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Orphan Nuclear Receptors: From Gene to Function*

VINCENT GIGUÈRE

Molecular Oncology Group, McGill University Health Centre and Departments of Biochemistry, Medicine, and Oncology, McGill University, Montréal, Québec, Canada H3A 1A1

Address reprint requests to: Dr. Vincent Giguère, Molecular Oncology Group, Room H5.21, McGill University Health Centre, 687 Pine Avenue West, Montréal, Québec, Canada H3A 1A1. E-mail: vgiguere@dir.molonc.mcgill.ca

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I. Introduction

THE RECOGNITION that the steroid, thyroid, and retinoid receptors constituted only a small subset of a large number of related gene products is around 10 yr old (1). The year 1988 witnessed the identification of the first cDNA clones encoding polypeptides with structural features suggestive of cryptic steroid hormone receptors (2), and at that time one could identify about a dozen or so distinct nuclear receptor-like proteins. There are now more than 50 identified in various species and, mainly through the various genome-sequencing projects, the number is now increasing at a rapid pace. Because the discovery of all these putative nuclear receptors had not been anticipated by previous physiological studies and therefore not linked with the biological effects of a particular hormone or ligand, these new gene products were referred to as orphan nuclear receptors. That the activity of orphan nuclear receptors could be potentially regulated by natural ligands led to the tantalizing suggestion that new hormone response systems remained to be discovered (2). Interest in orphan nuclear receptor research was also stimulated by the knowledge that classic members of the superfamily of nuclear receptors and their ligands play crucial roles in development, homeostasis, and disease. The possibility that nuclear receptors' activity might be regulated by the direct action of natural and synthetic compounds makes orphan receptors good targets for drug discovery. Therefore, the existence of a large number of potential new receptors offers the exciting opportunity to develop novel therapeutic agents, even in the absence of known natural ligands. Recent advances in the field have shown that such drugs could be used to treat a variety of illnesses, including diabetes, lipid disorders, and cancer. For these reasons, the study of orphan nuclear functions has regrouped scientists from a wide variety of fields, and consequently the information being generated on this subject is vast, diversified, and often very confusing. In this review, I will attempt to regroup some of this information in a format accessible to nonspecialists and specialists alike. I will also try to demonstrate how the study of orphan nuclear receptors has revealed new modes of action for nuclear receptors that often challenged previous dogma and highlights the discovery of new hormone response systems as well as regulatory pathways controlling cell fate, organogenesis, and basic metabolic functions.

II. Nuclear Receptors: General Concepts

Nuclear receptors provide multicellular organisms with a means to directly control gene expression in response to a wide range of developmental, physiological, and environmental cues. It is now recognized that nuclear receptor activity can be controlled by at least three distinct mechanisms: 1) binding of a small lipophilic ligand by the receptor or its partner in heterodimer complexes; 2) covalent modification, usually in the form of phosphorylation regulated by events at the cellular membrane or during the cell cycle; and 3) protein-protein interactions, generally through contacts with other transcription factors including nuclear receptors themselves. All three mechanisms can either work individually or in concert with each other to modulate a specific signal (Fig. 1). It should also be noted that some nuclear receptors mediate nongenomic effects that are too rapid to involve changes in gene transcription. This subject has been reviewed recently in this journal (3).

To place current studies on orphan nuclear receptors into their proper context, this review begins with a brief overview of the structural features and molecular mechanisms shared by nuclear receptors.

A. Anatomy of nuclear receptors

Nuclear receptors are composed of four independent but interacting functional modules (Fig. 2A). These are the modulator domain, the DNA-binding domain (DBD), the hinge region, and the ligand-binding domain (LBD). For some nuclear receptors, the sequence of the protein extends beyond the LBD at the carboxy-terminal end, but no specific role has been assigned to these additions when present.

1. Modulator domain. The modulator domain, also referred to as the A/B domain, displays the most variability both in terms of length and primary sequence. A large number of transcriptional units encoding nuclear receptors use alternative splicing, different promoters, and distinct translational start sites to generate multiple modulator domains, leading to the expression of many receptor isoforms from a single gene (Fig. 2B). This phenomenon is best exemplified by the family of retinoic acid receptors for which three genes produce at least eight receptors with similar DNA- and ligand-binding properties but distinct biological functions (reviewed in Refs. 4 and 5). The modulator domain usually contains a transcriptional activation function, referred to as AF-1. Studies of the estrogen and progesterone receptors have clearly demonstrated that the modulator domains possess promoter- and cell context-dependent activities (6–8), suggesting that the amino-terminal region of nuclear receptors may interact with cell-specific cofactors. Although no significant amino sequence homology exists between any members of the superfamily within this domain, unrelated modulator domains have been shown to confer similar properties to distinct receptors. For example, while the amino termini of estrogen receptor α and β share little sequence similarity, AF-1 activity of both receptors is enhanced through phosphorylation by the mitogen-activated protein kinase (MAPK) (9–11). In contrast, the domain confers responsiveness to the mixed agonist/antagonist 4-hydroxytamoxifen to ERα (12), while basal ERβ activity is unaffected by the synthetic compound (11, 13). In addition to MAPK, both cyclin-dependent protein kinase (14–16) and pp90^{rsk1} (17) have also been shown to phosphorylate the amino-terminal domains of specific nuclear receptors. The modulator domain can also interact directly with steroid receptor coactivators (SRCs) (see below) to enhance the activity of the receptor complex (18–21).

2. DBD. Nuclear receptors bind DNA as monomers, homodimers, and heterodimers (Fig. 3A) (reviewed in Ref. 22). While most heterodimeric complexes contain one of the ret-

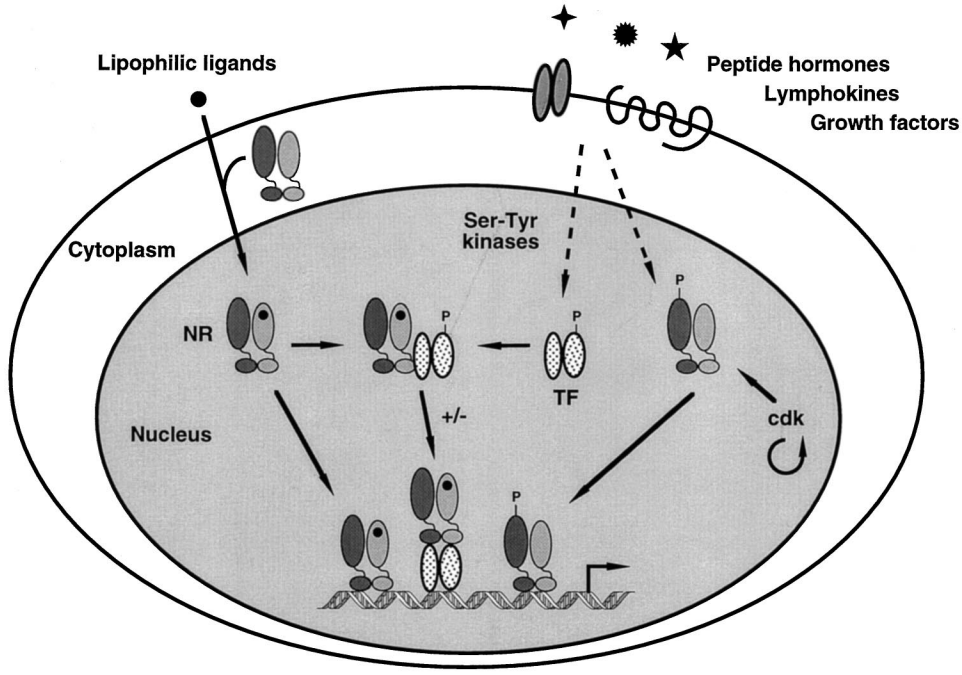

FIG. 1. Signaling pathways regulating genomic actions of nuclear receptors. Nuclear receptor activity can be regulated by direct binding of small lipophilic ligands, protein-protein interactions with other transcription factors, or by covalent modification such as phosphorylation after stimulation of cell surface receptors or by cyclin-dependent kinases.

