT. Within target cells, testosterone, or its 5α-reduced metabolite DHT, binds to the high-affinity androgen receptor (AR) located in the cytoplasm and/or nucleus. AR, like other steroid receptors, acts as a nuclear transcription factor in cells. Binding of androgen to its receptor produces a conformational (or allosteric) change that results in dissociation of the cytosolic macromolecular chaperone complex and formation of an activated ligand-

Table 1.
Human Steroid 5α-Reductase Isozymes

	Type 1	*Type 2*
Amino acids (mol wt)	259 (29.5 kDa)	254 (28.4 kDa)
pH optima	6.5–8.0	5.0
Gene, chromosome	SRD5A1, 5p15	SRD5A2, 2p23
Gene structure	5 exons, 4 introns	5 exons, 4 introns
Substrate (testosterone)	K_m = 1–5 μ*M*	K_m = 0.1–1.0 μ*M*
Enzyme deficiency	None known	Various mutations
Tissue expression	Liver, skin	Urogenital tract
Prostate expression	Epithelium (low)	Stroma (high)
Finasteride inhibition	$K_i \geq 300$ n*M*	K_i = 3–5 n*M*

bound receptor with high affinity for specific DNA-binding sites. Ligand-bound ARs form homodimeric complexes on specific DNA regulatory elements to activate (or repress) androgen-regulated gene transcription by RNA polymerase leading to altered levels of specific mRNAs. Translation of mRNAs on cytoplasmic ribosomes synthesizes the appropriate proteins that can alter cell function, growth, or differentiation. In addition, aromatization of testosterone to estradiol provides a source of estrogen that can bind to nuclear estrogen receptors (ERs) and promote estrogenic actions on the hypothalamus-pituitary, bone, lipids, and cardiovascular system in men.

This chapter focuses on the molecular biology of the steroid 5α-reductase and AR genes and the critical roles that these genes play in the processes of male sex differentiation, development, and reproduction *(2)*. The human conditions of androgen insensitivity and 5α-reductase deficiency are presented to illustrate how naturally occurring mutations in these key genes can alter the cellular mechanisms of androgen responsiveness and lead to aberrant developmental features. Molecular studies have also implicated AR in prostate cancer, spinal bulbar muscular atrophy, infertility, and breast cancer in men.

STEROID 5α-REDUCTASE ENZYME

The conversion of testosterone to a variety of 5α- and 5β-reduced metabolites was known prior to the discovery that DHT was actually the principal intracellular androgen concentrated within nuclei of androgen target tissues such as the prostate *(3)*. DHT proved to be twice as potent as testosterone in bioassays, and its physiological importance was confirmed by the abnormal sexual differentiation that occurred in human subjects with decreased serum concentrations of DHT owing to genetic defects in 5α-reductase activity *(4,5)*. The enzyme activity in tissue homogenates is characterized by two different pH optima. In the prostate and genital tissues, the primary peak of enzymatic activity is detected at pH 5.0, whereas in other nongenital tissues activity is maximal around pH 8.0. Indeed, different cDNAs derived from separate genes were isolated and found to encode the two isoforms of steroid 5α-reductase. Each gene contains 5 exons and 4 introns and there is 50% identity of their nucleotide sequences *(6)* (Table 1). The isoform with a pH optima of 8.0 was termed steroid 5α-reductase 1 and the pH 5.0 isoform was termed steroid 5α-reductase 2. The 28 to 29-kDa enzyme proteins are localized to the endoplasmic reticulum and nuclear membranes. Binding

of testosterone as a substrate is determined by both carboxy- and amino-terminal portions of the molecules, whereas the required binding of NADPH as a cofactor occurs within the carboxy-terminal half of the proteins. The human type 1 isoform is present at low levels in prostate, is encoded by a gene on the short arm of chromosome 5, has an optimal activity across a broad pH range from 6.5 to 8.0, a higher K_m (1–5 μ*M*) for testosterone, and is relatively insensitive (K_i = 300–500 n*M*) to the 4-azasteroid inhibitor, finasteride. The type 1 isozyme is the major 5α-reductase present in skin and liver. The type 2 reductase isozyme is encoded by a gene on the short arm of chromosome 2, has an acidic pH (5.0) optimum, a lower K_m (0.1–1.0 μ*M*) for testosterone, and is more sensitive to finasteride inhibition (K_i = 3–5 n*M*). The type 2 isozyme is expressed at high levels in prostate, predominantly in stromal cells and basal, but not secretory, epithelial cells.

Steroid 5α-Reductase Deficiency

Steroid 5α-reductase deficiency as a cause of male pseudohermaphroditism was first reported by Imperato-McGinley et al. *(4)* and Walsh et al. *(5)* in 1974. Serum and tissue DHT concentrations were reduced, and inadequate virilization of the external genitalia occurred in affected infants with a 46,XY karyotype. The condition has an autosomal recessive mode of inheritance and only homozygous males are affected. Infants with the enzyme deficiency have ambiguous genitalia at birth. Although there is considerable variation in the extent of masculinization of the genitalia, the phallus is typically quite small and appears as a normal or slightly enlarged clitoris. The labioscrotal folds are bifid and generally empty, but normal-appearing testes can be found in the inguinal canals. A urogenital sinus with a blind-ending vaginal pouch opens onto the perineum, hence the descriptive terminology of pseudovaginal perineoscrotal hypospadias applies to this condition. Wollfian duct development is normal and Müllerian ducts are absent. Examination of the phenotype and pubertal development in subjects with steroid 5α-reductase deficiency has provided valuable information about the roles of testosterone and DHT in normal development. For example, the incomplete masculinization of the external genitalia in affected subjects indicates that DHT is critical for normal development of male external genitalia in utero. By contrast, testosterone alone is sufficient for proliferation of Wolffian ducts. At puberty, the development of muscle mass, deep voice, phallic growth, and sperm production can be promoted by testosterone alone, whereas conversion of testosterone to DHT is apparently necessary for the development of other male secondary sexual characteristics such as hairline recession and prostatic enlargement. Although spermatogenesis has been reported in a few affected subjects, mature sperm are generally absent, either as a direct effect of the low levels of DHT or more likely as a secondary consequence of cryptorchidism. Gynecomastia does not occur. The differential effects of testosterone and DHT, as well as the underlying mechanisms, continue to intrigue investigators.

Mutations of the steroid 5α-reductase 2, but not steroid 5α-reductase 1, gene are responsible for the low levels of serum DHT and the inadequate virilization of the external genitalia in subjects with steroid 5α-reductase deficiency *(7,8)*. In an early study, deletion of the entire coding sequence for the steroid 5α-reductase 2 gene was discovered as the molecular basis for this disorder in a tribe residing in the New Guinea Highlands (Fig. 2). Other subjects with the clinical diagnosis of 5α-reductase deficiency were also known based on phenotype, endocrine findings, pedigree analyses, and

Fig. 2. Mutations of the human steroid 5α-reductase 2 gene. The five exons of the steroid 5α-reductase 2 gene are represented schematically along with those mutations that have been identified in subjects with 5α-reductase deficiency. Above each exon, the location of the mutations are indicated by codon number preceded by the normal wild type and followed by the substituted amino acid residue using the single-letter code at each position. An asterisk indicates the presence of a stop codon. Two mutations that occur as small deletions or insertions are indicated below the appropriate exon. In addition, subjects from a tribe in New Guinea were determined to have a deletion (del) of the gene as indicated by the black bar. (Reproduced from ref. *8*.)

measurements of 5α-reductase activity in cultured skin fibroblasts. Further molecular analyses of DNA using polymerase chain reaction amplification, single-strand conformational polymorphisms, and nucleotide sequencing revealed a heterogeneous array of additional gene mutations among subjects from more than 20 different ethnic groups. Most mutations occur as amino acid substitutions, splice-junction alterations, nonsense codons, or small deletions within the coding sequence (Fig. 2). Functionally, the mutations affect the binding of testosterone or NADPH to the enzyme, or the synthesis of nonfunctional or unstable proteins, or they reduce the level of protein synthesis. In many families the mutations are homozygous, often the result of consanguineous marriages, but others are compound heterozygotes. The absence of detectable mutations or detection of a mutation in only a single allele among clinically affected subjects suggests that additional mutations may map outside the coding sequence and the immediate intron flanking sequences surrounding the exons.

Subjects with steroid 5α-reductase deficiency display variable degrees of virilization that may be related to differences in residual type 2 activity as well as contributions to the overall production of DHT by the type 1 isozyme. For example, subjects from New Guinea with complete deletions of the type 2 gene, and hence a complete absence of steroid 5α-reductase 2 activity, do have measurable serum DHT. Other subjects with absence of functional type 2 enzyme, owing to a splice-junction abnormality, were able to convert exogenously administered testosterone to supraphysiological levels of DHT. These results suggest that steroid 5α-reductase 1 may contribute significantly to the overall production of serum DHT. This may have particular relevance following the apparent induction of the type 1 enzyme activity that coincides with puberty in males. The increasing androgen environment at puberty also causes further virilization among affected subjects. Interestingly, individuals with 5α-reductase 2 deficiency within

the Dominican Republic population were originally raised as females but subsequently changed their gender role behavior to male at the time of puberty. This served to reinvigorate the argument as to the relative roles of biological determinants and psychological factors in the development of gender identity. By contrast, similar behavioral changes do not occur among subjects with androgen insensitivity owing to mutations in the AR gene, a condition in which gender behavior conforms to the predominant anatomical development, and hence to gender assignment.

Animal models have also provided some further insight into the role of 5α-reductase activity, especially in females *(9,10)*. When steroid 5α-reductase 1 null mice were produced by homologous recombination in mouse embryonic stem cells, male mice appeared normal, but female mice exhibited a parturition defect that was maternal in origin. The parturition defect was reversed by the administration of 5α-androstan-3α,17β-diol, a 5α-reduced androgen formed from DHT whose formation is enhanced in the uterus at 17 to 18 ds of gestation. A decrease in litter size of homozygous steroid 5α- reductase 1–deficient females was also observed, and reversal of fetal wastage in these mothers could be effected by blocking excess estradiol formation, or its actions, during midgestation. Therefore, the 5α-reduction of androgens in female animals plays a crucial role in guarding against estrogen toxicity during pregnancy.

ANDROGEN RECEPTOR

AR shares homology and conservation of structure and function with the superfamily of nuclear ligand-dependent transcription factors that includes all of the steroid receptors among its members *(11)*. The human AR gene locus, which includes 8 exons and 7 introns, spans more than 90 kb of DNA in the q11-12 region of the X-chromosome *(12,13)*. Transcription of the AR gene is initiated from one of two sites within a 13-bp region of a single promoter *(14)*. The AR promoter does not contain a TATA- or a CCAAT-box. However, it does contain a GC-box near the initiation site that binds Sp1 and functions to recruit the TFIID complex, an adjacent long homopurine/homopyrimidine stretch and an active cAMP response element (CRE) *(15)*. Transcription from the initiation site at +13 (TIS-II), but not TS-I (+1), is dependent on binding of Sp1 to the GC-box *(14,16)*. The purine/pyrimidine region can bind Sp1 in its normal double-stranded B-DNA conformation, but is capable of binding a novel single-strand-specific protein as well *(16)*. Two mRNA species, a predominant 10.6- and a minor 8.5-kb mRNA, have been detected in various tissues. The 10.6-kb transcript consists of a 1.1-kb 5′ untranslated region (UTR), a 2.7-kb open reading frame (ORF), and a relatively long 3′ UTR of 6.8 kb. The shorter mRNA species contains the normal ORF and results from differential splicing in the 3′ UTR. The 5′ UTR of AR mRNA has also been suggested to play a role in the induction of AR translation *(17)*. The various cloned human AR cDNAs encode an approx 110-kDa receptor protein (AR-B isoform) containing a variable number of amino acids, from 910 to 919 residues, with the variations owing to polymorphisms within two stretches of amino acid repeats in the N–terminus. (All references to amino acid number in this chapter are to the human AR protein with 919 residues encoded by the cDNA originally cloned by Lubahn et al. *[12]*.) Posttranslational modification of AR occurs via phosphorylation of several serine residues and results in a shift in the apparent mol wt to 112–114 kDa *(11)*. An 87-kDa (AR-A) minor isoform of the receptor is translated relatively inefficiently from an

alternative initiation methionine codon at position 189 *(18)*. The AR-A isoform represents 20% or less of the total AR expressed in tissues. Its activity could not be distinguished from the more prominent AR-B isoform when transactivation was examined in cell transfection studies. Like other nuclear steroid receptors, the AR has distinct functional domains for ligand binding, DNA binding, and transcriptional activation, as well as for its nuclear localization *(19)*.

Amino-Terminal Transactivation Domain

Exon 1 of the AR gene is the largest and encodes the NH_2-terminal 538 amino acids *(20)*. Among the steroid receptors, the amino-terminus is the least conserved region with the greatest variation in length, but it plays an essential role in gene transactivation by AR *(19)*. Deletion of amino acids 142–239 in the NH_2-terminal domain causes a decrease in target gene transactivation, whereas a deletion confined to amino acids 199–239 leads to a significant increase in transactivation when compared to full-length AR *(11)*. This region is referred to as the activator function-1 (AF-1) domain. Neither the location of AF-1 nor its amino acid composition is conserved among steroid receptors, suggesting its role in determining the specificity of target gene regulation by receptors. A second, highly conserved ligand-dependent activation function (AF-2) domain resides within the carboxy-terminus of steroid receptors. A functional interaction has been demonstrated to occur between the NH_2-terminal activation and carboxyl ligand-binding domains *(11)*. Within the amino-terminus of AR, the regions encompassing amino acid residues 14–36 and 371–494 have been implicated in these interactions with the steroid-binding domain *(21)*. A unique, polymorphic polyglutamine stretch encoded by (CAG)nCAA and a polymorphic polyglycine stretch encoded by (GGN)n are present in the human AR amino-terminus, in addition to polyalanine and polyproline regions. Within the normal population, the polymorphic glutamine repeat varies from 9 to 33 residues, and the glycine stretch ranges between 16 and 27 residues. Polymorphic polyglutamine stretches are also present in the NH_2-terminus of the rat and mouse ARs, but their relative locations are not conserved. Acidic polyproline and polyglutamine sequence motifs are generally believed to confer a transcriptional activation function when present in various proteins. Indeed, recent findings related to prostate carcinoma and spinal bulbar muscular atrophy (SBMA) suggest that genetic alterations in the length of the polyglutamine stretch may sufficiently modify AR transcriptional activity so as to have pathological implications.