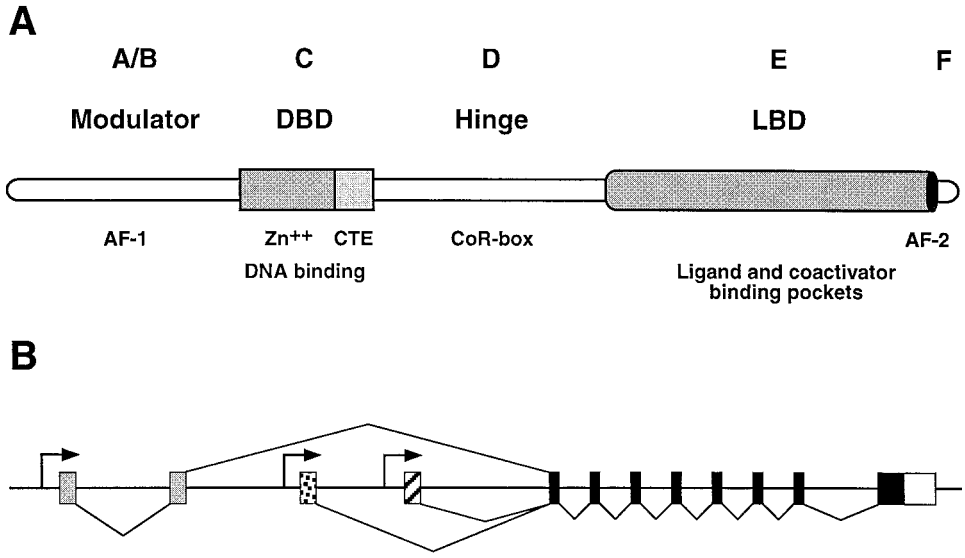

FIG. 2. Anatomy of nuclear receptors and typical gene structure. A, Nuclear receptors are composed of independent functional domains that include the DBD and LBD, the primary functions of which are to recognize specific DNA sequences and ligands, respectively. Nuclear receptors generally possess two transcription activation functions (AF-1 and -2) located at the amino and carboxy termini. The division of nuclear receptors into domains A–F is based on the degree of amino acid sequences conservation between the same receptor in different species. B, Schematic representation of the exon-intron organization of a typical nuclear receptor gene. The modulator domain is usually encoded by one or two exons. Distinct modulator domains can be generated by alternative promoter usage (*arrows*) and splicing (*linked exons*). The two zinc finger modules are generally encoded by distinct exons while the hinge and LBD are encoded by 6 to 10 exons. Additional alternative splicing may generate nuclear receptors with modified LBDs.

inoid X receptors (RXRs) as a common partner (23) (see below), alternative heterodimeric interactions between nuclear receptors have been reported and may be of physiological significance (24–32). Nuclear receptor DNA recognition sites, referred to as hormone response elements (HREs), contain one or two consensus core half-site sequences. For dimeric HREs, the half-sites can be configured as inverted, everted, or direct repeats. Steroid receptors recognize the half-site consensus sequence AGAACA while the estrogen receptors and other nuclear receptors bind to the half-site consensus sequence AGGTCA. For monomeric HREs, a single half-site is preceded by a 5′-flanking A/T-rich sequence. Half-site sequences can deviate quite considerably from the consensus sequences, especially for dimeric HREs in which a single conserved half-site is usually sufficient to confer high-affinity binding to the homo- or heterodimer complexes. Natural HREs rarely contain two perfect consensus half-sites.

The DBD of nuclear receptors is the most conserved domain. It is composed of two zinc finger modules encoded by 66–70 amino acid residues and a carboxy-terminal extension (CTE) that spans approximately 25 residues (Fig. 3B). On the basis of mutagenesis experiments, the DBD has been further divided into subdomains involved in direct recognition of the core half-site sequences (P-box) (33) and dimerization determinants (D- and DR-boxes) (34, 35). However, the crystal structure of the RXRα-thyroid hormone (T_3) receptor β ($T_3R\ \beta$) DBD complex and computer modeling have shown that each heterodimeric complex may utilize partner-specific dimerization determinants (36). The CTE plays dual roles in providing both protein-DNA and protein-protein interfaces (36, 37). Finally, due to the asymmetric nature of direct repeat HREs, RXR and its partner bind DNA in a fixed orientation. In T_3R-, vitamin D receptor (VDR)-, and all-*trans*-retinoic acid (atRA) receptor (RAR)-RXR heterodimer complexes, RXR occupies the upstream half-site and its partner the downstream half-site (34, 38–41). The site occupied by a receptor on a direct repeat HRE may regulate its ability to recognize its ligand (42).

3. The hinge region. This region of the nuclear receptors is also highly variable in both length and primary sequence: as its name indicates, its main function is to serve as a hinge between the DBD and LBD. The hinge has to be very flexible to let the DBD rotate 180° to allow some receptors to bind as dimers to both direct and inverted HREs (22). Recent studies have also demonstrated that the hinge region may serve as a docking site for corepressor proteins (43, 44).

4. LBD. The LBD is a multifunctional domain that mediates ligand binding, dimerization, interaction with heat shock proteins, nuclear localization, and transactivation functions. Although quite variable in primary sequence, nuclear receptor LBDs can be defined by a signature motif overlapping with helix 4 (45). In addition, ligand-dependent transactivation is dependent on the presence of a highly conserved motif, referred to as activation function-2 (AF-2), localized at the carboxy-terminal end of the LBD (Fig. 2A). X-ray crystallographic experiments suggest that LBDs have similar structures: they are formed by the folding of 11–13 α-helices into three layers that bury the ligand-binding site within the core of the LBD (46–52). Ligand-dependent transactivation involves the recruitment of coactivators (see below), a process in which the AF-2 plays an obligatory role. Comparison of holo- and apo-LBD structures has led to the mouse trap model in