Central DNA-Binding Domain

The cysteine-rich DNA-binding domain (DBD) is the most highly conserved region within the steroid receptor superfamily. The centrally located AR DBD, encoded by exons 2 and 3 of the AR gene, consists of 66 amino acids and shares 60–70% homology of amino acid sequence with the same domain of the glucocorticoid receptor (GR) and progesterone receptor (PR) *(20)*. By structural analogy based on nuclear magnetic resonance and crystallographic analyses of other steroid receptors, the DBD of AR consists of two zinc fingers incorporating perpendicularly oriented α-helices in which four cysteine residues at the base of each finger coordinate zinc in a tetrahedral array *(22)*. The residues, Gly577, Ser578, and Val581, within the proximal (P) box at the carboxy-terminus of the first zinc finger in AR, are conserved in GR and PR, and probably account for their common recognition of a steroid response element (SRE) consisting of the consensus palindromic

nucleotide sequence, -AGAACAnnnTGTTCT-. The α-helix containing the P-box is positioned in the major groove of DNA where the three amino acid residues of the P-box make base-specific contacts with DNA. The distal (D) box, consisting of five amino acids (ala-ser-arg-asn-asp) in the second zinc finger, is thought to play a role in AR homodimerization through a symmetric dimerization interface that is formed on binding to a palindromic SRE, as previously demonstrated for GR. However, the critical factors, including chromatin structure, that determine specificity of AR, GR, or PR binding to DNA-regulatory sequences, and hence their discriminatory function in steroid-specific gene regulation, remain to be elucidated.

Nuclear Localization Signal and Hinge Region

In the absence of ligand, AR is distributed within the cytoplasm and nucleus, where the addition of androgen induces its rapid migration to the nucleus. A bipartite nuclear targeting signal sequence encoded at the junction between exons 3 and 4 functions to shuttle the AR through nuclear pores. The signal sequence consists of two clusters of basic amino acids, separated by 10 amino acids, that reside within the region that joins the DBD and hinge region *(23)*. Amino acid substitutions or deletions in this region bounded by residues 617–633 cause an almost complete cytoplasmic localization of the receptor and loss of its transcriptional activity. The hinge region accommodates conformational flexibility between the DBD and steroid-binding domain of the receptor.

Steroid-Binding Domain

Androgen agonists and antagonists bind to the carboxy-terminus of AR encoded by exons 4–8 *(20)*. In the absence of ligand, steroid receptors repress gene transactivation, but this repression is relieved by the binding of steroids *(11,19)*. This concept is supported by the constitutive transactivating function of AR that results from deletion of the steroid-binding domain from the expressed protein *(19)*. On binding of androgen by AR, the previously inactive macromolecular complex, composed of AR and the chaperone proteins that include heat-shock proteins 90 and 70, dissociates *(24)*. Activation of the newly formed receptor-steroid complex coincides with alteration of the AR molecular conformation. The active conformation of the receptor is stabilized by the presence of androgen and is capable of forming a homodimer in antiparallel orientation with dyad symmetry that binds to androgen-responsive elements (AREs) to promote gene transcription *(25,26)*. Androgen binds to both nonphosphorylated and serine phosphorylated forms of AR, but the significance of this posttranslational modification on AR activity has not been clearly defined *(27,28)*. A series of α-helices are conserved in the steroid-binding domains of receptors. Crystallographic analyses of the steroid-binding domains of several receptors have been conducted, in the presence and absence of hormone *(29,30)*. The crystallographic maps have provided structural evidence for hormone contact with amino acid residues in helices 3, 5, 7, 11, and 12. A longer helix 12 and a C-terminal extension of the steroid-binding domain are structural features unique to PR, GR, and AR that are essential for binding of the relevant 3-oxosteroids *(30)*. A conserved AF-2 region is present among steroid receptors, including AR, and resides within helix 12. When the receptor is occupied by a steroid agonist, helix 12 closes over the binding pocket to form an interface for interaction with transcriptional coactivators; by contrast, binding of antagonists prevents this interaction interface from forming. Conformational changes that accompany the binding of steroid agonists also

promote the intramolecular interactions that occur between the amino-terminal transactivation and carboxy-terminal steroid-binding domains in AR, interactions that may further modulate receptor dimerization, DNA binding, and/or transactivation *(21,25)*.

SREs in DNA

SREs minimally contain a core recognition motif of 6 bp, but generally consist of two core motifs (half-sites) separated by a spacer of variable length *(31,32)*. The nucleotide sequence of the core motif is specific for subgroups of receptors; AR, GR, and PR all bind to hexamer half-sites with the consensus sequence TGTTCT. The ARE sequence differs from that for the ER (TGACCT) at positions 3 and 4, which are critical for receptor-specific recognition. The consensus SRE for AR is organized as an imperfect inverted or palindromic repeat of core motifs, although AR has been shown to bind also to a direct repeat *(33)*. The SRE is also typically characterized by the spacing of the two half-sites, which in the majority of known SREs for AR involves three nonspecific nucleotides. The consensus SRE for AR binding is therefore represented by the nucleotide sequence AGAACAnnnTGTTCT, but this sequence in isolation also binds GR and PR. The sequence- and cell-specific factors that distinguish AR-dependent gene regulation from that of GR and PR remain to be elucidated. Features of chromatin structure as well as nucleotide sequence may also influence receptor-specific transactivation.

Transcriptional Activation

Sequence-specific transcription factors such as AR interact with other general transcription factors in the control of gene activation *(32)*. These general factors in turn interact with the core promoter elements to induce basal transcription. RNA polymerase II and the general transcription factors assemble a transcription initiation complex along with the TATA-box binding proteins, TATA-binding protein (TBP), and TBP-associated factors (TAF_{II}s). Steroid receptors may enhance basal gene transcription, either by direct interaction with general transcription factors or with TAF_{II}s. The NH_2-terminal domain of AR was shown to interact with TFIIF *(34)*. In addition to these direct interactions between AR and the transcriptional machinery, other intermediary factors or coactivators may also be involved in the regulation of transcriptional activity. For example, AR interacts with sequence-specific DNA-binding transcription factors, such as AP-1 and NFκB, that further modulate gene transcription *(35,36)*.

Coactivators and Corepressors

Coactivators are cellular proteins that interact with the agonist-activated steroid receptor complexes to enhance transactivation of target genes *(37,38)*. Coactivators may have several roles including possession of intrinsic histone acetyltransferase (HAT) activity, recruitment of other proteins with HAT activity, and as integrators that enable regulatory molecules to be recruited and assembled at sites of transcriptional activity. A number of coactivators are either directly or indirectly involved in chromatin remodeling. The coactivators, transcriptional intermediary factor 2 (TIF2), steroid receptor coactivator-1 (SRC-1), and GR-interacting protein 1 (GRIP1), interact with the ligand-activated AF-2 regions of different steroid receptors, including AR, to enhance transcriptional activity *(39,40)*. SRC-1, in turn, can interact with integrators such as p300/CBP (CRE-binding protein) and pCAF (p300/CBP-associated factor) proteins that possess intrinsic HAT activity, with the overall effect being synergistic for transcriptional

activation *(41)*. ARA-70 (AR-associated protein) interacts in a ligand-dependent manner with AR to increase its transcriptional activity *(42)*.

By contrast, corepressors act to repress basal promoter activity in the absence of hormone. Cloning of the cDNAs for two corepressor proteins, nuclear receptor corepressor (NCoR) and silencing mediator for retinoids and thyroid hormone (SMRT), have provided general insights into the activities of corepressors *(43,44)*. Initially, the repressor protein associates with the receptor on chromatin to maintain a transcriptionally inactive structure by tethering of histone deacetylases to the DNA at sites close to the responsive element of the receptors. Subsequently, binding of hormone by the receptor causes release of the corepressor and recruitment of acetyltransferases, which disrupt the chromatin template. Finally, interaction between the activating domains of both the receptors and the recruited coactivators with the basal transcription factors results in gene transcription. Whereas the aforementioned mechanisms may be generally applicable, steroid receptors, in particular, are bound by a complex of heat-shock proteins in the absence of steroid, and therefore are probably not involved in tethering of histone deacetylase activity to DNA. However, when steroid antagonists bind to receptors they inhibit transcription, suggesting that corepressors may play a role in this repression.

Androgen Insensitivity

In 1950 Lawson Wilkins observed a female-appearing subject who had a 46,XY karyotype that excreted urinary steroid metabolites indicative of male gonadal function and who was insensitive to pharmacological doses of testosterone. Today, we understand this condition, known as androgen insensitivity (AIS), to be caused by inactivating mutations of the AR gene that result in partial or total loss of target gene responses to androgens in tissues *(45,46)*. In individuals with complete AIS owing to total inactivation of AR, the androgen-dependent internal Wolffian ducts are absent and the external genitalia is phenotypically female. Testes are present within the abdomen or inguinal canal and Müllerian ducts regress in response to secretion of Müllerian-inhibiting substance from Sertoli cells. At puberty, AIS is reflected at the level of the hypothalamus/pituitary by the high levels of luteinizing hormone (LH) with subsequent hyperplasia of Leydig cells causing serum testosterone levels to be at or above normal values for men. Aromatization of testosterone to estradiol and the ensuing unopposed estrogenization is responsible for breast development and the typical female body habitus observed with complete AIS. Seminiferous tubules contain immature Sertoli cells and primitive spermatogonia. Pubic and axillary hair is absent or sparse. The diagnosis of complete AIS is most often made either in infants, with abdominal testes that present the appearance of inguinal hernias, or at puberty, because of primary amenorrhea. By comparison, partial AIS includes a spectrum of phenotypes ranging from a predominantly female appearance (external female genitalia and pubic hair at puberty or with mild clitoromegaly and some fusion of the labia) to ambiguous genitalia, or even to individuals with a predominantly male phenotype. Subjects in the latter group appear with a micropenis, perineal hypospadias, and cryptorchidism. Wolffian duct derivatives may be nearly fully developed or rudimentary in partial AIS, depending on the residual androgenic activity. At puberty, elevated LH, testosterone, and estradiol levels are observed, but the partial virilizing effects of androgen cause the degree of feminization to be less than that seen among individuals with complete AIS.

In 1970 Lyon and Hawkes *(47)* noted the similarity between the X-linked inheritance

pattern for the condition they termed *testicular feminization* (*Tfm*) in the mouse and its parallel to AIS in humans. Further studies demonstrated the absence of biological response to androgens in *Tfm* mice, and a reduced binding of DHT in kidney cytosol was observed *(48)*. Meanwhile, Stanley et al. *(49)* reported a *Tfm* rat that was unresponsive to physiological doses of androgens but in which a biological response was generated with 10-fold higher doses. Binding of androgens in kidney nuclei and preputial gland cytosol from the *Tfm* rat was negligible *(50)*. These observations led to the conclusion that the *Tfm* mouse had a more absolute insensitivity to androgens, whereas insensitivity in the *Tfm* rat was relative *(50)*. Subsequent molecular analyses identified mutations in the AR gene of both species. In the mouse *(51)*, a single base deletion in exon 1 causes a frameshift that introduces a premature termination codon downstream at codon 412, although reinitiation of protein translation occurs from an internal start site with synthesis of a low level of truncated receptor that still binds androgen but lacks transcriptional activity; in the rat *(52)*, a single nucleotide missense mutation in codon 734 results in an amino acid substitution (Arg→Gln) in the AR steroid-binding domain that causes reduced androgen binding and impaired transcriptional activity.

Spurred by the in vitro biochemical studies of androgen binding in tissues from the *Tfm* rodent models, during the 1970s, our research group pioneered androgen-binding studies in cultured human genital skin fibroblasts that elucidated the biochemical and cellular basis of human AIS *(53)*. Androgen binding in fibroblasts from subjects with the clinical diagnosis of AIS was not uniformly absent but, rather, was classified into several categories that included normal (AR+), deficient (AR±), and absent (AR−). More recently, cloning of the AR cDNA has led to molecular characterization of lesions in the AR gene of subjects with AIS *(12,45,46)*. Presently, more than 100 different mutations have been identified in subjects with AIS, and these are primarily point mutations or small deletions or insertions *(54)*. Absence of androgen binding in genital skin fibroblasts is associated only rarely with complete and partial deletions of the AR gene, but more often with single base mutations that introduce premature termination codons, disrupt mRNA splicing, or cause substitution of amino acids in the steroid-binding domain. Less than 5% of the AR gene mutations causing AIS are owing to deletions encompassing one or more exons. Two subjects with complete AIS and deletion of the entire AR gene have been identified, and hence these individuals represent the null phenotype. In addition, normal androgen binding is present in cultured fibroblasts of some subjects with AIS. Although the molecular defect in these latter subjects was originally hypothesized to occur at some point in the androgen pathway distal to the receptor, many actually have a mutation in the DBD of the AR gene that permits normal binding of steroid, but interferes with AR binding to DNA. In cases of partial AIS, androgen binding in fibroblasts was either quantitatively normal or partially deficient related to mutations that cause less critical amino acid substitutions in the DBDs or steroid-binding domains of AR.