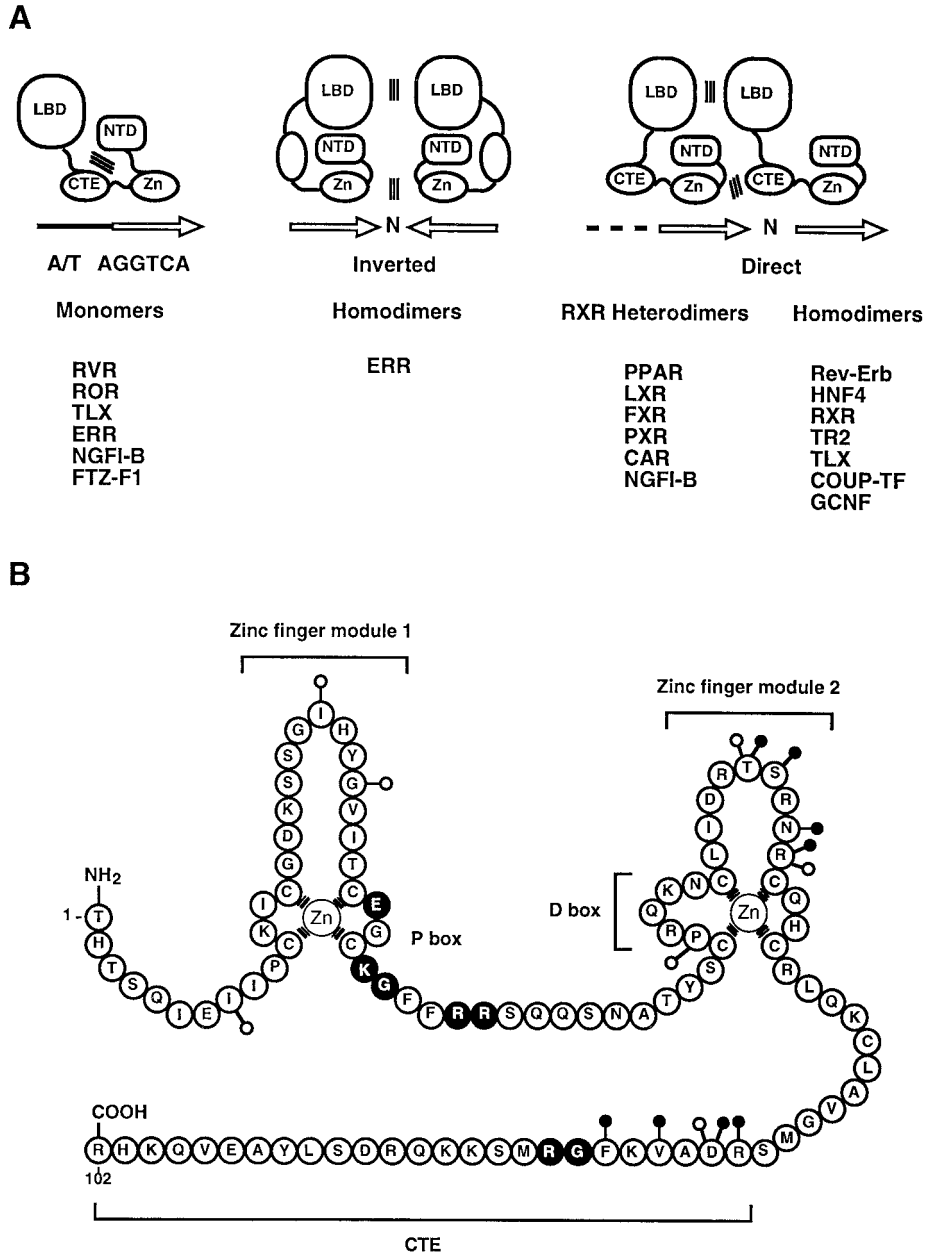

FIG. 3. DNA binding by nuclear receptors. A, Nuclear receptors can bind DNA as monomers, homodimers, and RXR heterodimers. Nuclear receptor binding sites are composed of one or two half-core motifs, generally AGGTCA or a close variant that could be preceded by a 5′-flanking A/T-rich sequence. The half-core motifs and 5′-flanking A/T-rich sequences are recognized by the first zinc module and the CTE, respectively. Half-core sequences in dimeric sites can be arranged as inverted, everted (not shown), or direct repeats. Intra- and intermolecular protein-protein interactions can influence DNA binding specificity and receptor dimerization. B, Schematic structure of a nuclear receptor DBD. The two zinc finger modules as well as the CTE are identified. Residues in *black* have been shown to make direct contacts with DNA. *Closed and open symbols* linked to certain residues represent residues that have been shown to mediate dimerization in distinct receptor complexes.

which ligand binding induces a conformational change in the LBD allowing coactivators to bind (48). In this model, the AF-2 motif folds back against the core LBD upon ligand binding, closing the ligand-binding pocket and forming a novel interface involving residues from the AF-2 itself and at least three other helices (53). While transcriptionally competent interfaces are induced by receptor agonists, binding of antagonists to the LBD leads to the formation of a nonfunctional interface preventing interaction between the nuclear receptor and coactivator proteins (49).

B. Mechanisms of action

The textbook model of nuclear receptor action is often represented by an inactive cytoplasmic receptor in a complex with heat shock proteins which, upon ligand binding, translocates to the nucleus and activates gene expression. Although this model is valid for some steroid receptors (54), most nuclear receptors are constitutively nuclear and often bound to DNA in the absence of their ligand. It is also now widely recognized that in the absence of ligands, many nuclear receptors can act as a strong repressor of gene expression (43, 44, 55–58). To modulate transcription of their target genes, nuclear receptors interact with coregulatory proteins. Nuclear receptors have been shown to associate with various components of the general transcription machinery, corepressors, coactivators, and the cointegrator CBP (CREB-binding protein)/p300 (reviewed in Refs. 59–63). Corepressor proteins may function by recruiting histone deacetylases, an activity that keeps the chromatin in a repressive state (64–66). Upon ligand binding, the repressor complex dissociates from the receptor, which is then free to interact with the coactivator complex. The receptor-coactivator complex may

contain one or more coactivators, including an RNA coactivator referred to as SRA (67), p/CAF (p 300/CBP-associated factor), CBP/p300, and other uncharacterized components. SRC-1, p/CAF, and CBP have been shown to possess intrinsic histone acetylase activity leading to a derepression of the chromatin structure (68–73). Taken together, these results delineate a new model for transcriptional regulation by nuclear receptors that includes three chromatin states: 1) normal chromatin in the absence of receptor that displays basal levels of histone acetylation and transcription; 2) repressive chromatin with deacetylated histones and no transcription in the presence of the unliganded receptor; and 3) active chromatin with high levels of histone acetylation and transcription in the presence of liganded receptor (reviewed in Ref. 74). However, chromatin disruption alone is not sufficient for transcriptional activation, indicating that additional interactions between nuclear receptors and the general transcription machinery are required to regulate gene expression (75).

Finally, nuclear receptors can also regulate gene transcription via direct interactions with other transcription factors, a process that does not depend on DNA binding by the nuclear receptor (reviewed in Refs. 76 and 77). In particular, the glucocorticoid receptor (GR) has been shown to antagonize AP-1 and nuclear factor-κB activities via transcriptional interference (78–82). Recent *in vivo* experiments that used reverse genetics to engineer a mutant mouse carrying a DNA-binding deficient GR have demonstrated that development and survival of mice do not require HRE-mediated gene regulation (83). These observations emphasize the multifaceted control of nuclear receptor activities and the independence of each functional domain in carrying out physiological roles.

III. Orphan Nuclear Receptors

A. Definition

Classic members of the superfamily of nuclear receptors were originally cloned on the basis that known hormones were transducing their physiological functions through binding to proteins referred to as receptors (1, 84). This is true for cortisol and aldosterone, estradiol, progesterone, and testosterone, vitamin D, T_3, and, to a certain extent, for atRA, for which the mechanism of action had been postulated to resemble that of steroid hormones (85), and for the insect hormone ecdysone (86). Therefore, the existence of these receptors was well recognized, and the combined efforts of biochemical and cloning experiments revealed their common structure and mode of action as defined above. Once investigators realized that nuclear receptors shared extensive homology at the amino acid and nucleotide sequence levels, a search for new members was undertaken using low-stringency screening of cDNA libraries with well conserved DBD fragments as probes. This cloning exercise led to two unexpected results. First, individual ligands, such as T_3 and atRA, were shown to regulate development and physiology through multiple receptors. This finding was first exemplified by the characterization of a second receptor for T_3 (87) and later by the cloning of three RAR genes, each encoding multiple isoforms (reviewed in Refs. 4 and 5). The recent identification of a second estrogen receptor in various species (11, 88, 89) demonstrates that even for classic ligands, the hunt for new receptors is still very active. Second, the search for new members of the superfamily led to the isolation of multiple cDNAs encoding proteins with structural features found in nuclear receptors. However, since ligands could not be linked to these putative receptors based on structural studies alone, these and other new members of the superfamily identified using various cloning strategies were referred to as "orphan nuclear receptors." For the purpose of this review, orphan nuclear receptors are defined as gene products that embody structural features of nuclear receptors that were identified without any prior knowledge of their association with a putative ligand. Using this definition, orphan nuclear receptors remain in this category even after the subsequent identification of specific ligands.

B. Nomenclature

The element of randomness associated with the cloning of orphan nuclear receptors led to a great diversity in the naming of these new genes. No common nomenclature or even a basic naming scheme was ever followed, and often the same receptors cloned in different species or by different groups were given unrelated names. Recently, a unified nomenclature system for the nuclear receptor superfamily has been adopted (90). The nomenclature is based on the well known system used for the cytochrome p450 superfamily. In this system, the gene subfamilies are designated by Arabic numerals, groups by capital letters, and individual genes by a second set of Arabic numerals. Receptor isoforms generated from the same gene by alternative promoter usage or differential splicing are designated by a lowercase letter at the end of the name. The introduction of this nomenclature system is not designed to replace the use of trivial names, but only to clearly identify which nuclear receptor was studied in a particular set of published experiments. The use of this system should be enormously helpful to both nuclear receptor aficionados and nonspecialists in attributing functional properties to each receptor.

A list of known vertebrate orphan nuclear receptors is presented in Table 1. In this table, orphan nuclear receptors are first classified in seven groups (0 to VI) according to the molecular phylogeny analysis performed by V. Laudet (91). Each group is divided into families referred to by their most commonly used trivial names, and each family member is identified by a Greek letter. Receptor isoforms generated from a single gene are identified by an Arabic numeral. Each receptor is then identified by its official name, and a list of other known trivial names is also provided. In this review, for simplicity and clarity, orphan nuclear receptors will be referred to by their family names (most commonly used trivial names), and subtypes will be referred to by a Greek letter. Readers are asked to use Table 1 as a guide to relate these names to other trivial names and to the official nomenclature.

Table 2 displays a list of published *Drosophila* orphan nuclear receptors together with their corresponding vertebrate homologs, when identified. Note that each group contains at least one *Drosophila* gene. Invertebrate orphan nuclear re-

TABLE 1. Vertebrate orphan nuclear receptors

Groups	Families	Subtypes	Isoforms	Nomenclature	Trivial names	Species[a]	References
I	PPAR	α		NR1C1	PPARα	h, m, r, l, g, x	(176, 182, 214, 569–573)
		β		NR1C2	PPARβ, PPARδ, NUC1, FAAR	h, m, r, l, x	(181, 182, 572, 574–576)
		γ	1, 2	NR1C3	PPARγ	h, k, b, p, m, r, l, x	(182, 577–581)
	Rev-Erb	α		NR1D1	RevErbAα, EAR-1	h, r	(370, 371)
		β		NR1D2	RVR, RevErbAβ, BD73, HZF2	h, m, r, c	(110, 372–375, 582)
	ROR	α	1, 2, 3, 4	NR1F1	RORα, RZRα	h, m	(109, 342, 391, 412)
		β		NR1F2	RORβ, RZRβ	m, r, c	(111)
		γ	1, 2	NR1F3	RORγ, TOR	h, m	(392, 393, 396)
	LXR	α		NR1H3	LXRα, RLD1	h, r	(148, 271)
		β		NR1H2	LXRβ, UR, NER, RIP15, OR1	h, m, r	(272–275)
	FXR			NR1H4	FXR, RIP14, HRR1	h, m, r	(274, 281)
	PXR		1, 2	NR1I2	PXR.1, PXR.2, SXR, ONR1, xOR6, BXR	h, m, x	(257, 258, 264)
	CAR	α		NR1I3	hCAR1, MB67	h	(266)
		β		NR1I4	mCAR1	m	(267)
II	HNF4	α		NR2A1	HNF4	h, m, r, x	(287–289, 291, 583–585)
		β		NR2A3	HNF4β	x	(289, 290)
		γ		NR2A2	HNF4γ	h	(291)
	RXR	α		NR2B1	RXRα	h, m, c, x, f	(125–128, 586–588)
		β		NR2B2	RXRβ, H2RIIBP	h, m, r, x, f	(127, 128, 589–592)
		γ	1, 2	NR2B3	RXRγ	h, m, r, x, c, f	(127, 128, 132, 587, 593)
	TR2	α		NR2C1	TR2, TR2-11, xDOR2, aDOR1	h, m, x, a	(426, 594, 595)
		β		NR2C2	TR4, TAK1, TR2R1	h, m, r	(427, 428, 596, 597)
	TLX			NR2E1	T1x, TLL, xTLL	h, m, c, x, f	(443, 444, 598, 599)
	COUP-TF	α		NR2F1	COUP-TFI, COUPTFA, EAR3, SVP44	h, m, r, x, f	(447, 453, 600–602)
		β		NR2F2	COUP-TFII, COUPTFB ARP1, SVP40	h, m, r, c, x, f	(448, 449, 451, 454, 499, 603)
		γ		NR2F4	xCOUP-TFIII, COUP-TFγ	x	(450, 451, 601, 604)
				NR2F5	SVP46	f	
				NR2F6	EAR2	h, m, r	
III	ERR	α		NR3B1	ERRα, ERR1	h, m	(2, 511, 605)
		β		NR3B2	ERRβ, ERR2	h, m, r	(2, 509)
		γ		NR3B3	ERRγ	h	(504)
IV	NGFI-B	α		NR4A1	NGFI-B, NUR77, N10, TR3, NAK1, TIS1	h, d, r, m, x	(514–516, 522, 606, 607)
		β		NR4A2	NURR1, NOT, RNR1, HZF-3, TINUR, TR3β	h, m, r	(518, 527, 528, 546, 608, 609)
		γ		NR4A3	NOR-1, MINOR, TEC, CHN	h, m, r	(520, 529, 530, 610, 611)
V	FTZ-F1	α	ELP1, 2, 3	NR5A1	FTZ-F1, SF1, ELP, AD4BP	h, b, m, r, c	(339–341, 612–616)
		β		NR5A2	FTF, LRH1, PHR1, CPF, FFLR, FF1rA	h, m, c, x, f	(342–344, 613, 616–618)
VI	GCNF			NR6A1	GCNF, RTR	h, m, x	(552, 619–623)
0	DAX			NR0B1	DAX1, AHCH	h, p, m, r	(367, 624)
	SHP			NR0B2	SHP	h, m, r	(566, 567)

[a] h, Human; k, monkey; b, bovine; p, pig; l, rabbit; d, dog; m, mouse; r, rat; g, guinea pig; c, chicken; f, fish; a, axolotl; x, *Xenopus laevis*.

ceptors will be mentioned in this review only to make points relevant to the functions of vertebrate receptors. Readers particularly interested in the functions of orphan nuclear receptors in *Drosophila* are referred to recent reviews on the subject (92–94).

C. Structural and functional diversity

The vast majority of orphan nuclear receptors possess all the functional domains that characterized classic nuclear receptors (Fig. 4). Some receptors have a very short modulator domain, and therefore lack an AF-1, while Rev-Erbα and -β lack the conserved AF-2. In addition, the nuclear receptor superfamily includes members possessing either a conserved DBD or LBD, but not necessarily both in the same molecule. Both DAX-1 and SHP lack a nuclear receptor-like DBD, while *Drosophila* EGON, KNIRPS, and KNRL, as well as numerous nuclear receptor-like gene products encoded in the *Caenorhabditis elegans* genome show no homology with nuclear receptors in their LBDs (95–103). However, these proteins can bind DNA or a ligand using these unrelated domains. DAX-1 has been shown to bind hairpin loop structures in DNA via its unique amino-terminal domain (104), while other intracellular receptors (*e.g.*, aryl hydrocarbon receptor) and serum and cellular binding proteins (such as retinol-binding protein, cellular retinoic acid-binding proteins) bind small lipophilic ligands using structures unrelated to the LBD of the nuclear receptors.

With the exception of DAX-1, orphan nuclear receptors recognize specific HREs through their well conserved DBD in a manner similar to that of classic nuclear receptors (37,

TABLE 2. *Drosophila* orphan nuclear receptors

Group	Trivial names	Official name	Vertebrate	References
I	E75	NR1D3	Rev-Erb	(625, 626)
	E78	NR1E1	Rev-Erb	(626)
	DHR3	NR1F4	ROR	(627)
	ECR	NR1H1	LXR	(628)
	DHR96	NR1J1	VDR	(629)
II	DHNF4	NR2A4	HNF4	(630)
	USP	NR2B4	RXR	(129–131)
	DHR78	NR2D1	TR2	(629)
	TLL	NR2E2	TLX	(631)
	DSF	NRE3	TLX	(632)
	SVP	NR2F3	COUP-TF	(633)
III	DERR	NR3B4	ERR	(634)
IV	DHR38	NR4A4	NGFI-B	(629)
V	FTZ-F1	NR5A3	FTZ-F1	(635, 636)
	DHR39	NR5B1	FTZ-F1	(637, 638)
0	KNI	NR0A1		(96)
	KNRL	NR0A2		(95, 97)
	EGON	NR0A3		(97)
	TRX	NR0A5		(639)

105). Functional studies of orphan nuclear receptors have considerably changed previously held dogma on how nuclear receptors can bind DNA. First, the identification of RXR led to the discovery that a large subset of classic and orphan nuclear receptors bind DNA as heterodimers (reviewed in Ref. 23). Second, several orphan nuclear receptors can bind DNA with high affinity as monomers (105–116). Monomeric nuclear receptors utilize the CTE (Fig. 3) to recognize distinct A/T-rich sequences located upstream of a single core half-site. The CTE-DNA interactions provide additional protein-DNA contacts in monomeric sites necessary for specific and high-affinity binding (107, 108, 113, 117, 118). The distinct amino-terminal domains contained in orphan nuclear receptor RORα (RAR-related orphan receptor) have been shown to interact with a common CTE to regulate the receptor's binding site specificity. The hinge and amino-terminal domain appear to orient the zinc finger modules and the CTE relative to each other and are required to achieve proper interactions with the core AGGTCA half-site and the specific A/T-rich moiety (109, 113, 118). The observations that mutations in the amino-terminal domain of the T_3R change the DNA-binding specificity of this receptor (119) and that the CTE contributes to the specificity and polarity of PPAR (peroxisome proliferator-activated receptor) binding to DNA (120) suggest that the participation of these two domains in DNA recognition could be widespread among nuclear receptors.