Specific mutations in the AR gene of subjects with AIS that were identified in our laboratory and the functional properties of some of these mutant ARs are presented next (Fig. 3). Relatively few mutations occur in exon 1 of the AR gene, and most of these represent single nucleotide substitutions, insertions, or deletions. They almost uniformly result in frameshifts and premature stop codons. The majority of mutations occur in exons 2 and 3 encoding the DBD and in exons 4–8 encoding the steroid-binding domain. Mutations in these regions are responsible for the entire spectrum of

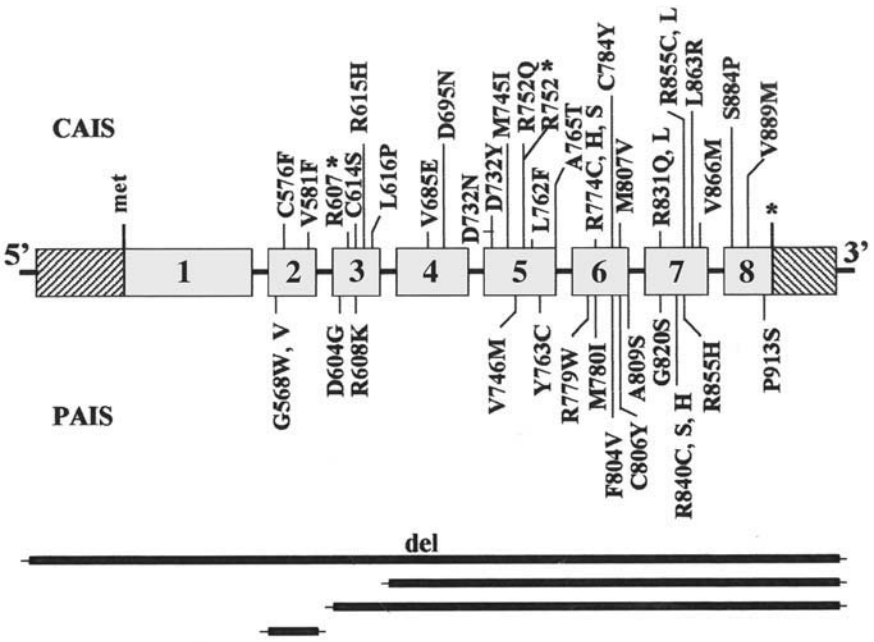

Fig. 3. Mutations of the human AR gene. The eight exons of the AR gene are represented schematically along with those mutations that have been identified in our laboratory among subjects with complete (CAIS) and partial (PAIS) AIS. The location of each mutation is indicated by the codon number preceded by the normal wild type and followed by the substituted amino acid residue using the single-letter code at each position. An asterisk indicates the presence of a stop codon. In addition, the black bars indicate various deletions (del) within the gene that have been identified among subjects with CAIS. Additional AR gene mutations are indexed in the AR gene mutation database, (*see* ref. *54*.)

phenotypic variation that occurs in both partial and complete AIS, and no region is more or less represented in either form of AIS. In exon 2, substitutions of Gly568 by either Val or Trp led to partial AIS, and replacement of Cys576 or Val581 with Phe occurred in subjects with complete AIS. DNA binding of AR was decreased with the conservative replacement of Gly568 by Val in the loop of the first zinc finger, but a complete loss of DNA binding accompanied substitution of Cys576 and Val581, which are critical for formation of the tetrahedral complex with zinc and the direct contact of the P-box with DNA, respectively. As a consequence of the effects of these mutations on DNA binding by AR, transactivation of reporter-gene activity was deficient with Gly568→Val and absent with Cys581→Phe. Point mutations in exon 3 resulted in substitution of Asp604 by Gly, a stop codon at Arg607, and replacement of Arg615 by His in the second zinc finger motif. The mutation of Asp604 caused partial AIS, whereas the latter two mutations occurred in subjects with complete AIS. Arg615 is in the region of the second zinc finger that forms an α-helix involved in DNA binding.

Mutations in the steroid-binding domain affect the level of androgen binding, steroid specificity, and association/dissociation kinetics of ligand-receptor interaction. In exon 4, a point mutation caused substitution of Asp695 by asn, leading to complete AIS owing to a mutant receptor with decreased stability and accelerated steroid dissociation rate. Substitutions in >50% of the amino acid residues encoded by exon 5 have been

reported among subjects with partial or complete AIS. The amino acid sequence encoded by exon 5 is the most highly conserved portion of the steroid-binding domain among members of the AR/GR/PR subfamily of steroid receptors. For subjects studied in our laboratory, complete AIS resulted from mutation of amino acid residues 732 (Asp to Tyr or Asn), 745 (Met to Ile), 752 (Arg to Gln or Stop), 762 (Leu to Phe), and 765 (Ala to Thr). The substitution of Arg752 by Gln is identical to that which occurs in the *Tfm* rat at the homologous residue, Arg734. Although a stop codon in exon 5 (Arg752→Stop), 3′ to the region encoding the DBD, might be hypothesized to allow permissive translation of a truncated AR protein with constitutive transcriptional activity, analogous to earlier transfection experiments that used deletion mutants, the phenotype is, however, that of complete AIS. Presumably, instability occurs within the AR mRNA or the truncated AR peptide, leading to absence of in vivo biological activity. Substitutions of Val746→Met and Tyr763→Cys were associated with partial forms of AIS.

A few relative "hot spots" for mutation have been found to recur in multiple unrelated families, and although CpG-dinucleotides are subject to a higher relative rate of mutation, the overall heterogeneity of mutations remains as an impressive feature of AIS. CpG dinucleotides are subject to a high frequency of C-T transitions by methylation of cytosine to 5-methylcytosine, followed by spontaneous deamination to thymidine. Such mutational events occur for arginine residues at positions 774, 831, 840, and 855, and valine at 866, where the relative frequency of mutations in the AR gene is high and observed in multiple families. Arg is completely conserved in AR, PR, and GR at positions 774, 831, and 855, but is unique to AR at position 840. Consequently, substitution of Arg774 by Cys or His and Arg831 by Gln or Leu occurs in subjects with complete AIS. ARs with Arg774→Cys and Arg831→Leu failed to bind androgen and were unable to induce transcriptional activity even at supraphysiological concentrations of androgen. By comparison, mutant ARs with His774 and Gln831 retained some steroid-binding capacity and a very low level of transcriptional competence at high concentrations of androgen, despite their association with complete AIS. Replacement of Arg840 by Cys, His, or Ser caused partial AIS. The phenotype of complete or partial AIS depends on the specific amino acid substitution at Arg855 and Val866. When Arg855 was replaced by Cys or Leu and Val866 by Met, the phenotype was invariably complete AIS. By contrast, Val866→Leu caused partial AIS and Arg855→His was variably associated with either partial or complete AIS, even within the same family.

The variable phenotypic presentation resulting from substitution of Arg855 by His suggests the possibility that individual variability in other factors that influence androgen action, such as the level or timing of testosterone synthesis and cellular specificity mediated by protein coactivators or integrators, could be involved during development of the fetus. Interestingly, both mutations of Val866 to Met or Leu retained steroid binding despite their differential effects on phenotypic presentation as complete or partial AIS, although in each case the receptor was reported to be thermolabile and exhibited an altered dissociation constant for androgen binding.

Relatively few mutations occur within the most C-terminal region of the steroid-binding domain encoded by exon 8. However, the occurrence of AIS associated with these mutations demonstrates the critical nature of this region for maintenance of steroid binding and transcriptional activity of AR. Replacement of Ser884 by Pro, Val889 by Met, and Phe916 by Leu each cause complete AIS, whereas the most distal naturally occurring mutation associated with partial AIS results from substitution of Ser for Pro913.

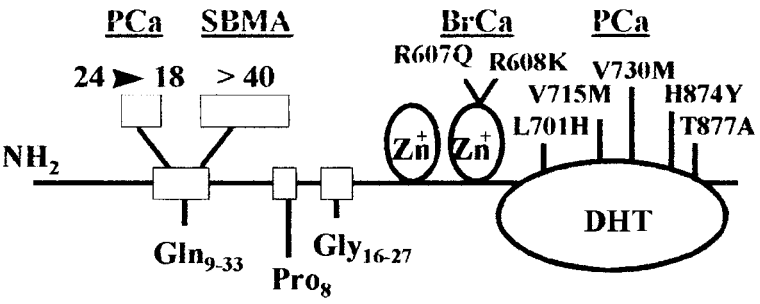

Fig. 4. Mutant forms of the AR associated with the human pathologies of prostate cancer (PCa), spinal bulbar muscular atrophy (SBMA), and male breast cancer (BrCa). The human AR protein is represented schematically showing the amino-terminal (NH_2) polymeric amino acid repeats, the central two zinc fingers, and the carboxy-terminal (COOH) steroid-binding domain occupied by dihydrotestosterone (DHT). Contraction of the polyglutamine region has been reported in PCa whereas expansion of this region occurs in SBMA. Male breast cancer has occurred in subjects with partial androgen insensitivity and amino acid substitutions in the DBD. Paradoxical responses of prostate cancer cells to endocrine therapy have been associated with several somatic cell mutations in the AR gene affecting the steroid-binding domain.

The clinical and pathophysiological features of AIS provide a human model for understanding the role of androgen and its receptor in the induction and maintenance of male sex differentiation and function. Although severe forms of hypospadias represent a feature of AIS, AR mutations are a very infrequent cause of isolated hypospadias. Mutant receptors may have reduced specificity and/or affinity for androgen binding and DNA binding, altered association and/or dissociation kinetics for steroid binding, and inherently decreased stability to proteolytic degradation. In several AIS subjects with the same AR gene mutation, phenotypic variation has been observed. The evidence suggests that receptor activity is also influenced by the length of the polymorphic polyglutamine stretch in the transcriptional activation domain, and additional factors such as androgen synthesis or expression of coactivators or integrators may account for modulation of receptor activity among individuals. Amino acid substitutions owing to single nucleotide substitutions in the AR gene are most frequently observed, and, in general, conservative mutations appear to have less deleterious effects than nonconservative mutations. The diverse nature of the mutations involved in the phenotypic spectrum of AIS and the heterogeneous distribution of these mutations throughout the coding sequence of the AR gene is impressive. However, the number and diversity of these naturally occurring mutations and their associated clinical and biochemical phenotypes provide a significant resource for understanding the structure-function relationships of AR from the in vivo expression and activities of the mutant receptors in cells.

Spinal Bulbar Muscular Atrophy

Several polymorphic repeats are located in exon 1, which encodes the amino-terminus of AR. An expansion of the polymorphic, polyglutamine stretch, encoded by (CAG)nCAA, is the molecular basis of SBMA (Kennedy's disease) *(55)*. In normal individuals, the (CAG)nCAA repeat contains 9–33 CAG triplets, whereas 38–75 CAG codons are associated with SBMA (Fig. 4). Disease severity is inversely correlated with the length of this repeat. SBMA is characterized by progressive muscle weakness

and atrophy with clinical symptoms manifested in the third to fifth decade of life. The pathology is associated with a severe depletion of lower motor nuclei in the spinal cord and brain stem, and distal axonopathy of the dorsal root ganglion cells is observed. In addition, subjects with SBMA frequently exhibit endocrine abnormalities including testicular atrophy; reduced or absent fertility; gynecomastia; and elevated follicle-stimulating hormone, LH, and estradiol levels similar to observations in mild forms of AIS. Sex differentiation occurs normally, and characteristics of mild androgen insensitivity appear later in life. This may be related to a reduced AR expression and reduced testosterone level in older men. SBMA is an X-linked disease and occurs only in men. At present it is not known whether disease progression involves the ligand-activated or ligand-free AR. In two cases, extended testosterone therapy had neither a beneficial nor a harmful effect.

The molecular mechanisms underlying SBMA remain somewhat speculative at present. In fact, the disease appears to be a combination of the loss of normal AR function in androgen-dependent tissues coupled with a gain of function (GOF) mechanism in motor neurons. Several other similarly progressive neurodegenerative diseases—Huntington's disease, dentatorubal-pallidoluysian atrophy, and spinocerebellar ataxis—are caused by an expanded CAG repeat located in the coding region of the respective genes. Although the proteins encoded by each of these genes are widely expressed throughout the body, neuronal tissue is specifically affected. Theoretically, intragenic expanded CAG repeats could be pathogenic at the DNA, RNA, or protein level. Increased binding of RNA-binding proteins to RNAs containing expanded CAG repeats has been observed. These RNAs might disrupt normal transport in cells or competitively affect the interactions of these proteins with other cytoplasmic proteins. Alternatively, the expanded polyglutamine stretch could serve as a better substrate for transglutaminase, an ubiquitously expressed enzyme that catalyzes coupling of glutamine and lysine residues. Polyglutamine stretches might also function as polar zippers to form protein aggregates that are unable to be processed normally by the ubiquitin-mediated proteolytic degradation pathway, as well as form intranuclear inclusions, as seen in Huntington's disease. By comparison to a GOF mechanism, the endocrine abnormalities present in subjects with SBMA reflect a loss of function for AR. Because many transcription factors commonly contain polymorphic glutamine stretches, the question arose, does the length of the polyglutamine stretch in AR modify its transcriptional activity? In cotransfection studies, the length of the CAG repeat was inversely proportional to the transactivation function of AR *(56)*. However, conflicting data have suggested that this was related to reduced stability of AR mRNA and decreased expression of AR protein *(57)*.

Prostate Cancer

Androgens, predominantly DHT, are involved in the growth and development of the prostate, but also play a role in the evolution of prostate cancer. At the time of detection and surgery, tumors are often androgen dependent, and therefore endocrine abalation therapy that combines castration and antiandrogen and/or GnRH antagonist administration is often implemented. About 80% of subjects initially respond to endocrine ablation therapy, but, ultimately, the majority show tumor recurrence and progression. Failure of endocrine therapy can be explained by several molecular mechanisms, including androgen dependence or independence.

Somatic mutation of the AR gene represents one potential mechanism for androgen independence in which activation of AR occurs owing to other steroids and antiandrogens (Fig. 4). The first mutation (Ala877Thr) of this nature was detected in the human prostatic carcinoma cell line, LNCaP, that showed an increased growth rate in response to estradiol, progesterone, and the antiandrogens, hydroxyflutamide and cyproterone acetate *(58)*. Additional somatic cell mutations were observed in primary tumors, but only at a very low frequency *(59)*. The majority of mutations have been identified in hormone refractory tumor samples and metastatic lesions *(60,61)*. These mutations most often involved amino acid substitutions in the steroid-binding domain that altered the steroid specificity for binding; however, there are also reports of mutations occurring in the 5′ UTR, as well as in the amino-terminal and DNA-binding regions. A large number of tumors have been screened for AR gene mutations, but the frequency of genetic alterations at this level appears to be quite rare.

Amplification of the AR gene was also observed in a number of hormone recurrent tumors, but not in primary tumors *(62)*. AR gene amplification resulted in higher levels of AR mRNA and a corresponding increase in the levels of AR protein expression. The increase in AR levels within tumors might provide a growth advantage to cells when androgen levels are low, such as after androgen ablation therapy. Although theoretical at present, amplification of growth-related gene targets for AR activity or for genes of AR coactivators in prostate may also represent potential mechanisms for androgen-independent growth of tumors.

Recent studies have suggested that cross talk between AR pathways and other signal transduction pathways involving growth factors and autocrine/paracrine peptides could lead to receptor activation in the absence of androgen *(63)*. In addition, these alternative pathways could also potentiate the activity of AR in the presence of low concentrations of androgen. Whether these alternative pathways are initiated owing to changes in kinase activity that affect the phosphorylation of AR or other phosphoproteins remains to be clarified.

A somatic variation in the polymorphic (CAG)nCAA-repeat in exon 1 of the AR gene was observed in a prostate tumor specimen and was suggested to have a role in prostatic carcinoma *(64)* (Fig. 4). Studies related to subjects with SBMA showing that the increased length of the polyglutamine stretch was inversely correlated with AR transactivation prompted additional epidemiological studies among normal and prostate cancer patients to determine the relationship between the occurrence of prostate tumors and variation in (CAG)nCAA polymorphisms. Several recent studies have shown a correlation between shorter CAG-triplet repeats in exon 1 and an increased risk for prostate cancer. In addition, these tumors tended to be more aggressive and were related to a younger age at diagnosis *(65,66)*. Population distribution analyses have shown that African-Americans have a lesser mean number of CAG-triplet repeats in the AR gene than Caucasians, further suggesting a correlation between a shorter polyglutamine stretch and a higher incidence of prostate cancer within the African-American population *(67)*.