All vertebrate orphan nuclear receptors possess a highly recognizable LBD (Fig. 5). The presence of a conserved LBD is often interpreted as a strong indication that all vertebrate orphan nuclear receptors possess the intrinsic ability to bind a specific ligand. However, since the LBD mediates multiple functions (such as dimerization and coactivator interaction), its presence may only be required for these activities, which could be regulated via covalent modifications or protein-protein interactions (see above). Moreover, widely phylogenetically divergent receptors can bind similar ligands, suggesting that the ligand-binding function of nuclear receptors has evolved independently several times during evolution (121). This hypothesis implies that a certain number of orphan nuclear receptors may never have acquired the ability to bind ligands. However, this hypothesis would also imply that the LBD possesses the intrinsic ability to bind ligands, and that only a few mutations would be necessary to modify an ordinary transcription factor into a ligand-modulated one. The reverse hypothesis seems more plausible, *i.e.*, the ancestral nuclear receptor was a ligand-dependent transcription factor and that mutations during the course of evolution changed the ligand-binding specificity of novel nuclear receptors generated through gene duplication according to the increasing needs of more complex organisms. Some nuclear receptors may have lost their ligand-binding properties during evolution, but more drastic changes in their primary structures may have been expected, such as the loss of the AF-2 domain involved in ligand-dependent transactivation. While evolutionary studies are useful for stimulating speculative debates, well designed biochemical, molecular, and physiological experiments are more likely to provide answers on the roles and functions of orphan nuclear receptors and their putative ligands.

IV. Novel Hormone Response Systems: RXR and Its Heterodimeric Partners

It has now been demonstrated that RXR and its heterodimer partners, with the exception of nerve growth factor induced gene B (NGFI-B), are liganded (122–124). The next section will review how the search for ligands associated with orphan nuclear receptors, a concept now referred to as "reverse endocrinology," has led to the discovery of novel hormone response systems.

A. RXR: rexinoids

RXRα was originally cloned as a result of its homology with the RARα DBD (125). Three RXR gene products referred to as RXRα, -β, and -γ were identified in mammals (125–128), as well as a *Drosophila* homolog, ultraspiracle (129–131). RXRs as a family are ubiquitously expressed, although individual RXR genes display unique but overlapping pattern of expression during development and in adult tissues (127, 132–134).

RXRα could be activated by supraphysiological doses of atRA, suggesting that the natural ligand for RXRα might be a metabolite of atRA (125). Starting with this hypothesis, two groups independently identified 9-*cis*-retinoic acid (9cRA) as the RXR ligand (Fig. 6) (135, 136). The identification of 9cRA as the natural RXR ligand was the first demonstration of reverse endocrinology, where the discovery of a receptor leads to the identification of a novel hormone. As 9cRA was also found to be a high-affinity ligand for RAR (136), several groups began a search for natural and synthetic RXR-specific ligands. Two noncyclic terpenoids, methoprene acid and phytanic acid, were found to bind and activate RXRs in a specific manner (137–139). Phytanic acid is a natural chlorophyll metabolite present in normal human diet, while methoprene acid is an environmental contaminant. While both ligands have the potential to regulate or disrupt natural RXR responses, the physiological significance of these find-

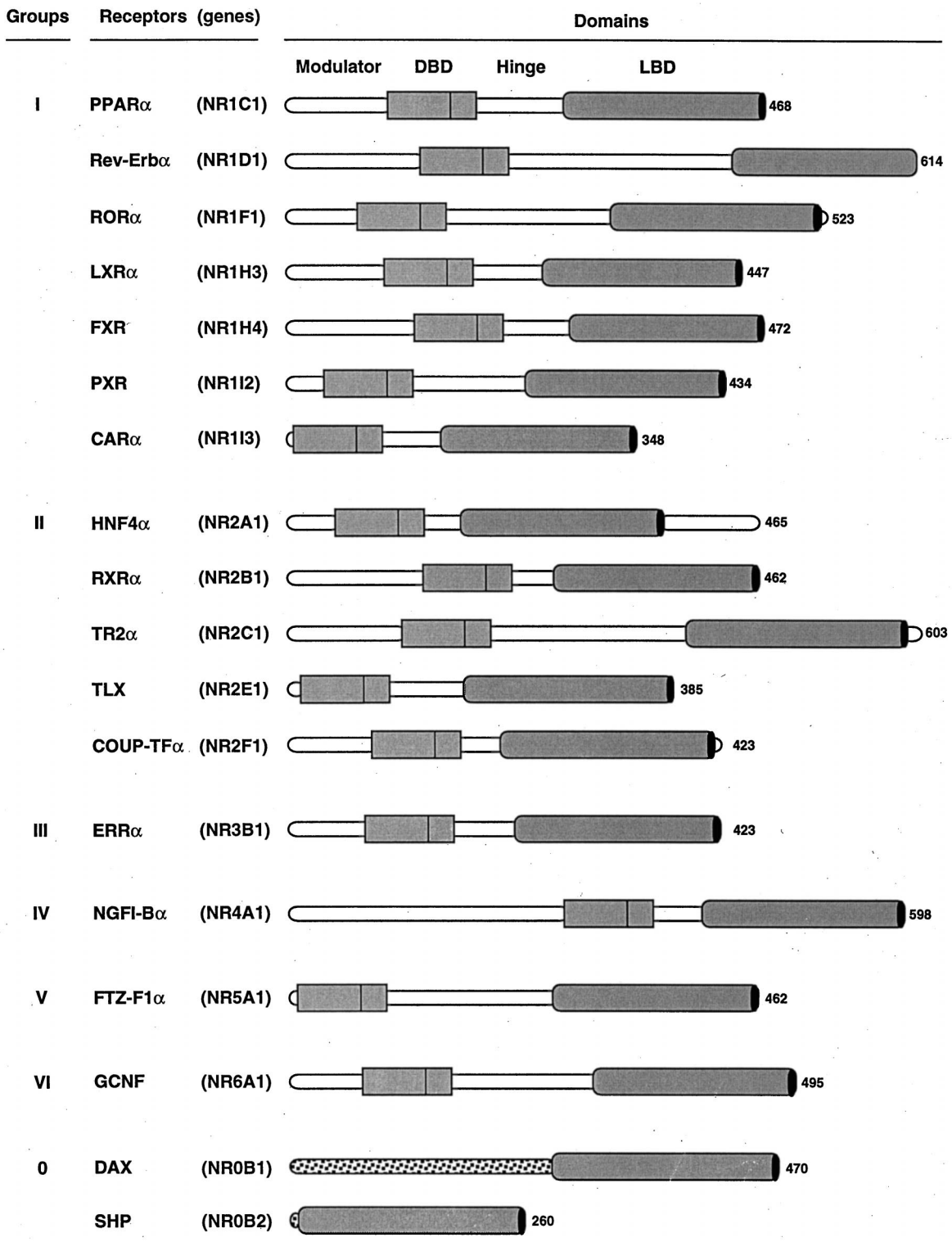

FIG. 4. Schematic representation of vertebrate orphan nuclear receptors. One member of each family is shown. The presence of a ligand-dependent AF-2 domain is indicated in *black*. Atypical sequences in DAX-1 and SHP are represented by a *dotted* domain.

ings remains to be proven, especially in view of the relative low affinities of these compounds for RXRs. Several synthetic compounds have now also been characterized that bear the characteristics of RXR-selective ligands, including both agonists and antagonists (140–144).

As introduced above, RXRs participate in a wide range of hormone response systems via their association with other nuclear receptors as heterodimeric partners. Two types of RXR heterodimeric complexes exist: nonpermissive heterodimers that can be activated only by the partner's ligand

FIG. 5. Sequence alignment of vertebrate orphan nuclear receptor LBDs. The putative secondary structure adopted by nuclear receptors is represented by α-helices and β-strands according to crystal structure of PPARγ (52).