Breast Cancer in Males

Breast cancer occurs infrequently in males, but clinical conditions of reduced androgen activity predispose subjects to gynecomastia, and possibly to further breast cell proliferation. Therefore, it was not surprising that two research groups reported muta-

tions in the AR gene of male subjects with breast cancer in association with partial AIS *(68,69)* (Fig. 4). However, further studies among men with breast cancer that were not associated with abnormal sex differentiation and AIS have failed to confirm a correlation with mutations in the AR gene.

Male Infertility

In several studies, a reduction in AR binding in genital skin fibroblasts cultured from men with azoospermia and oligospermia, but otherwise normal virilization, was reported to represent a mild form of AIS. However, each of these studies predated access to molecular techniques for AR gene analysis, and the presence of molecular defects in AR have not been documented *(45)*. Infertility in a man with deletion of exon 4 in the AR gene was reported *(54)* but remains an enigma since a gross dysfunction of this mutant receptor would be expected, but was never tested. A mutation in exon 5 was reported in one subject with severe oligospermia, and an extended (CAG)nCAA repeat in the AR gene was reported in another subject associated with impaired spermatogenesis *(54)*. At present, evidence to support a role for mutations of the AR gene in subjects with isolated infertility remains inconclusive.

SUMMARY

Androgens play a key role in male sex differentiation and development and in the maintenance of male reproductive function, and the effects of these hormones are an important component in the development of several pathological conditions. Testosterone and its 5α-reduced metabolite, DHT, are potent androgens that act on target cells to initiate and maintain the masculine phenotype. Germ-line mutations in AR and 5α-reductase genes cause AIS syndromes and 5α-reductase deficiency, respectively. The effects of these genetic mutations on male sex differentiation and development have played a key role in elucidating the pathways of androgen action. ARs transduce the steroid signal within cells, but attempts to correlate differences in receptor levels with various disease states have been relatively unsuccessful. However, molecular studies of AR gene structure have recently provided new insights toward defining a molecular and genetic basis for the pathology associated with diseases—including SBMA, breast carcinoma, and prostate cancer—affecting middle-aged and older men. Further studies at the molecular level to define the steroid- and DNA-binding properties of ARs, as well as the transcriptional activity and interactions of the receptor with coactivators, corepressors, and integrators within the transcriptional complex, will provide additional insight into the complex nature of androgen action.

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18 Steroid Receptor Regulation by Phosphorylation and Cell Signaling Pathways

Nancy L. Weigel, PhD

CONTENTS

INTRODUCTION

The steroid receptors are members of a large family of ligand-activated nuclear receptors that encompasses the classical steroid receptors as well as the vitamin D receptor (VDR), retinoid receptor and thyroid receptor *(1)*. Additionally, there are numerous members, originally termed *orphan receptors* because their functions and/or ligands were unidentified *(2)*, whose ligands are also small, hydrophobic molecules such as oxysterols *(3)*, leukotriene B-4 *(4)*, prostaglandin J(2) *(4)*, and farnesol metabolites *(5)*. The nuclear receptors share common structures with carboxy-terminal ligand-binding domains (LBDs), DNA-binding domains (DBDs) containing two zinc finger motifs, and amino-terminal domains containing transactivation functions that are extremely heterogeneous in length *(6)*.

Functionally, the family can be separated into multiple groups. In the absence of an activating signal, the classical steroid receptors (androgen [AR], estrogen [ER], glucocorticoid [GR], mineralocorticoid [MR], and progesterone [PR]) are found associated with heat-shock proteins *(7)*. Activation leads to homodimerization and binding to specific DNA response elements. Although the consensus sequences for the DNA-binding sites have been identified (AGAACA–TGTTCT for AR, GR, MR, and PR; and AGGTCA–TGACCT for ER) *(8)*, natural sequences may deviate substantially and promoters may contain clusters of half-sites in addition to the classical palindromic binding sites.

The second group of nuclear receptors includes the vitamin D receptor (VDR), retinoic acid receptor (RAR), thyroid hormone receptor (TR) as well as some of the

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orphan receptors. These receptors form heterodimers with retinoid X receptors (RXRs) *(9–11)* and bind to their cognate DNA response elements in the absence of activating signals *(12)*. Typically these receptors repress basal activity of target genes in the absence of ligand *(13)*. Data published in the last few years suggest that the inactive receptors bind corepressors such as nuclear receptor corepressor or silencing mediator for RAR and TR and that ligand binding releases the corepressors, allowing binding of coactivators and stimulation of transcription of the target gene *(14,15)*. The receptors are activated by their respective ligands; whether 9-*cis* retinoic acid, the RXR ligand, enhances or inhibits the receptor response appears to be dependent on both the receptor and the promoter *(16,17)*.

Finally, there are family members that can act as monomers *(18)*. In addition, some of the family members, such as Nur77, are also strongly regulated at the transcriptional level *(19,20)*. As the orphan receptors are better characterized, other modes of regulation may be found.

In addition to regulation by ligands, coactivators, and corepressors, there is ample evidence that signal transduction pathways leading to phosphorylation of the receptors themselves, or to associated proteins, regulate the functions of the steroid receptors. Two approaches have been used to elucidate the roles of phosphorylation in receptor function. First, the phosphorylation sites have been identified and their roles examined by site-directed mutagenesis. Second, various signal transduction pathways have been activated or inhibited and the consequences of these manipulations measured using receptor-mediated transcriptional activation as an end point. Using the second approach, the phenomenon of receptor-dependent, ligand-independent activation of steroid receptors was first detected *(21)*.

RECEPTOR PHOSPHORYLATION

All of the nuclear receptor family members studied to date are phosphoproteins. In many cases, there are multiple phosphorylation sites phosphorylated by different kinases; recent studies suggest that the various phosphorylations play quite different roles in the activity of the receptors.

Phosphorylation Sites

The steroid receptors typically are multiply phosphorylated, and, in most cases, exhibit enhanced phosphorylation in response to hormone; the majority of the sites reside in Ser-Pro motifs implicating proline-directed kinases in the regulation of steroid receptor phosphorylation. Although studies of the other members of the family are less complete, typically the receptors which are phosphorylated on fewer sites and the sites are more often not Ser-Pro consensus sites. The phosphorylation sites identified in some of the better characterized receptors are summarized next.

Androgen Receptors

Whereas phosphorylation of endogenous human AR in LNCaP prostate cancer cells has been reported to be enhanced by hormone treatment with an accompanying decrease in mobility on sodium dodecyl sulfate (SDS) gels (an apparent increase in size from 110 to 112 kDa) *(22)*, the phosphorylation level of transiently expressed AR in COS cells has been found to be constitutive *(23)*. Three phosphorylation sites have been identified in AR, all containing Ser-Pro motifs *(24)*. Ser^{81} and Ser^{94} are in the amino-

terminal region whereas Ser^{650} is located in the hinge region between the hormone and DBDs. Peptide-mapping studies indicate that there are additional unidentified sites. The kinases that phosphorylate these sites have not been identified.

Estrogen Receptors

Phosphorylation sites in human and mouse ER have been characterized. In the case of human ER, several groups have found that Ser^{118} in the amino terminus of the protein is the major hormone-dependent phosphorylation site *(25–27)*, but others have reported that Ser^{167} is the major hormone-dependent site in the endogenous ER of MCF-7 cells *(28)*. Additional phosphorylation sites in ER include Ser^{104} and/or Ser^{106}, both of which are found in Ser-Pro motifs as is Ser^{118} *(25)*. In addition to the serine sites, there is good evidence that ER is also phosphorylated on Tyr^{537} in the LBD *(29,30)*. Peptide-mapping studies suggest that there are additional phosphorylation sites in the carboxyl terminus of ER *(25)*. Ser^{118} has been identified as a mitogen-activated protein kinase (MAPK) site *(31,32)*, Ser^{167} as a casein kinase II site *(28)*, and there is evidence that ER is also phosphorylated by cyclin-dependent kinases (CDKs) *(33)*. Tyr^{537} can be phosphorylated in vitro by src kinase. Collectively, these studies demonstrate that ER is regulated by multiple kinases.

Phosphorylation studies of mouse ER have shown that analogous sites including Ser^{122} (analogous to Ser^{118}) and possibly Ser^{171} are phosphorylated as well as two sites, Ser^{156} and Ser^{158}, which do not contain Ser-Pro motifs *(34)*. In addition, Ser^{298}, in the hinge region, is phosphorylated *(34)*. This site appears to be analogous to the hinge sites in chicken PR and in AR.

Glucocorticoid Receptors

Phosphorylation of GRs is complex, with seven sites (six serines and one threonine) identified *(35)*. All reside in the amino-terminal domain. Although several contain Ser-Pro motifs, one is a casein kinase II consensus sequence and the others do not conform to known consensus sequences. The phosphorylation of one of the sites, Ser^{220}, is preferentially increased by hormone treatment *(36)*. Thr^{171} and Ser^{246} can be phosphorylated by MAPK whereas Ser^{224} and Ser^{232} are substrates for CDK *(37)*. Based on studies in yeast, it appears that phosphorylation of the cyclin-dependent sites is stimulatory whereas MAPK sites are inhibitory *(37)*.

Progesterone Receptors

Unlike the other steroid receptors, PR is expressed as two forms, PR-A and PR-B, that differ by the presence of 128–164 additional amino acids at the amino terminus of PR-B. In the chicken PR, all of the phosphorylation sites are in the domains common to PR-A and PR-B. Four sites have been identified: three in the amino terminus and one in the hinge region *(38,39)*. Each site contains a Ser-Pro consensus. Two of the sites, Ser^{211} and Ser^{260} are basally phosphorylated, but the extent of phosphorylation is increased on hormone treatment. Phosphorylation of Ser^{367} and Ser^{530} is detected after hormone treatment.

Phosphorylation of human PR is less well characterized. Seven sites have been identified in human PR. Three of these sites, Ser^{81}, Ser^{102}, and Ser^{162}, are unique to PR-B *(40)*. Of the sites identified, four (Ser^{81}, Ser^{162}, Ser^{190}, and Ser^{400}) are basal sites whose phosphorylation is increased in response to hormone *(40,41)*, and the phosphorylation of three sites (Ser^{102}, Ser^{294}, and Ser^{345}) is induced by hormone *(42)*. With the exception

of Ser^{81}, which is contained in a casein kinase II consensus site, all of the sites are found in Ser-Pro motifs. Mapping studies suggest that there are additional sites *(40)*. Ser^{81} is phosphorylated by casein kinase II *(40)* whereas Ser^{162}, Ser^{190}, and Ser^{400} can be phosphorylated by cyclin A Cdk2 complexes *(41)*.

Phosphorylation of Other Family Members

Many of the other nuclear receptors are also phosphorylated. The VDR is phosphorylated at Ser^{208} in the hinge region; this site is a casein kinase II site *(43,44)*. Thyroid receptors are also phosphoproteins *(45–49)*. Phosphorylation of RARs and RXRs is dependent on both the isoform and the splice variant. For example, RARβ1 and RARβ3 exhibit ligand-dependent phosphorylation whereas RARγ and RARβ2 do not *(50)*. Although most of the phosphorylation is found on serine residues, RARβ also contains a phosphotyrosine *(50)*. Ser^{369} is phosphorylated in RARα1 *(51)*, and Rochette-Egly et al. *(52)* have recently shown that Ser^{77} of RARα is phosphorylated by cdk7. PPARγ is phosphorylated by MAPK *(53,54)*.

Phosphorylation and Receptor Function

For the most part, the role of individual phosphorylation sites has not been determined in great detail, but the limited studies to date suggest that the sites may have different and even opposing roles.

Ligand Binding

Although there have been some reports that Tyr^{537} in ER plays a role in ligand binding *(55,56)*, a recent study using site-directed mutagenesis shows that phosphorylation of this residue is not required for high-affinity ligand binding *(57)*. Identified sites in other steroid receptors lie outside of the LBD and do not appear to alter ligand binding.

DNA Binding

There are numerous studies implicating phosphorylation in DNA binding. Phosphatase treatment of ER reduces DNA binding *(58)* and chicken and human PR, isolated from tissue or cells treated with hormone, bind DNA more strongly than receptor treated with hormone in vitro *(59,60)*. In the case of TRs, phosphorylation enhances the DNA binding of some forms *(47,48)*, but reduces DNA binding of the TRα2 variant, a nonligand-binding form of the receptor *(49)*. Arnold et al. *(30)* reported that Tyr^{537} of ER plays a role in ER dimerization and subsequent DNA binding and that phosphorylation of Ser^{167} also enhances DNA binding *(61)*. The contribution of other sites to DNA binding has not yet been reported.

Transcriptional Activation

Most of the studies examining the role of phosphorylation in receptor function have been directed toward determining whether phosphorylation alters transcriptional activation. Mutation of the hinge site in AR reduces activity by 30% *(24)*. Mutation of Ser^{118} in ER also reduces activity, and the extent of reduction is dependent on the promoter *(25,26)*. Mutation of Tyr^{537} in ER increases basal activity when a negatively charged amino acid is substituted for tyrosine and decreases basal activity when phenylalanine is substituted for tyrosine *(57)*. In all cases, the mutants exhibit activity in response to hormone. Initial functional studies with GR mutants suggested only small changes in activity *(62)*, but more recent studies demonstrate that mutation of phosphory-

lation sites greatly alters activity on a simple promoter *(63)* as well as plays a role in the stability of the receptors *(63)*.

The sites in chicken PR have been most extensively characterized. Mutation of either Ser^{211} or Ser^{260} to alanine greatly reduces the transcriptional activity of the receptor *(64)*, and, at least in the case of Ser^{211}, the extent of the response is strongly dependent on the promoter and cell type *(65)*. Substitution of alanine for Ser^{530} decreases the transcriptional response to low concentrations of hormone without changing the hormone binding affinity *(66)*. There is some evidence that substitution of alanine for Ser^{367} increases the transcriptional activity of the receptor. Reports of the role of phosphorylation in human PR function are more limited. Mutation of Ser^{190} reduces the activity of the receptor by about 50% *(67)*.

Taken together, these limited studies suggest that phosphorylation plays roles in receptor stability, DNA binding, and interactions with other proteins altering transcriptional activation.

LIGAND-INDEPENDENT ACTIVATION

In studies to determine whether the protein kinase A (PKA) pathway modulated the activity of chicken PR, Denner et al. *(21)* first found that chicken PR can be activated by treating cells with 8-Br cAMP, an activator of PKA, in the absence of hormone. Since that first report, there have been a number of reports showing ligand-independent activation of a subset of the steroid receptors by several different cell-signaling pathways. Although several receptors respond to similar stimuli, the mechanism by which ligand-independent activation is achieved may depend on both the receptor and the activation pathway.

Signal Transduction Pathways that Activate Steroid Receptors

A variety of stimuli activate various steroid receptors. Although there is good evidence that some of the pathways are distinct, it is not yet clear whether or not some of the seemingly diverse signals converge in one or two common pathways to cause activation of receptors.

Androgen Receptors

The data describing ligand-independent activation of AR show that human AR can be activated in the absence of ligand in response to specific signals and that the response is dependent on the promoter and factors that have not yet been identified *(68)*. Culig et al. *(68)* first reported that AR, transfected into DU 145 prostate cancer cells that lack endogenous AR, can be activated by growth factors including insulin-like growth factor-1 (IGF-1), keratinocyte growth factor (KGF), and epidermal growth factor (EGF), but that the extent of the response depended on the growth factor and the promoter. With more complex promoters such as the prostate-specific antigen (PSA) promoter, only IGF-1 elicited a strong response. That an endogenous receptor can activate an endogenous target gene was demonstrated in LNCaP cells in which treatment with IGF-1 induced PSA expression that was blocked by AR antagonists *(68)*. Nazareth and Weigel *(69)* subsequently reported that forskolin, an activator of adenylyl cyclase, activates AR transfected into either CV1 or PC3 prostate cancer cells; this activation can be blocked by the AR antagonists, casodex and flutamide. Moreover, inhibition of

the PKA pathway reduced hormone-dependent activation of AR without altering AR expression levels or diminishing general transcription.

By contrast, several groups have reported that signaling pathways stimulate the hormone-dependent activity of AR, but do not find evidence for ligand-independent activation. Ikonen et al. *(70)* demonstrated that the hormone-dependent activity of rat AR transiently transfected into CV1 cells was stimulated by treatment with 8-Br cAMP or okadaic acid (a phosphatase inhibitor), or by activation of PKC. Growth factors also enhance hormone-dependent activation of rat AR *(71)*. The hormone-dependent activity of human AR, stably transfected into Chinese hamster ovary cells, can be stimulated by activation of PKC, but not PKA *(72)*.

Estrogen Receptors

Transfection Studies. Evidence that estrogen receptors can be activated by signal transduction pathways both in cells and in vivo is more extensive than for the other steroid receptors. However, the activation is cell, activator, and promoter dependent. For example, although ligand-independent activation of ER was observed in 3T3 cells transfected with an ER expression plasmid and an ERE-vit-CAT reporter in response to elevated cAMP levels (treatment with cholera toxin and isobutylmethylxanthine), there was no activation when an $(ERE)_2$-tk-CAT reporter was used *(73)*.

Results from transfection studies show that ER can be activated by diverse signals. Activation of a variety of Ser/Thr kinase pathways leads to activation of ER. Smith et al. *(74)* found that dopamine activates ER transfected in HeLa cells. Ignar-Trowbridge et al. *(75,76)* found that activators of PKA and PKC pathways were capable of activating ER in ovarian adenocarcinoma cells, and Ince et al. *(73)* showed that, under some conditions, ER transfected into 3T3 cells can be activated by elevation of cAMP levels.

Growth factor pathways, which are initiated through tyrosine phosphorylation, also activate ER. Ignar-Trowbridge et al. *(77,78)* showed that mouse ER transfected into Ishakawa cells can be activated by EGF treatment as can endogenous ER in BG-1 human ovarian cancer cells. The activity is blocked by ICI 164,384. EGF activates human ER transfected into HeLa cells *(79)*. Other growth factors such as IGF-1, insulin, and heregulin also activate ER under appropriate conditions *(80,81)*. Other studies show that overexpression of cyclin D1 induces activation of ER in the absence of ligand *(82)*.

The diversity of factors that activate ER raise the question, Are there many separate pathways that lead to activation or do the activators converge on one site? Analysis of the response of ER mutants indicates that there are a minimum of two distinct pathways. Collectively, the data show that the EGF-mediated pathway requires the amino-terminal portion of the receptor whereas some of the other pathways require the LBD. For example, the Val^{400} ER mutant is not activated by dopamine treatment in the absence of hormone *(74)*, but can be activated by the EGF pathway *(79)*. Other studies with deletion of AF-2 mutants implicate the amino terminus in the EGF pathway *(75,79)*. Finally, chimeras between GR (which does not exhibit ligand-independent activation) and ER implicate the amino terminus of ER in the EGF-induced activation *(79)*. By contrast, studies by Patrone et al. *(83)* show that the carboxy terminal of ER is required for insulin-dependent activation of ER in neuroblastoma cells.

Endogenous ER and Natural Target Genes. Although the phenomenon of ligand-independent activation is intriguing, a key question is, is this a physiological response

inducing endogenous receptors to activate transcription of target genes? Evidence that this is so comes from both studies of cells containing endogenous ER and in vivo studies. The first demonstration of ligand-independent activation of endogenous ER was the finding of Aronica and Katzenellenbogen *(84)* that increases in levels of rat uterine cell PR (whose expression is regulated by ER) through treatment with IGF-1 or cAMP can be blocked by ER antagonists. In vivo evidence was first provided by Ignar-Trowbridge et al. *(77)*, who found that EGF treatment of ovariectomized mice caused induction of markers of estrogen action such as phosphatidylinositol lipid turnover and uterine DNA synthesis; the ER antagonist, 164,384, partially blocked the induction. Moreover, this treatment resulted in increased nuclear retention of ER and an apparent increase in phosphorylation of ER as judged by mobility on SDS gels. The studies of Curtis et al. *(85)* in the ER knockout mouse provide further support that some of the actions of EGF are mediated by ER. In these mice, treatment with EGF does not induce PR mRNA expression or DNA synthesis as it does in wild-type mice.

Glucocorticoid Receptors

In contrast to most of the other steroid receptors, there is no evidence that GR can undergo ligand-independent activation. Numerous studies, under conditions that activate other steroid receptors in a ligand-independent manner, have failed to demonstrate ligand-independent activation of GR. However, there is good evidence that signal transduction pathways enhance the activity of GR. Activation of either PKA or PKC enhances the response to hormone *(86,87)*. Despite the failure of GR to be activated in the absence of ligand, Nordeen et al. *(88)* found that administration of the antagonist RU486 in combination with 8-Br cAMP induces activation of GR. That this effect shows some specificity for signal transduction pathways is evidenced by the failure of PKC activation to induce a similar response.

Progesterone Receptors

Whether PRs exhibit ligand-independent activation appears to be heavily species dependent. One of the best characterized receptors, chicken PR, responds to a variety of signals. There is also evidence that the rodent receptors exhibit ligand-independent activation, but human PR is unresponsive or responds only under very restricted conditions.

Chicken PRs. Chick PR was the first receptor shown to exhibit ligand-independent activation *(21)*. Treatment with 8-Br cAMP activates cPR transfected into a variety of cell types including CV1 and HeLa, and activation occurs whether the promoter is a very simple one, such as the GRE_2E1bCAT promoter, which contains two GRE/PREs and the TATA box from the E1b promoter, or very complex such as the MMTVCAT, which contains multiple sites including half-sites that contribute to optimal activation *(21,89)*. In addition to cAMP, chicken PR is activated by dopamine *(90)*, phosphatase inhibitors such as okadaic acid or vanadate, as well as growth factors such as EGF *(21,89)*. That there are at least two pathways to ligand-independent activation of chicken PR is illustrated by the finding that a mutation at amino acid 628 of chicken PR-A eliminates ligand-independent activation through the dopamine pathway, but not the response to okadaic acid *(90)*.

Rat PRs. Evidence from cell-based studies as well as in vivo studies demonstrates that rat PR undergoes ligand-independent activation. Turgeon and Waring *(91)*, using primary rat pituitary cells transfected with a reporter, demonstrated GnRH- or 8-Br cAMP–dependent transcriptional activation that was blocked by the progesterone antagonist, RU486.

Mani et al. *(92)*, using a lordosis response assay, have shown that rat PR can be activated in vivo by direct injection of dopamine agonists into the third ventricle; this response can be blocked either by the progesterone antagonist, RU486 *(92)*, or by preadministration of PR antisense oligonucleotides *(93)*.

Human PRs. In contrast to the studies of rodent and chicken PRs, most studies with human PR do not show ligand-independent activation even when conditions identical to those used for chicken PR are used *(94)*. As with GR, treatment with activators of signal transduction pathways stimulate hormone-dependent activity *(60)*. Moreover, RU486 in combination with 8-Br cAMP does activate PR, and inhibitors of PKA activation reduce hormone-induced transcription *(95,96)*. Despite a number of studies failing to show ligand-independent activation of PR, there are at least two reports describing ligand-independent activation. Kazmi et al. *(97)* described ligand-independent activation of PR in transfected COS-1 cells, and Philpott and Shahid *(98)* have reported that dopamine can activate hPR-B transfected into CV1 cells. Therefore, ligand-independent activation of human PR may occur under more restricted conditions than have been found for chicken PR.

Other Steroid Receptor Family Members

Several reports have described ligand-independent activation of other members of the nuclear receptor family, but for the most part, these receptors have not been studied as extensively as the steroid receptors. VDR *(99)*, RARα, RARβ, RXRα, RXRβ, and RXRγ *(100)*, but not RARγ *(100)*, have all been shown to exhibit ligand-independent activation under some conditions.

Mechanism of Ligand-Independent Activation

Phosphorylation of Steroid Receptors

Because all inducers of ligand-independent activation directly or indirectly alter phosphorylation of proteins, the simplest explanation would be that altered phosphorylation of the receptors is responsible for ligand-independent activation. Despite the finding that many receptors are activated in the absence of hormone, the mechanisms by which this occurs appear to be dependent on pathway and receptor. For example, treatment with 8-Br cAMP causes activation of chicken PR, and two of the phosphorylation sites, Ser^{211} and Ser^{260}, are important in achieving maximal activation, but are not absolutely required *(64)*. The treatment does not change phosphorylation of cPR on the known sites, nor does it induce phosphorylation of novel sites *(64)*.

By contrast, treatment of ER-containing cells with EGF causes ligand-independent activation and increased phosphorylation of Ser^{118}. Mutation of Ser^{118} to alanine eliminates the response to EGF *(79)*. However, substitution of a glutamic acid at the position restores response to EGF, indicating that a negative charge at Ser^{118} is necessary, but not sufficient to induce EGF-dependent activation of ER *(79)*. Activation of PKA also induces phosphorylation of ER, but in the carboxy-terminal region of the receptor *(25)*. Whether this phosphorylation plays a role in ligand-independent activation is not known.

Finally, ligand-independent activation of AR may be accompanied by dephosphorylation. Treatment with forskolin produces predominantly the faster mobility form of AR on SDS gels *(69)*, which has been associated with a less phosphorylated state.

Role of Coactivators and Corepressors

The evidence previously outlined suggests that in many cases altered phosphorylation of the receptors is insufficient to induce ligand-independent activation. With the recent finding that coactivators and corepressors modulate receptor action, these proteins appear to be likely targets of signal transduction pathways. Although these proteins were initially described as stimulators of hormone-dependent activation, recent studies have shown that CREB-binding protein and steroid receptor coactivator-1 (SRC-1) can play a role in ligand-independent activation of PR *(101)* and ER *(102)*. Mutation of Tyr^{537} to an acidic residue is sufficient to induce hormone-independent interaction between ER and SRC-1 *(103)*. A preliminary report by Rowan et al. *(101)* shows that treatment with 8-Br cAMP alters the phosphorylation of SRC-1 at specific sites. It is tempting to speculate that this altered phosphorylation plays a key role in inducing ligand-independent activation of steroid receptors through the PKA pathway.

SUMMARY

It is evident from the studies described herein that the activities of the nuclear receptor families are regulated by signal transduction pathways. Phosphorylation of the receptors contributes to the hormone-dependent activity of the receptors and may also play a role in downregulation. The novel finding that some of the receptors can be activated by signal transduction pathways in the absence of hormone highlights the importance of these pathways in integrating the steroid hormone signals with the activity of membrane receptors to produce the appropriate cellular responses.

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19 Steroid Receptor Actions

Agonists and Antagonists and the Role of Coactivators and Corepressors

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and Margaret A. Shupnik, PhD

CONTENTS

INTRODUCTION

Steroid hormones, after binding to their receptors, regulate a large number of physiological processes including development, growth, metabolism, and reproduction and fertility *(1,2)*. Target tissues include those involved in sexual development and reproduction such as the gonads, uterus, epididymis, and pituitary, as well as the brain, bone, and liver. Steroid hormones are hydrophobic and circulate in serum bound to carrier proteins such as globulins and albumins. The steroids then pass through the cell membrane and bind directly to protein receptors, which act as dimers in the nuclei of target cells to influence the transcription rate of responsive genes. The cell- and tissue-specific effects of steroid hormones have been observed for many years, but advances in

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molecular biology and structural biology combined with new pharmaceutical tools have begun to provide a framework to understand these differences.

Steroid receptor antagonists, known commonly as antiestrogens or antiprogestins, bind to the nuclear receptors and either fail to permit or prevent the biological effects normally occurring as a result of ligand binding *(3)*.The antagonists could thus prevent activation of responsive genes in target tissues in the presence of endogenous steroids. However, in vitro experiments performed in parallel with different cell types demonstrated that some putative receptor antagonists were in fact partial agonists depending on the cellular context. Thus, a compound such as the estrogen receptor (ER) antagonist tamoxifen acts to inhibit cellular responses in breast and breast cancer cells, but is a partial agonist in uterus and bone *(3)*. The wide degree of responses for these compounds in different contexts has led to the use of the term *selective estrogen receptor modulators* (SERMs), which more accurately describes the biological effects. Because of the use of several steroid receptor antagonists in treating steroid-dependent cancers of the breast and prostate, and the general importance of the steroid hormones in maintaining good health, there is great interest and clinical significance in understanding the general processes underlying the specificity and diversity of biological responses. Recent studies have demonstrated that the process by which ligand binding influences gene transcription, or transactivation, can be influenced by several factors. These include the existence of several steroid receptor isoforms expressed at different levels in various cell types, the presence of specific cellular proteins that bind to the ligand-bound or unbound receptor and stimulate (coactivators) or suppress (corepressors) activity, and the modification of the receptor or interacting proteins by kinases activated by growth factors and other peptides. In each case, the conformation of the ligand-bound receptor may differ with specific steroid receptor antagonists, and could be influenced by these factors to a greater or lesser extent. Because the most information is available for the estrogen and progesterone receptor (PR), this review concentrates on those proteins.