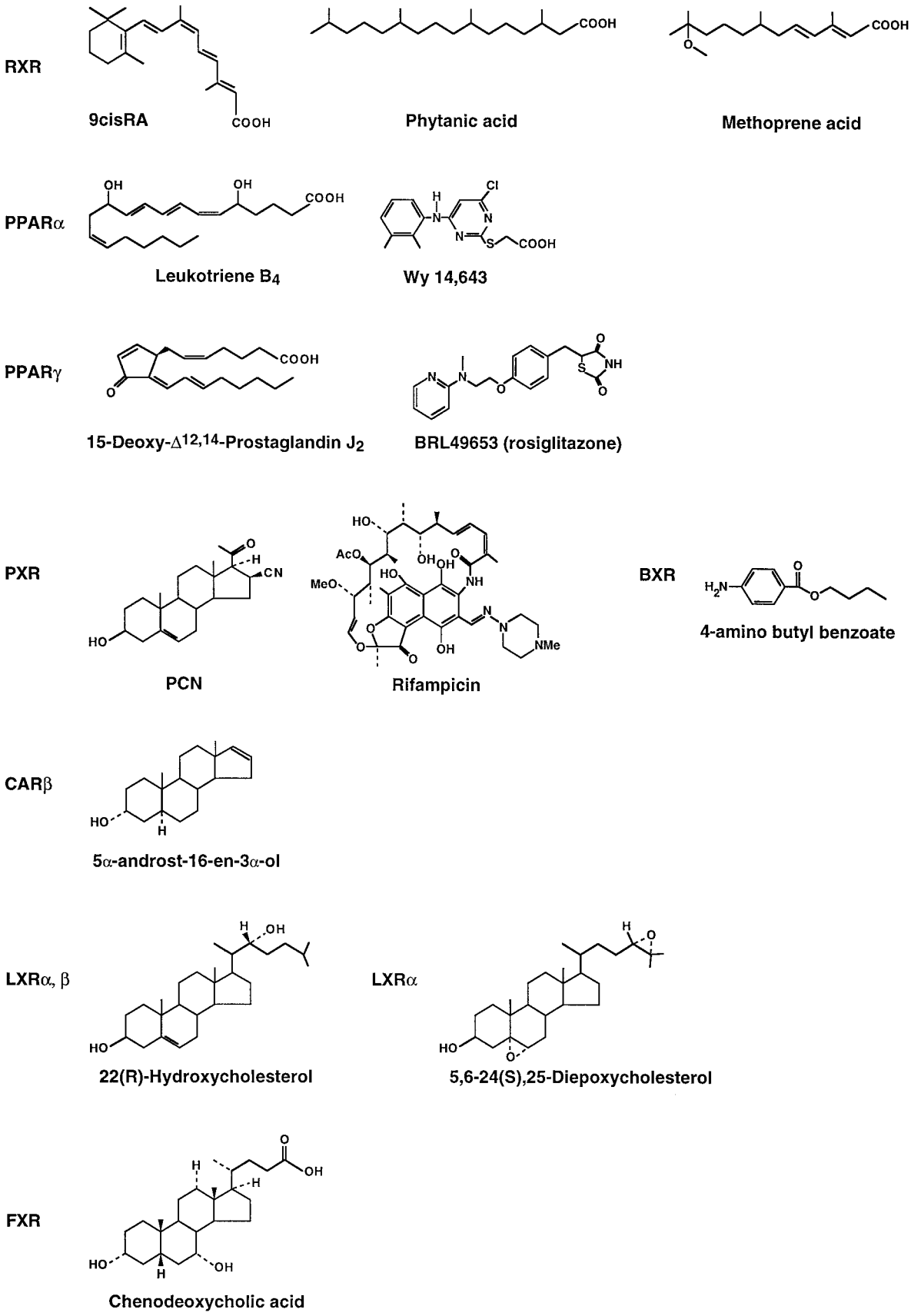

FIG. 6. Representative ligands and activators shown to modulate the activity of RXR and its heterodimeric partners.

(42, 145, 146), and permissive heterodimers that can be activated either by RXR or by the partner's ligand (147–150). Nonpermissive heterodimers include RAR/RXR, T_3R/RXR, and VDR/RXR, although RXRs are only completely silent in the T_3R/RXR and VDR/RXR complexes. Both RAR and RXR ligands can activate the RAR/RXR complex (151, 152); however, RXR ligands are effective only in the presence of a RAR ligand (146, 153, 154). The ability of each heterodimeric complex to allow RXR ligand binding may be explained in part by distinct intermolecular interactions between the RXR AF-2 domain with the coactivator-docking site of its partner (154). Since RXR-selective compounds can elicit a response from both retinoid- and non-retinoid-related pathways, the term rexinoids is now being used to distinguish RXR-specific activators from other vitamin A derivatives and synthetic analogs acting as RAR ligands. The development of rexinoids has considerably extended the therapeutic repertoire of vitamin A derivatives. Rexinoids have recently been shown to inhibit the growth of atRA-resistant human breast cancer cells (155), act as chemopreventive agents and even cause regression of mammary carcinoma in the rat (156), and sensitize diabetic and obese mice to insulin (157, 158).

Because RXRs play a dual role in nuclear receptor signaling (as receptor for 9cRA and as a heterodimer partner for several nuclear receptors), it has been difficult to assess the precise contribution of RXR-selective pathways in developmental and physiological processes. Two lines of evidence point to the fact that RXR could act as a specific receptor in its own right. First, 9cRA binding to RXRs promotes the formation of RXR homodimers (159), demonstrating that RXRs can function independently of other signaling pathways when bound to a DR-1 HRE (160). Second, transgenic mice expressing a chimeric RXR protein in which the RXR LBD was fused to the DBD of the yeast activator Gal4, together with a β-galactosidase reporter gene driven by Gal4 upstream-activated sequences, showed a receptor-specific activation pattern in the developing spinal cord consistent with a role for endogenous RXR ligands *in vivo* (161). This technique offers the potential to investigate the activation pattern of any nuclear receptors and should be particularly useful in studying orphan nuclear receptor functions.

Genetic ablation experiments have revealed that RXRα plays a primary role in placenta, heart, and eye morphogenesis (162–170). The putative functions of RXRα in adult animals are unknown due to the embryonic lethal phenotype. RXRβ mutant mice have abnormal spermatogenesis (171), while RXRγ null mice are apparently normal (172). While generation of RXR and RAR compound mutants have clearly demonstrated that the RXR/RAR heterodimer complex transduces the retinoid signal for a number of RA-dependent processes during development (162, 173, 174), surprisingly, there is no genetic evidence yet available indicating that RXRs actively participate in other hormone response pathways *in vivo*.

B. PPAR: multiple ligands, multiple functions

Three PPAR genes generating a number of isoforms have been identified in mammals: PPARα, -β, and -γ (Table 1) (reviewed in Ref. 175). The members of the PPAR family have been cloned by techniques including screening of a cDNA library with a mixture of oligonucleotides directed against a conserved region of the DBD, low-stringency screening, expression library screening using radiolabeled HREs, and DNA affinity purification and microsequencing of nuclear proteins. The primary sequences of PPARα, -β, and -γ are more divergent than members of other families, reflecting a rapid evolution from the ancestral PPAR gene. Each PPAR gene displays a distinct expression pattern during development and in adult animals. PPARα is highly expressed in heart, liver, kidney, intestine, and brown fat, tissues that demonstrate high rates of fatty acid β oxidation (176, 177). Hepatic PPARα expression levels have been observed to vary widely in individual animals (177), possibly due to hormonal modulation of PPARα expression by glucocorticoids (178), physical stress (179), or changes in serum insulin levels (180). PPARβ is more widely expressed in adult tissues: high levels of PPARβ transcripts are detected in the brain, kidney, small intestine, and Sertoli cells (177, 181). Interestingly, PPARγ isoforms are expressed in a tissue-specific fashion: PPARγ1 transcripts are abundantly expressed in the spleen, intestine, and white adipose tissue (177), while the PPARγ2 isoform is preferentially expressed in white and brown fat.

PPARs bind to DR1 HREs as a heterodimer with RXR (147, 182, 183). However, analysis of natural PPREs has clearly shown that nucleotides that are present 5′ of the two consensus half-sites regulate the efficiency with which specific PPAR isoforms recognize PPREs (120, 184, 185). PPREs have been identified in genes controlling all aspects of carbohydrate and lipid metabolism (147, 182–184, 186–211). In addition to directly binding to their cognate response element, it has been suggested that PPARs may regulate gene expression by forming heterodimers with other nuclear receptors such as $T_3R\beta$ and liver X receptor (LXRα) (27, 212, 213).