STEROID RECEPTOR STRUCTURE AND TRANSACTIVATION

General Structural Features and Receptor Isoforms

All steroid receptors share the same general structure (Fig. 1), consisting of five structural and functional domains: A/B, C, D, E, and F *(1,2)*. Several of the regions are modular in nature and have been shown by domain-swapping studies to contain all the necessary information for biological specificity within the defined amino acid sequence. These include the central C region, or DNA-binding domain (DBD), consisting of two zinc fingers that directly contact the DNA sequences of target genes, and the C-terminal E region that binds ligand. The conformation of the E region after ligand binding is clearly critical in the subsequent biological response, and this portion of the molecule forms a hydrophobic core that completely surrounds the lipophilic steroid *(4–6)*. Other biological functions, most notably receptor transactivation and the regulation of gene transcription, require cooperation between more than one receptor region. Full transcriptional activity requires both the activation function-1 (AF-1) and AF-2 regions contained in the N- and C-terminal protein regions, respectively *(7)*. The N-termini of the receptors encode the A/B regions and the AF-1 functions, in which there is the most diversity in length and amino acid sequence, even among related receptors or receptor isoforms. These regions may not have a high degree of structure or order,

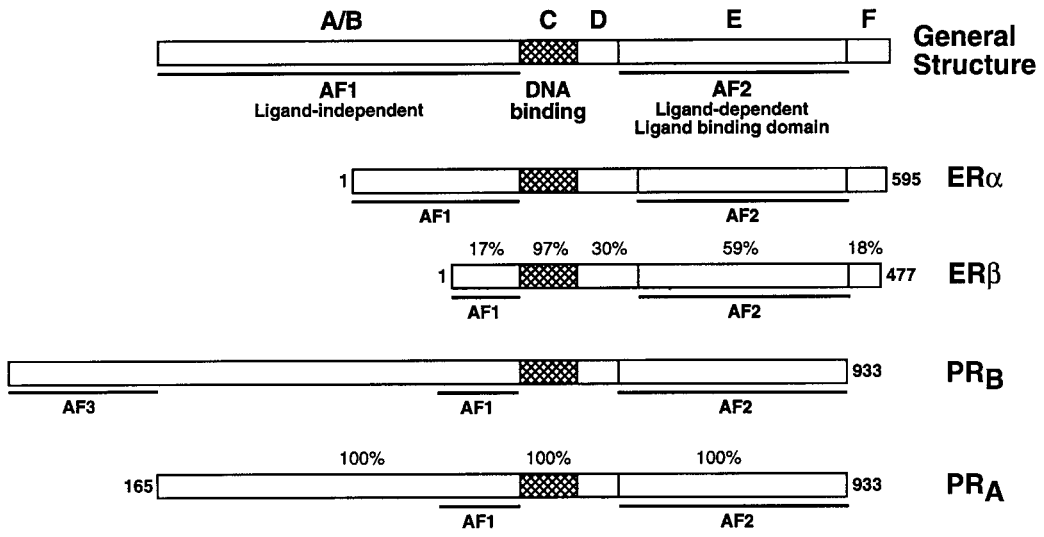

Fig. 1. Modular domain structure of steroid receptors. The general structure of steroid receptors is shown on top with corresponding domains A–F, a central DBD (shaded), and two transactivation functions (AF-1 and AF-2). Structures of the human estrogen receptors α and β (ERα and ERβ) and PRs A and B are shown below aligned by their DBDs. Amino acid numbers are displayed at each end of the receptors, and percentage of domain homologies of ERβ and ERα and PR-A with PR-B are shown.

and do not appear, at this point, to have defined common motifs that confer biological responsiveness. The AF-1 function, as discussed subsequently, is ligand independent, but may be influenced by modification through protein kinase pathways. The AF-2 function is generally encoded within the E hormone-binding region, consisting of a series of 12 α-helices, and is ligand dependent *(7–9)*. Appropriate transactivation requires the correct sequence and conformation of the region encoded by helix 12, which undergoes a conformational change on ligand binding necessary for the biological response *(6,9–14)*. However, removal of the F region at the extreme C-terminus of the receptors can reduce the transcriptional response to hormone and can modify the ER responses to receptor antagonists or to protein kinase activators *(15)*. Finally, removal of or mutation of the D or hinge region can also modify receptor responsiveness *(16)*. Thus, the appropriate transcriptional response is the result of multiple cooperative interactions.

Cloning of the ER cDNA and gene from human and rodent cells and tissues confirmed the general steroid receptor structure in proteins of approx 64–68 kDa *(2,17)*. The ER mRNA and proteins appeared at highest levels in uterus, with lower levels of expression in pituitary, ovary and other reproductive tissues, and liver, and very low levels in brain. The mRNA levels are regulated by estrogen in a tissue-specific manner both positively and negatively *(18,19)*, suggesting that modulation of ER levels contributes to tissue-specific responses. Given the tissue distribution and ligand-binding profile of this protein, it was assumed that there was only one ER gene and protein. However, cloning of closely related cDNAs from rat prostate *(20)* and human tissue *(21)* demonstrated the existence of a second ER form, called ERβ, with the earlier

form designated as ERα. ERβ (51–58 kDa) is present in some of the same tissues as ERα, including uterus, pituitary, and brain, but appears to be expressed in both overlapping and distinct cellular patterns *(22,23)*. In the ovary, ERβ is highly expressed preferentially in granulosa cells of small, growing, and preovulatory ovarian follicles, suggesting that ERβ could play a critical role at distinct stages of the ovarian cycle *(24)*. Finally, ERβ is present at moderate to high levels in some tissues that contain no detectable ERα, such as lung, bladder, and spleen. ERβ mRNA levels do not appear to be regulated by estrogen, although gonadotropin and cyclic adenosine monophosphate (cAMP) may suppress ERβ in ovarian cells *(24)*. Thus, the estrogen response in a given tissue can be modulated by the expression of both ER isoforms, and the differential regulation of the receptors by specific physiological conditions. The observed phenotypes of mice in which either the ERα *(25)* or ERβ *(26)* genes were preferentially disrupted suggest that ERα is required for both male and female fertility and reproductive behavior, as well as breast development and normal uterine and pituitary function. A homozygous mutation of the human ERα gene in a male human, encoding a truncated protein, resulted in normal male development but with osteoporosis and failure of bone epiphysis fusion *(27)*.

ERβ knockout mice are fertile, but females have reduced ovarian efficiency and smaller litter size. Older mutant males display signs of prostate and bladder hyperplasia. Additional studies will be required to determine which receptor isoform is the most critical in brain function and cognition, cardiovascular function, and bone homeostasis. The ERs share high homology (>90%) in the DNA-binding region (Fig. 1), 50–55% homology in the ligand-binding domain (LBD) clustered in discrete areas, and very low homology in the AF-1 (A/B region) domains. Both receptors bind DNA and estrogen response elements (EREs) similarly, and bind to natural estrogen metabolites in the same rank order and with the same general affinities *(28)*. An additional complicating factor is the description of an ERβ2 isoform, with an 18 amino acid in phase insertion in the LBD *(29)*. ERβ2 binds ligands with much lower affinity compared to the original ERβ (or ERβ1), and has a correspondingly poorer ability to transactivate genes. Levels of ERβ2 are detectable in several cell types, but translated protein has yet to be demonstrated in normal cells, and the true physiological significance of this variant remains to be proven. However, binding affinities for the synthetic antiestrogens and SERMs can differ widely between ERα and ERβ, particularly with estrogen compounds substituted at the 17α position. ERα binds to many classical antiestrogens with higher affinity *(22,28)*. Recently, novel compounds that function as selective estrogens or antiestrogens for the ER isoforms, with differences in both binding and transcriptional efficacy, were described *(30)*. An aryl-substituted pyrazole was an ERα potency-selective agonist, with higher binding affinity for ERα, and 120-fold higher potency in stimulation of ERα vs ERβ in cellular transactivation assays. A tetrahydrochrysene compound had a four-fold preferential binding affinity for ERβ and was an agonist on ERα but an antagonist on ERβ.

These data, coupled with the tissue distribution and cellular expression of the ER isoforms, suggest that it will be possible to target defined tissues or cells with specific synthetic compounds for the desired biological effect. Overall efficacy in cells and living animals, however, will be influenced by several considerations, including the facts that ligand-binding affinity is not always linked directly with transactivation efficiency of the ligand-bound ER with some compounds *(31)*. In addition, somewhat

different biological effects can be observed using different reporter genes with different ERE sequences, as discussed subsequently. Finally, ERα and ERβ can form heterodimers, with the heterodimer formed preferentially to ERβ homodimers *(32)*. Therefore, the binding and transcriptional effects noted with synthetic ligands on individual receptor isoforms will be influenced in individual cells and tissues by the overall expression and ratios of ERα and ERβ.

PR does not have distinct isoforms encoded by two separate genes, but does exist as two closely related cDNAs and proteins that differ only in their N-terminal AF-1 region. The larger human receptor, PR-B, is approx 114 kDa, whereas the smaller PR-A is 94 kDa and lacks the N-terminal 164 amino acids contained in PR-B *(33)*. The two proteins are identical in the LBD and, as expected, bind to a variety of agonists and antagonists with similar or identical affinity. However, they differ considerably in the AF-1 region, and do differ in their transactivation capability on different promoters and in response to synthetic agonists and antagonists. In many tissues and breast cancer cell lines, the two proteins are present in approximately equal ratios, but regulation of receptor levels and alterations in the ratios of PR-A to PR-B can occur under some conditions. Estrogen can stimulate PR gene transcription and mRNA levels in both rat and human cells, and estrogen pretreatment is required for PR expression in several tissues such as uterus in vivo *(34)*. PR-B expression appears to be most sensitive to E levels and also declines in uterine cell lines that are poorly differentiated *(35)*. The ratio of human uterine PR-A to PR-B varied during the menstrual cycle from >10:1 from d 2 to 8 to about 2:1 between d 14 and 16 near ovulation *(36)*. Exogenous estrogens enhanced expression of PR-B preferentially. Therefore, the steroid environment can influence the ratio of the two receptors and thus the potential response to progestins. Knockout mouse models establish a critical role for PR in the development of mammary glands, sexual differentiation, and fertility, but do not distinguish between the relative importance of PR-A and PR-B forms *(37)*.

AF-1 and AF-2 Domains: Ligand-Independent and Ligand-Dependent Transactivation

Early studies by several groups *(7,8,11,38)* established that both N- and C-terminal regions of the steroid receptors were required for full transcriptional activity, and that the contribution of both regions to this response was more than additive. Both the AF-1 and AF-2 domains have cell- and promoter-specific effects, suggesting that cell-specific proteins might bind to the receptors and contribute to the transcriptional response, and that specific DNA sequences that comprise or surround the response element might also influence receptor conformation or binding to additional proteins. Direct physical interactions between the AF-1 and AF-2 domains or proteins binding to these regions are postulated, and have been demonstrated for some receptors such as the androgen receptor (AR) *(39)*. Thus, in some studies, the activity of the AF-1 domain is measured in a full-length receptor in which the AF-2 activity is abolished by specific amino acid mutations *(38)*. Specific sequences in the AF-1 domain of the ER, particularly amino acids 120–150, are necessary for cooperative activity between AF-1 and AF-2 on agonist binding, and activation of transcription by antiestrogens may also require amino acids 41–64 *(40)*. AF-2 requires ligand binding to manifest its activity and occurs as a result of conformational changes that recruit coactivator proteins such as steroid receptor coactivator-1 (SRC-1), glucocorticoid receptor interact-

ing protein-1 (GRIP-1), or amplified in breast cancer (AIB-1) *(41–43)*. These proteins bind within a hydrophobic pocket formed by helices 3, 5, 6, and 12 in the LBD, and require movement or ordering of helix 12 on ligand binding *(4–6,13,14)*. Mutations within this region, particularly those that disrupt the hydrophobic pocket, have severe consequences on transactivation through AF-2 *(9,44)*. Binding of antagonists such as antiestrogens effectively obliterates the activity of AF-2, and residual or partial agonist activity noted in some cell types is believed to be a result of AF-1 activity in those cells *(38)*.

As predicted, the biological response of the AF-1 domain in the N-terminal region of the receptor is ligand independent, but can be modulated through direct protein modification through phosphorylation cascades. Several kinases, notably those stimulated by growth factors or Ras-mediated signaling cascades, can act on AR, PR, and ER to stimulate transcription from transfected reporter genes independent of the hormone ligands; addition of hormone often results in an additive or synergistic effect *(34)*. Mitogen-activated protein kinase phosphorylates Ser^{118} and Ser^{167}, and these phosphorylation events are directly linked to stimulated transcription *(45,46)*. Stimulated phosphorylation of several distinct serine residues can be demonstrated in ER, PR, and AR, in response to numerous kinases such as protein kinase A (PKA), PKC, and cyclin-dependent kinases (*see* Chapter 18), as well as in response to steroid agonist and antagonist binding *(47–53)*. Mutation of these sites diminishes but does not abolish transactivation of the receptor, and the physiological role of phosphorylation is under active investigation in many laboratories. However, the potential for cellular growth factors and other peptide signals to influence steroid receptor activity is important and may assume critical significance in actively growing cells such as those found in steroid-dependent cancers. The additional observation that kinase-mediated processes, particularly the actions of PKA, can alter the agonist/antagonist activities of some steroid receptor ligands suggests that this mechanism can have grave biological consequences. The additional AF-1 region contained in the PR-B form has been called AF-3 by some investigators *(54)*. This region can modulate the activity of the full-length receptor and in a chimeric construct when fused to the PR DBD. The PR-A form functions as a transcriptional repressor in human breast cancer and cervical carcinoma cells, and its effects predominate in PR-A:PR-B heterodimers *(33,35)*. PR-B responds inappropriately to antiprogestins in breast cancer cells to stimulate transcription, and the overall response will thus result from the ratio of the isoforms *(56)*.

ER ANTAGONISTS

ER antagonists have been divided into two classes. Type I antagonists are triphenylethylene compounds that include tamoxifen, its metabolites, and several analogs. These compounds antagonize the actions of estrogen but also have partial agonist actions under some circumstances. This partial agonist activity is the basis for the use of tamoxifen in the treatment of breast cancer because tamoxifen acts as an antagonist to estrogen's proliferative actions in breast but maintains agonist actions in bone, uterus, and on lipid metabolism *(3)*. Because of the potential of uterine hypertrophy and the loss of sensitivity to tamoxifen in breast cancer, another class of antiestrogens was developed.