The initial characterization of PPARα led to the observation that peroxisome proliferators, a group of structurally unrelated compounds that cause proliferation of hepatic peroxisomes, liver hyperplasia, and hepatic malignancies in rodents (reviewed in Refs. 175 and 203), could stimulate its activity when employed at pharmacological doses (176). A search for more potent and natural PPARα activators first demonstrated that fatty acids could activate PPAR (214). PPARα activity was subsequently shown to be induced by eicosanoids (215), carbaprostacyclin (216), nonsteroidal anti-inflammatory drugs (NSAIDs) (217), and leukotriene β_4 (LTB_4) (218). Although PPARβ and -γ can be activated by common PPAR ligands such as docosahexenoic acid and certain prostaglandins (215), PPARγ was shown to specifically bind to thiazolidinediones (TZDs), a class of antidiabetic drugs (219, 220). Other PPARγ ligands include the natural prostaglandin metabolite 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (PGJ2) (219, 221), polyunsaturated fatty acids (222), and NSAIDs such as ibuprofren (223). Specific synthetic PPARβ ligands have been identified by screening biased chemical libraries (224); however, a natural high-affinity PPARβ ligand has yet to be characterized. Representative PPAR ligands and activators are shown in Fig. 6. In addition to direct activation by PPAR ligands, PPAR-RXR heterodimers can be activated by RXR-specific ligands. Transient transfection studies showed that maximal activation of

a PPRE-containing gene promoter was achieved by simultaneous treatment with the synthetic PPAR ligand WY14,643 and 9cRA (225). As mentioned above, this synergism is also observed *in vivo*, where the efficacy of TZDs in reducing fasting hyperglycemia and hypertriglyceridemia in *db/db* obese mice is potentiated by dual treatment with rexinoids (157). Finally, PPARγ isoforms display isoform-specific transactivation potential due to their distinct ligand-independent AF-1 domain (226, 227). In particular, the PPARγ2 AF-1 domain contains a consensus MAPK site, and phosphorylation of that site after stimulation of the MAPK pathway by epidermal growth factor, and insulin inhibits the adipogenic potential of liganded PPARγ2 (226, 228), presumably because phosphorylation of this site reduces ligand-binding affinity via an intramolecular communication between the AF-1 and the LBD (229). The PPARγ2 AF-1 domain has also been shown to interact with a coactivator protein termed PGC-2 (230). Ectopic expression of PGC-2 in preadipocytes leads to an increase in fat cell differentiation, suggesting that PGC-2 may be a limiting cofactor for the adipogenic action of PPARγ2.

The identification of ligands and target genes linked to lipid metabolism greatly facilitated the analysis of PPARγ functions. Numerous *in vitro* studies have provided strong support for a crucial role for PPARγ in adipogenesis (reviewed in Refs. 203, 231, and 232). In particular, differentiation of fibroblasts into adipocytes is accompanied by increased expression of PPARγ (233), and overexpression of PPARγ2 is sufficient to induce fibroblasts to undergo adipocyte differentiation in the presence of ligands (234). It has been shown that activation of PPARγ induces cell growth arrest in fibroblast cell lines, which suggests that PPARγ may play an important role in cell cycle withdrawal during adipogenesis *in vivo* (235). Although PPARγ was first thought to be an adipocyte-specific modulator (236), PPARγ transcripts have now been detected in many tissues including normal mammary epithelia and breast adenocarcinomas (237), colon (238–241), and macrophages (242, 243). Remarkably, treatment of human breast cancer cells with TZDs led to a series of molecular and morphological changes that are associated with a more differentiated state and to the induction of apoptotic pathways, suggesting that PPARγ-induced cell differentiation may offer a novel therapeutic approach to breast tumors (237, 244). A small clinical trial involving three patients with advanced liposarcoma also suggests that troglitazone (Parke-Davis, Ann Arbor, MI) could be effective at promoting the differentiation of this type of solid tumor (245). PPARγ ligands have also been demonstrated to slow the growth and induce the differentiation of human colon cancer cells in culture or implanted tumors (246). Somatic PPARγ mutations that impaired the function of the protein were also found in sporadic colon cancers, suggesting that loss of function of PPARγ may contribute to the etiology of human colon cancer (247). However, mice genetically susceptible to develop polyps in the colon treated with PPARγ ligands show an increased frequency of colon adenocarcinomas (240, 241). These apparently contradicting results may reflect a distinct role for PPARγ in the context of normal colon epithelium (proliferation) and tumor cells (growth arrest) (248). These observations raise a warning flag to long-term use of TZDs and beg for additional investigation of the role of PPARγ in normal and abnormal colon physiology. PPARγ has also recently been indirectly implicated in the regulation of monocyte functions (reviewed in Ref. 249). At relatively high doses, PPARγ ligands were shown to be effective in reducing the levels of inflammatory cytokines and production of nitric oxide by isolated monocytic cells (242, 250). In addition, PPARγ appears to be involved in the maturation of monocytes along the macrophage lineage, more specifically in the conversion of monocytes to foam cells, which can be induced upon exposure of monocytes to oxidized LDL. It has been proposed that two oxidized derivatives of linoleic acid present in LDL, 9- and 13-hydroxyoctadecadienoic acid (9- and 13-HODE), can act as PPARγ ligands once internalized into foam cells by oxidized LDL receptor-mediated endocytosis (243, 251). PPARγ activation by 9- and 13-HODE then leads to foam cell maturation and directly enhances the expression of the CD36 lipoprotein scavenger receptor gene promoter, resulting in increased macrophage LDL and oxidized LDL uptake, which promotes cholesterol deposition in atherosclerotic plaques. Again, both findings have direct implications for use of TZDs as therapeutic agents. On one hand, TZDs, which are well tolerated in patients with non-insulin-dependent diabetes mellitus, could replace the less well tolerated NSAIDs to treat inflammatory diseases. In contrast, prolonged use of TZDs could accelerate the formation of atherosclerotic plaques and increase cardiovascular diseases.

The study of PPARα null mice have revealed three important functions for PPARα *in vivo* (198). First, it has been demonstrated that PPARα is an essential mediator of the hepatic response to peroxisomal proliferators such as Wy-14,643, clofibrate, and DHEA-S (198, 252). However, PPARα is not essential for peroxisome biogenesis as normal numbers of hepatic peroxisomes are present in PPARα$^{-/-}$ mice. Second, PPARα appears to orchestrate the expression of genes encoding mitochondrial, peroxisomal, and cytochrome P450 enzymes involved in cellular fatty acid utilization in response to changes in intracellular levels of fatty acid metabolites (209, 253, 254). Third, PPARα null mice show a prolonged inflammatory response when challenged by its natural ligand, LTB_4 (218). It has been proposed that the prolonged response to LTB_4 is due to the disruption of the normal feedback mechanism controlling the degradation of this chemotactic inflammatory agent, implying that liganded PPARα regulates transcription of genes involved in this catabolic pathway. Finally, PPARα ligands have been shown to inhibit the inflammatory response of aortic smooth muscle cells *in vivo*, which participate in plaque formation and post-angioplasty restenosis (255). Thus, in contrast to PPARγ ligands, activators of PPARα may have beneficial vascular effects in atherosclerosis.

While the role of the widely expressed PPARβ remains elusive, studies using cyclooxygenase-2 (COX2) null mice suggest that the essential role played by the COX2-derived prostacyclin PGI_2 in implantation and decidualization is transduced by this receptor in the uterus (256). PPARβ, RXRα, COX2, and PGI synthase are coexpressed in stromal cells surrounding the implanting blastocysts, and PPARβ agonists (PGI_2, carbaprotacyclin, and L, 165,041) that can

specifically promote PPARβ/RXRα heterodimerization and transactivate the receptor complex *in vitro* restore implantation and decidualization in COX2 null mice. Once again, the potential role of PPARβ ligands in implantation suggests that the development of PPAR-directed drugs should be vigilantly monitored to avoid unwanted side effects.

C. PXR: pregnanes, xenobiotic compounds, and benzoate derivatives

The characterization of PXR may well represent the first identification of a steroid hormone-based response system in many decades. Murine PXR was identified through a computer search of expressed sequence tags (ESTs) derived from a liver cDNA library (257). PXR is predominantly expressed in the liver and intestine of embryos and adult animals and is most closely related to the VDR at the structural and amino acid sequence levels. Using a Gal4-PXR chimeric protein to perform an initial search for PXR activators, Kliewer *et al.* (257) found that synthetic pregnanes (C21 steroids) and both glucocorticoid agonists and antagonists were potent inducers of PXR activity. Interestingly, some of these compounds, in particular dexamethasone and pregnenolone 16α-carbonitrile (PCN), were well known to induce the expression of the cytochrome p450 *CYP3A* gene in rodent liver and intestine and cultured hepatocytes (see Refs. 257 and 258). CYP3A is involved in the hydroxylation of steroid hormones as well as various toxic xenobiotics. Induction of *CYP3A* and other members of the rodent p450 3A family is believed to confer protection against drugs and toxic xenobiotics by increasing their catabolism (259). Like its close relative VDR, PXR was found to recognize DR-3 HREs as a heterodimer with RXR, and a DR-3 motif previously demonstrated to be responsible for the activation of the CYP3A promoter by dexamethasone and PCN (260–262) was also found to mediate PXR activation of the CYP3A promoter by these compounds (257). Interestingly, mouse and human PXR display important differences in their activation profile by certain drugs (258, 263). While PCN acts as a potent inducer of mouse PXR, it has only a weak inductive effect on human PXR. Conversely, rifampicin is a strong activator of human PXR but has very little activity on mouse PXR. These species-specific activation profiles were not entirely unexpected as marked interspecies differences had been observed in the induction of CYP3A genes. The comparative analysis of human and mouse PXR function provide an elegant molecular explanation for these species-specific responses. Taken together, these results provide convincing evidence that PXR is responsible for the induction of *CYP3A* genes in response to treatments with PCN, dexamethasone, and various xenobiotic agents. However, these are synthetic compounds that must mimic the action of endogenous steroids whose normal physiological role could be to regulate steroid and sterol metabolism in liver and intestine. Based on the finding that PXR is best activated by pregnenolone and its derivatives, Kliewer *et al.* (257) proposed that the natural ligand for PXR is likely to be a pregnane; hence, the name pregnane X receptor (PXR). The broader activation profile and low affinities for human PXR activators has also been interpreted to mean that PXR could function as a steroid and xenobiotic sensor that directly regulates the activity of catabolic p450 enzymes in response to the presence of their substrates (123, 263). If this hypothesis is correct, a high-affinity ligand for PXR may not be required. Regardless of which hypothesis turns out to be correct, the first direct application to spring from these discoveries is likely to be the development of more rapid and accurate PXR-based assays to screen for the ability of drugs to induce CYP3A genes, an important component of the drug development process (258).