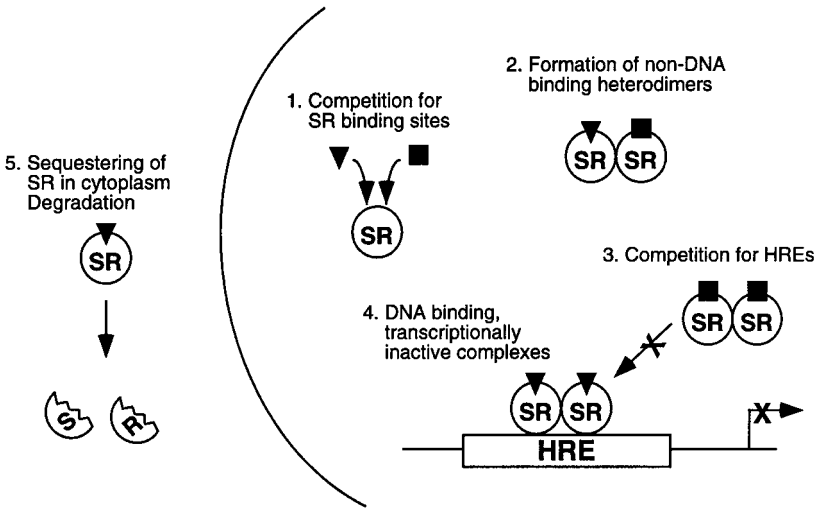

Fig. 2. Mechanisms of steroid antagonist action. General mechanisms of steroid antagonist action are shown: ■, agonist; ▼, antagonist. SR, steroid receptor; HRE, hormone response elements; curved line, the nuclear membrane.

Type II antagonists are "pure" antiestrogens and contain no agonist properties. These compounds (ICI 164,384 and ICI 182,780), developed by Wakeling and colleagues *(57)*, are 7α substitutions of estradiol and have shown clinical usefulness after the failure of tamoxifen *(3)*. Substitutions at the 11β position have also proved effective in creating pure antiestrogens *(3)*. Additional compounds, which some have classified as Type III antagonists *(38)*, have recently been developed that have little chemical similarity to tamoxifen or estrogen. However, these compounds make use of the structural features necessary for ER binding. These include raloxifene (also called keoxifene, LY 156,758, LY 139,481-HCI, and Evista®) and the structurally similar LY117018; they act as antagonists in breast and uterus but have partial agonist actions in bone and on serum lipids *(3)*. Other nonsteroidal compounds, such as EM800 (EM652), have also shown pure antagonist activities *(58)*. The search for new ER ligands rapidly continues, and the discovery of ERβ has further heightened the need for SERMs. Recently two novel nonsteroidal ligands with differential actions on ERα and ERβ were described *(30)*. The first is a triaryl-substituted pyrazole and is a selective ERα agonist. The second, a *cis*-diethyl-substituted tetrahydrochrysene called THC, is a selective antagonist of ERβ.

MECHANISM OF ACTION OF ER ANTAGONISTS

ER antagonists act via several mechanisms (Fig. 2); however, two principle mechanisms are involved. First, antagonists compete with estradiol or other estrogenic agonists for ER-binding sites. Second, on binding, antagonists induce various degrees of conformational changes in the receptor *(11,38)*, and it appears that the C-terminal 30 amino acids in the F domain are particularly important in determining the actions of antiestro-

gens *(11,15)*. These conformational changes provide the basis for all other mechanisms of action (Fig. 2). These actions include the following:

1. The ability to form dimers
2. The ability to bind DNA
3. The ability to interact with coregulatory proteins
4. The availability of phosphorylation sites
5. The ability to form an active transcriptional unit

Type I antagonists like tamoxifen block estrogen actions by competing for the LBD and preventing the AF-2 function of ER *(38,59)*. The agonist action of tamoxifen appears to result from a conformational change that allows AF-1 to enhance transcription *(38,59)*. Thus, tamoxifen-bound ER can bind DNA and activate transcription, but it does so in a less productive manner than estrogen. Type II antagonists such as ICI 182,780 induce different conformational changes in ER that not only prevent estrogen binding to the ER, but also lead to a receptor that is inactive in both AF-1 and AF-2 *(38,59,60)*. Nevertheless, ICI-bound ER still binds EREs, demonstrating a distinction between promoter binding and transcriptional activation *(61)*. In addition, the ICI compounds decrease ER protein levels by sequestering newly synthesized ER in the cytoplasm. The mechanisms underlying the actions of these and other antagonists is still being elucidated, but new studies suggest that antiestrogen effects are isoform, cell, and promoter dependent.

CELL- AND PROMOTER-DEPENDENT FACTORS THAT INFLUENCE ANTIESTROGEN ACTIONS

The effects of antiestrogens in a particular cell or tissue are dependent on several interacting factors in the cell background including the following:

1. The relative expression of ERα and ERβ
2. The specific promoter elements present in ER-regulated genes
3. The expression of transcriptional cofactors
4. Ligand-independent pathways that activate ERs

These differences are manifested in the observation that estrogen actions on the same promoter are cell-type dependent.

Cell-Specific Responses

The contribution of AF-1 and AF-2 to ERα transactivation is cell-type and promoter dependent *(38,59)*. In HepG2 human carcinoma cells, estrogen-induced transactivation by ERα could be completely recapitulated by a construct containing mutations in AF-2. However, in HS578T human breast cancer cells, estrogen activated transcription equally from the wild-type ERα, ERα with a mutated AF-2, and ERα lacking AF-1 *(59)*. Initial experiments with ERα showed that although ICI 164,384 inhibited both AF-1 and AF-2 activity *(38,58)*, in several different cell and promoter contexts, tamoxifen and raloxifene had very different profiles. In HepG2 human hepatocarcinoma cells, in which AF-1 activity appears to predominate, using the human complement C3 promoter, estrogen equally activates ERα and ERα with a mutated AF-2 *(38,59)*. Tamoxifen acts as partial agonist to activate the wild-type ERα, but the mutation in AF-2 reduces this effect *(38)*. These data show that although transcriptional activation

in this context occurs through AF-1, alterations in AF-2 can change the agonist properties of tamoxifen. Conversely raloxifene (keoxifene) completely antagonizes the wild-type ERα, but acts as an agonist on the mutated AF-2 construct *(38)*. Thus, the ability of antiestrogens to inhibit (or stimulate) transcription will be influenced by the cell milieu.

Receptor Isoforms in Model Systems

Another cellular factor that influences the actions of antiestrogens is the relative expression of ER isoforms. Although ERα and ERβ have similar affinities for estrogen and estrogen agonists *(22,28)*, there is very little homology in the A/B (AF-1) region of the receptors. Thus, factors that influence AF-1 activity may have significantly different effects on ERα and ERβ. In particular, partial agonists such as tamoxifen may have significantly different actions on estrogen action depending on which receptors are present. For example, in human endometrial cancer (HEC-1) or breast cancer (MDA-231) cells transfected with ERα, tamoxifen acts as an antagonist to estrogen but has partial agonist activity on an estrogen-responsive promoter *(62)*. However, tamoxifen and other antagonists show no activation of the ERβ *(62)*. Similarly, although tamoxifen completely blocks estrogen actions of both ERα and ERβ in COS-1 monkey kidney cells using a model promoter, partial agonist activity is only observed on ERα *(58)*. Similar results have been observed using model promoters in HeLa cells *(60)*. Pure antiestrogens such as ICI 182,780 and EM652 had no agonist activity *(58)*. New compounds may also be able to selectively antagonize ER isoforms, such as the ERβ-specific THC *(30)*.

Role of Promoter Elements

EREs in the promoter regions of regulated genes mediate the effects of ER binding. However, the consensus EREs used in model promoters do not necessarily reflect the estrogen responsiveness of natural promoters that may have imperfect EREs, or multiple or half EREs, and may respond to ERs binding other motifs or through ER interactions with other *trans*-acting factors. A well-known example is the ability of ERα to interact with Jun at activator protein-1 (AP-1) sites to enhance transcription *(63)*. In HeLa cells, estrogen can activate a model promoter containing an AP-1 element via ERα *(64)*. Tamoxifen retains full agonist activity in this context, and both raloxifene and ICI 164,384 have about 50% of the activity of estrogen *(64)*. Similarly, in Ishikawa endometrial carcinoma cells, tamoxifen, but not the pure antiestrogen RU 39,411, could activate an AP-1-containing promoter through ERα *(65)*. Other elements may also mediate ER actions. For example, a TA-rich region and a CCAAT sequence of the brain creatine kinase promoter can confer estrogen responsiveness through ERα in HeLa cells and primary rat fibroblasts *(66)*. In this context, ICI 164,384 and tamoxifen acted as pure antagonists *(66)*. By contrast, tamoxifen acts as a full agonist of the rat uterine calbindin-D 9k gene in vivo and in vitro whereas ICI 182,780 acts as a pure antagonist *(67)*. The human retinoic acid receptor α-1 (RARα-1) promoter can be activated by ERα by a mechanism that does not involve direct DNA binding; however, both tamoxifen and ICI compounds act as antagonists on ERα *(68)*. In addition, Montano and colleagues *(69,70)* have identified *cis*-elements in the PR distal promoter region and in the human quinone reductase gene that modulate the ability of estrogen and antiestrogens to alter transcription. Thus, the precise nature of the promoter may also alter the antagonist/agonist actions of antiestrogens.

Several interacting factors determine the efficacy of antiestrogen action and the ability of some antiestrogens to have agonist actions in some tissues or cells while completely blocking estrogen actions in others. The complexity of these issues is enhanced by the recognition that predictions about ligand actions in different cell and promoter contexts is nearly impossible, even for the "pure" antiestrogens. Recent studies comparing ERα and ERβ have reiterated this fact. In 1997, Paech et al. *(64)* first demonstrated that three classes of antiestrogens (represented by tamoxifen, raloxifene, and ICI 164,384) could activate AP-1 elements through ERα. Although these antagonists did not activate an ERE-containing model promoter in HeLa cells by ERα or ERβ, all three compounds strongly activated AP-1 sites through ERβ even though estrogen did not. Furthermore, estrogen antagonized raloxifene. Similar results were obtained in breast cancer and endometrial cancer cell lines *(64)*. Another striking example is the ability of several antiestrogens, including tamoxifen, raloxifene, and ICI 182,780, to activate the human RARα-1 promoter through ERβ, even though estrogen and ERα do not *(68)*. This effect is mediated through a promoter region containing stimulatory protein-1 (Sp-1) sites *(68)*. Thus, in these cases, even "pure" antiestrogens enhance transcriptional activation through ERs. Similar agonist actions of antagonists mediated through ERβ on non-ERE-containing promoters have been observed on the human quinone reductase gene *(70)*. These observations gain further complexity when coupled with the fact that ERα and ERβ form heterodimers. Thus, although ERβ homodimers are completely inhibited by tamoxifen in many systems, ERα and ERβ heterodimers can be activated *(60)*.

PR ANTAGONISTS AND MECHANISMS OF ACTION

Like ER antagonists, PR antagonists have been divided into two classes. However, the Type I and Type II classification is distinct from that of the antiestrogens. The distinction between classes results from the ability of Type I PR antagonists (ZK98299) to inhibit PR binding to DNA and the ability of Type II antagonists (RU486 and others) to promote it. The general chemical structure of PR antagonists does not denote type, since both ZK98299 and RU486 are 11β-phenyl-substituted steroid compounds. Similarly, chemically similar nonsteroidal PR ligands such as RWJ 47626 (antagonist) and RWJ 26819 (agonist) are both Type II ligands. In addition to acting as competitors with progesterone for PR-binding sites, these ligands bind PRs and induce conformational changes in the receptor *(71–74)*. However, with the exception of ZK98299, these changes actually promote DNA binding *(72)*. The principal conformational change induced by PR ligands appears to involve the C-terminus (similar to the ER). The PR agonists progesterone and RU5020 induce a conformational change that occludes the 14 C-terminal amino acids from binding by a specific antibody, whereas unliganded or RU486-bound PR is detected by the antibody *(73)*. Interestingly, proteolytic cleavage studies suggest that it is the position of the AF-2- containing helix 12 that is differentially exposed by the binding of different ligands *(72)*, an observation that agrees well with data for ER. Mutational studies suggest that full activation of PR requires cooperativity between the AF-1 and AF-2 domains *(74)*. Mutational studies suggest that RU486 and progesterone interact with slightly different regions of the PR to exert antagonist and agonistic actions, respectively *(74)*. Deletion of the 42 amino acids of the carboxy terminus of PR allows RU486, but not progesterone, to bind *(75)*. This mutation

functions to make RU486 an agonist, suggesting that antagonism of PR requires some type of inhibition by the C-terminus *(75)*. In addition, these conformational changes can alter the binding of other cofactors that alter PR function *(76)*. Furthermore, it has been observed in vitro that heterodimers of agonist-bound PR and antagonist-bound PR bind very poorly to DNA *(77)*. Thus, in addition to competing for progesterone response elements (PREs), antagonist-bound receptor may act to sequester agonist-bound receptor from DNA. A third class of partial agonist antiprogestins has been examined. These compounds are 16α-substituted analogs of RU486 and display cell-specific activity *(78)*.

CELL- AND PROMOTER-DEPENDENT FACTORS THAT INFLUENCE ANTIPROGESTIN ACTIONS

PRs exist in two isoforms—PR-A, and the N-terminally extended PR-B (see Fig. 1)—and the relative expression of these isoforms plays a critical role in the overall response to ligands. Both PR-A and PR-B can act as transcriptional activators on PREs *(79)*. However, although PR-B is a strong transcription activator, PR-A is active only in some contexts, and PR-A can repress PR-B transactivation. Thus, the relative expression of PRs in different tissues is critical to the overall response to PR agonists. In CV1 and HeLa cells, progesterone stimulates the activity of the mouse mammary tumor virus (MMTV) promoter in the presence of PR-B but not PR-A. In this context, PR-A strongly represses PR-B activity. By contrast, PR-A and PR-B both activate MMTV in HepG2 cells, and coexpression of these receptors leads to an additive effect. Nevertheless, under conditions in which PR-A inhibits transactivation, this inhibition was potentiated by PR agonists and antagonists alike *(79)*. This inhibition did not require DNA binding, consistent with the notion that part of PR repression is owing to the formation of inactive heterodimers *(56,79)*.

Antagonist-bound PR-B can also activate transcription through mechanisms that do not require DNA binding *(56)*. Like ERs, these actions are thought to occur through interactions with other *trans*-acting elements of tethering proteins. Apparently similar conformational changes induced by agonists and antagonists alike allow these interactions. However, PR-A can still inhibit this action, suggesting that PR-A inhibition of PR-B is a dominant effect in contexts in which PR-A itself is not a transcriptional activator.

STRUCTURAL CONSEQUENCES OF LIGAND BINDING

As ligand-activated transcription factors, the conformation of ER and PR as a result of ligand binding is critical in mediating the transcriptional responses. In general, the binding of natural ligands such as E are stimulatory and enhance ER activation, whereas the binding of antiestrogen, such as the partial agonist tamoxifen or the pure antagonist ICI 164,384, is inhibitory. The basis for the agonism or antagonism has been shown to be dependent not only on binding to the LBD, but on distinct conformational changes evoked by ligand binding.

Early studies on RARs *(4,5)* demonstrated that the interaction of the receptor LBD with a cognate ligand resulted in receptor activation, involving loss of heat-shock protein binding, followed by receptor dimerization and binding to DNA. ER does not require ligand for dimerization and interaction with DNA in vitro *(2)*. However, ER activity is ultimately dependent on conformation, which is dictated primarily by the

type of ligand bound. Limited proteolytic digestion of ER bound to E or E antagonists exhibited marked variations in digestion patterns, providing early experimental support for this hypothesis *(11,38)*. Chymotrypsin digestion of labeled ER bound to E or antiestrogens resulted in protected receptor fragments of 30 and 32 kDa. Subtle differences were apparent, because the intensity of the 32-kDa band was greater in the presence of E, and the 30-kDa band was more predominant in the presence of antiestrogens. The protected band was localized to the LBD by the demonstration that a protected band of 29 kDa was observed with an ER lacking the C-terminal 30 amino acids. In later studies, tryptic digestion of labeled ER bound to E resulted in a proteolytic fragment of 32 kDa, whereas digestion of ER bound to the partial agonist antiestrogens 4-hydroxytamoxifen, nafoxidine, and keoxifene (raloxifene) resulted in a novel and distinct 28-kDa peptide fragment. Binding to the complete or pure antagonist ICI 164,384 resulted in a slightly different digestion pattern, with a peptide doublet migrating at 30–28 kDa. These data indicate that variations among E and antiestrogen-induced ER conformations can exist, and that they may have biological relevance. This concept has been confirmed by intracellular localization and crystallographic studies.

Immunofluorescence studies have shown that pure antagonist antiestrogens, such as ICI 164,184 or RU58668, can alter the cellular localization of ER *(61,80)*. In transfected Cos-1 cells, ER is localized to the nucleus in the absence of ligand, and in the presence of E or tamoxifen. In the presence of the ICI or RU compounds, ER is present in the cytoplasm and forms perinuclear clusters. The cytoplasmic clustering of ER required only the LBD, and was dependent on the synthesis of new proteins. It was suggested that the antiestrogens induce a conformational change in the LBD that enhances interactions with specific proteins undergoing rapid turnover, and that these proteins force the receptor out of the nucleus or retain it in the cytoplasm.

Site-directed and regional mutational analysis of ER has provided information on specific receptor regions and amino acids critical for hormone binding, transactivation, and the interpretation of the agonist/antagonist character of specific ligands *(34)*. One especially important finding was that regions critical for transactivation and ligand binding and discrimination can be separated. Ligand binding requires the formation of a hydrophobic binding pocket within a large portion of the LBD, from C381 to C530. Mutations near cysteine 381, and between amino acids 520–530, had severe consequences on ligand binding, whereas mutations in the 520–530 region and the F domain resulted in ligand discrimination changes *(81–83)*. Alanine mutation substitution of residues 515–535 revealed four residues critical for E binding: G521, H524, L525, and M528 *(81)*. Many antiestrogens contain a bulky side chain with basic or polar functional groups that may interact with charged and polar amino acids near the hormone-binding sites of ER. Mutations around Cys530 that alter the charged and polar amino acids with minimal steric alteration (ER Lys529Asn, Lys531Gln, Asn532Asp) result in receptors with a 10-fold lower affinity for E binding, but unaltered affinity for and response to antiestrogens *(84)*.

The transactivation/AF-2 region requires amino acids downstream from hormone binding, located in a conserved core motif located in helix 12, amino acids 535–548 in human ER *(44)*. This core motif contains two pairs of hydrophobic amino acids (Leu539/540 and Met543/Leu544), a glutamic acid at E542, and two aspartate residues (D538 and D545). Point mutations in this region severely suppress or obliterate the transcriptional response to E without the elimination of E binding. Mutation of the

charged residues to neutral amino acids (E:A or D:N) had no effect on E binding or responses, whereas mutation of either pair of hydrophobic residues to alanines inhibited ER activity with no effect on hormone binding. However, these same mutations converted the antiestrogenic activity of ICI 164,384 and tamoxifen to complete agonists *(83)*. With these transfected mutant receptors, reporter gene activity was stimulated to a level comparable to that of the wild-type ER in the presence of E. A naturally occurring ER mutation isolated from a tamoxifen-sensitive breast cancer cell line contained an Asp351Tyr mutation *(82)*. Finally, a Leu540Gln mutation results in an ER that is still insensitive to E, but can distinguish between partial antiestrogens such as tamoxifen, which remain agonistic, and pure antiestrogens such as ICI 188,384 and RU54,876, which show a greatly reduced stimulatory activity. These latter mutations required an intact AF-1 domain and the presence of the F domain for the observed activities *(31)*. Such mutations could have grave consequences in E-dependent cancers, for which adjuvant antiestrogen therapy is common.

X-ray crystallographic studies have provided insight into the relevant conformational changes induced by agonists vs antagonists. The earliest studies were performed with the unliganded human retinoic X receptor-α, and the RAR-γ bound to all-*trans* retinoic acid *(4,5)*. Both receptor LBDs are similar in consisting of 12 α-helices folded into an antiparallel α-helical sandwich. The ligand fits into a hydrophobic pocket formed by interactions of helices 1, 3, 5, 11, and 12, and binding of ligand resulted in a repositioning of helix 12 to cover the hydrophobic pocket.

Similar structural changes occur in ligand-bound ER and PR. For ER, E binds in a hydrophobic groove formed by helices 3, 6, 8, 11, and 12; forms hydrogen bonds with Glu353, Arg394; has hydrophobic interactions with Ala350 and Leu387; and has nonpolar interactions with Ileu424, Gly521 and Leu525 *(12)*. With E binding, helix 12 forms a lid over the cavity and interacts with residues in helices 3, 5, 6, and 11. Although helix 12 does not come into direct contact with E, movement of the helix exposes specific conserved amino acids to potential interactions with additional regulatory proteins, such as coactivators. Binding of the ER LBD to the antiestrogen raloxifene dramatically alters the conformation of helix 12, which is rotated 130°. Helix 12 then lies in a groove composed of helices 3 and 5, resulting in the burial of residues required for additional protein-protein interactions.

Similar observations were made for PR bound to agonist or antagonist, in that the position of helix is altered on agonist binding *(6,13)*. PR has a 12 amino acid C-terminal extension compared to ER, and this region is essential for hormone binding in PR and AR. Ru486 is likely to displace helix 12 and this extension from this conformation. Mutations in the C-terminal extension, including deletions up to and including helix 12, allow the antagonist (RU486)-bound PR to activate transcription. This suggests that the antirepressive effects on the C-terminal extension are at least as important to transcriptional activation as the contribution of helix 12 to formation of the coactivator interface.

Recent crystallography studies have defined the sites on ER that directly interact with accessory proteins as a result of ligand-induced conformational changes, and point out the importance of the conserved core sequence of helix 12 *(14)*. Coactivator proteins such as SRC-1 and GRIP-1 bind in the hydrophobic groove formed by helices 3, 4, 5, and 12 of ligand-bound receptors, and mutations in helix 12 such as Glu542Lys decrease transactivation and eliminate binding of cofactors to the LBD. Interaction of

various coactivators occurs through a motif (Leu-X-X-Leu-Leu) known as the NR box. Crystallization of the ER LBD bound to diethylstilbesterol, an E agonist, with a 13 amino acid peptide corresponding to the GRIP-1 NR box from GRIP-1 shows this interaction occurs directly in the helix 3, 4, 5, and 12 hydrophobic groove *(6)*. When the ER LBD was bound to tamoxifen, the GRIP-1 peptide was no longer bound. Instead, helix 12 was repositioned such that it did not form the bottom of the groove and, in fact, competitively bound to the region occupied by the NR box peptide in the agonist-bound receptor. This probably occurs because the sequence Leu-Leu-Glu-Met-Leu (amino acids 539–544) is similar to the NR box motif and can occupy a similar site. These data provide a structural basis to understand the significance of receptor mutations and to predict potential conformations that could be fully or partially suppressive. Furthermore, alteration of protein structure or charge as a result of posttranslational modification, such as phosphorylations, could also alter conformation induced by agonist/antagonist binding and specific coactivator interactions. Such a situation may explain the alteration of antagonist responses after treatment with kinase activators.

COACTIVATORS AND COREPRESSORS

One potential mechanism for cell- and tissue-specific effects of steroid receptor agonists and antagonists would be the presence of cell-specific proteins capable of interacting with the receptors and modulating their activity. One such group of proteins is called coactivators, including the previously mentioned SRC-1, GRIP-1, AIB-1 and others *(41)*, that bind to agonist-bound receptor LBDs. Both coactivators and steroid receptors also bind to integrator proteins such as CBP and p300, which aid in bringing ligand-bound receptor into direct contact with the transcriptional machinery *(85)*. Both coactivators and CBP have histone deacetylase activity, which causes chromatin to have a more open structure and results in a higher level of transcriptional activation *(85)*. The studies we described previously have shown that agonist-bound receptors recruit coactivators, whereas antagonist-bound receptors do not *(14,86)*. To date, most coactivators studied occur in most or all cell types, although their levels may be regulated. SRC-1 levels, e.g., are modulated by steroid status and are stimulated by estrogen *(87)*, and AIB-1 was originally described based on increased levels observed in breast cancer cells *(43)*. ARA70, an androgen receptor coactivator isolated from human prostate cells, does appear to have greatly enhanced effects for this receptor, and other proteins with receptor-specific effects and cell-specific expression may exist as well *(88)*. There clearly is redundancy in coactivators, as demonstrated by the observation that the SRC-1 knockout mouse exhibited some specific diminished responses to steroids, but was viable and fertile *(89)*. Levels of transcriptional intermediator factor-2 (TIF-2), a related coactivator, were increased in these animals compared to wild-type siblings. Not all coactivators are equally effective at enhancing ligand-bound receptor activity. Overall transcriptional stimulation can be governed by the levels of specific coactivators and other molecules capable of interaction with the LBD *(90,91)*. For example, RIP140 confers a modest stimulatory response on receptor activation, but in the presence of more effective coactivators such as SRC-1 actually suppresses overall activation by competition for binding to the LBD *(90)*. Both orphan receptors *(92)* and receptor variants *(93)* that interact with steroid receptors can also competitively suppress SRC-1 stimulation or ER.

To date, no AF-1 specific coactivator has been isolated. This will clearly be of great interest, because partial agonists of estrogen and progesterone are believed to exert their actions through this domain. SRC-1 has been reported to interact with both the AF-1 and AF-2 domains, and may contribute to the AF-1 effect in at least some cases *(94)*. Thus, there may be coactivator or modulator proteins that interact with AF-2, AF-1, or both domains. A novel hinge domain–binding coactivator, L7/SPA, has been isolated from HeLa cells, and increased the partial agonist activity of TAM-bound ER and RU486-occupied PR *(76)*.

In general, unliganded ER does not bind to corepressors such as silencing mediator of retinoid and thyroid hormone receptors (SMRT) and nuclear receptor corepressor (NCoR), which bind to the hinge region of nuclear receptors, such as the thyroid hormone receptors and RARs, and prevent binding of the LBD regions to coactivators *(41)*. In these receptors, the corepressor proteins invoke a receptor conformation that actively represses transcription, owing partially to histone acetylase activity of the coactivator proteins. Specific conformations of ER, resulting from binding to receptor antagonists, may result in corepressor recruitment to the liganded receptor complexes. For example, in HepG2 liver cells, in which tamoxifen is a partial agonist, exogenous SRC-1 enhanced E and TAM-stimulated transcription, whereas overexpression of the corepressor SMRT strongly reduced basal and TAM-mediated transcription with no effects on E activity *(86)*. Similarly, PR bound to antiprogestins of the partial agonist class binds more effectively to corepressors N-CoR and SMRT than does PR bound to other ligands *(76,95)*, and this association can be suppressed by treatment of cells with cAMP *(95)*. Unliganded PR may bind to corepressors, and additional corepressors with more complicated or specific receptor requirements may exist. For example, human ER LBD bound to antiestrogens such as tamoxifen, but not bound to E, associates with at least one nuclear protein capable of acting as a corepressor *(96)*. Such molecules would not be isolated using only ligand-bound receptors as bait in typical two-hybrid or other protein interaction assays. Additional studies will undoubtedly focus on the types of accessory proteins bound to specific receptors with various ligands, as well as modifications of those proteins by intracellular signaling cascades.

SUMMARY

Overall, several factors including the character of the ligand, the steroid receptor isoform expressed in a specific cell type, and intracellular signaling pathways activated in a given cell or tissue may all be important in determining the character of partial steroid antagonists. At least some of these responses are directly related to the complement of coactivators and corepressors associated with ligand-bound receptor within a given context. As we have discussed, the levels of individual coactivators and corepressors may be modulated physiologically, and it is likely that posttranslational modifications will also occur in response to signaling cascades by growth factors and other bioactive peptides. Individual ligand binding to specific receptor isoforms confers distinct conformational changes and contours to the receptors, capable of interacting with the cellular accessory proteins. Based on both the identity and levels of coactivators and corepressors, the resulting receptor protein complex will have either a stimulatory or suppressive conformation and a resulting effect on model gene transcription. An additional layer of diversity will then be provided by the specific ERE or responsive promoter region

in the cellular target genes, since the receptor conformation may be altered as it binds to different DNA sequences, or contacts different proteins at nearby promoter regions. These interactions can alter the essential character of a given ligand, from antagonist to agonist or the reverse. Current and future studies will be focused on the essential mechanisms underlying such diversity and specificity, and how these processes can be regulated or manipulated for a given positive biological outcome.

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