The *Xenopus* PXR ortholog, referred to as BXR (264) and xONR1 (265), appears to play a completely different role in that organism. BXR is expressed early during *Xenopus* development, and biochemical purification of transcriptionally competent embryonic extracts tested in a BXR-dependent activation assay led to characterization of endogenous benzoate metabolites as BXR ligands (Fig. 6) (264). While the exact role of these compounds in vertebrate development is unknown, their identification as orphan nuclear receptor ligands suggests that this class of molecules may participate in previously unrecognized morphogenetic signaling pathways.

D. CAR (constitutive androstane receptor): androstanes and phenobarbital

Study of CAR function has recently introduced another new concept in nuclear receptor action. CAR was originally identified through screening of a cDNA library with a degenerate oligonucleotide based on a conserved region of the nuclear receptor DBDs (266). CAR was found to bind DR-5 HREs as a heterodimer with RXR, sites previously shown to be regulated by RAR-RXR complexes in the presence of retinoids or rexinoids. However, CAR was found to activate reporter genes driven by promoters containing DR-5 HREs (266, 267) or a complex HRE present in the *CYP2B* gene (268) in the absence of retinoids, rexinoids, or any other exogenously added ligands. Thus, the name CAR was referring to constitutively active receptor. Despite its constitutive behavior, the likelihood that CAR activity could be regulated by a ligand was considered high, mainly on the basis of its association with RXR. The search for a ligand revealed that CAR is, in fact, a steroid receptor for androstenol and androstanol (Fig. 6), but contrary to previous dogma concerning steroid receptor action, these ligands switch the activity of CAR off instead of on (269). A biochemical analysis of CAR function suggests that the two steroids act as inverse agonists, not antagonists, since addition of these ligands induces the dissociation between CAR and a coactivator protein *in vitro*. A legitimate question to ask is whether androstenol and androstanol are the best ligands for CAR. The affinities of both compounds for CAR (>400 nM) are well below normal physiological levels found in the plasma. Nonetheless, this discovery should stimulate studies on this previously unrecognized androstane-related signaling pathway and provide a new model to investigate the molecular and structural mechanisms underlying nuclear receptor activation and repression.

While the constitutive activity of CAR can be suppressed by androstanes, various phenobarbital-type inducers have been shown to reverse the negative effect of androstanes on

the human cytochrome P450 (CYP) 2B6 promoter (270). Phenobarbital is the prototype for xenochemicals that induce CYP2B genes. This observation suggests that CAR, together with PXR and PPARα, may participate in a nuclear receptor-based regulatory pathway controlling the expression of CYP genes in response to exogenous xenochemicals and endogenous compounds such as steroids and lipids.

E. LXR: control of cholesterol metabolism by oxysterols

LXRα was so named based on its initial isolation from a human liver cDNA library and the observation that its expression is enriched in that tissue (148). LXRα is also expressed at significant levels in other organs such as the intestine, kidney, and spleen (148, 271). A second member of the family, LXRβ, is ubiquitously expressed (272–275). Both LXRα and -β recognize DR-4 HREs as heterodimers with RXR (148, 271, 272, 274). LXR binding sites, referred to as LXREs, preferentially contain nonconsensus half-sites (AGTTCA). LXRα has also been found to heterodimerize with PPARα: this interaction does not lead to the formation of a transcriptionally active complex and has not yet been shown to be physiologically relevant (213). The LXR-RXR complex belongs to the class of permissive heterodimers as the RXR ligand 9cRA was found to be a potent activator of LXR transcriptional activity (148). Interestingly, RXR was shown to occupy the 5′-half-site on the LXRE, a position that does not allow ligand binding in other heterodimeric complexes. This suggests that RXR-ligand activation potential is not exclusively dictated by receptor binding polarity on DNA (42) but is rather dependent on the RXR partner and primary sequence of the HRE (150). Furthermore, activation of the LXR-RXR complex by 9cRA requires the LXR but not the RXR AF-2 domain, suggesting that ligand binding by one receptor induces conformational changes in its partner that lead to transcriptional activation (150, 276). Similar conclusions were reached in studies using the synthetic rexinoid LG100754, which can activate the nonpermissive RAR-RXR heterodimer via the unliganded RAR (277). This phenomenon was referred to as the phantom ligand effect.

The search for LXR ligands led to the discovery that endogenous oxysterols are potent and selective LXR activators (149). The most active compounds identified were 22(R)- and 24(S)-hydroxycholesterol, 24(S),25-epoxycholesterol, and 7α-hydroxycholesterol (Fig. 6) (149, 223, 278). Oxysterols are oxidized derivatives of cholesterol that serve as intermediary substrates in the rate-limiting steps of steroid hormone and bile acid synthesis (279). One prediction from these findings is that liganded LXRα could act as a sensor of cholesterol and regulate its metabolism. The phenotype of mice carrying a targeted null mutation in the LXRα gene confirmed this hypothesis (280). The absence of LXRα results in a block of cholesterol catabolism, leading to accumulation of hepatic cholesterol in mice fed a high cholesterol diet (2%) associated with mysregulation of *CYP7A*, which encodes a key regulatory step involved in bile acid synthesis. LXRα mutant mice also display abnormal fatty acid synthesis. A role for LXRα as a cholesterol sensor is strongly supported by the observation that LXRα knock-out mice do not up-regulate bile acid synthesis or diminish their cholesterol uptake in response to high cholesterol levels. However, since the phenotype is observed only when the mice are exposed to very high levels of cholesterol in their diet, the role of LXRα under normal physiological conditions remains to be elucidated. Nonetheless, these discoveries will certainly lead to the search for possible mutations in the LXRα gene in patients with defects in cholesterol metabolism as well as the development of therapeutic agents targeting the oxysterol response pathway.

F. FXR: bile acids receptor

FXR is most closely related to the *Drosophila* EcR and binds to EcRE (IR-1) and DR-4 HREs in a complex with RXR (274, 281). Transactivation assays have shown that rat FXR can be activated by high concentrations of farnesol (281). Farnesol is an isoprene intermediate in the mevalonate biosynthetic pathway and most likely activates FXR via its conversion into a higher affinity derivative (Fig. 6). However, the activity of the highly homologous mouse FXR (RIP14) is not induced by farnesol but rather can be stimulated by atRA and the synthetic retinoid TTNPB (282). As observed for the action of farnesol on rat FXR, high concentrations of atRA are required to activate mouse FXR, and no evidence of direct binding by atRA and TTNPB was obtained, again suggesting that FXR could serve as a receptor for an unknown metabolite of these activators. Because farnesol and retinoids share common metabolic pathways, it is expected that the activating compound would be a retinoid metabolite.

More recently, however, FXR was shown to be a receptor for bile acids (283–285). The combined results of three groups demonstrate that several bile acids are potent inducers of FXR transcriptional activity and promote FXR interaction with a coactivator at physiological concentrations. Bile acid activated FXR was also shown to enhance transcription from the intestinal bile acid binding protein (IBABP) promoter and inhibit transcription from the CYP7A promoter, most likely via antagonism of LXRα action. Taken together, these results suggest that FXR is a general regulator of bile acid metabolism, acting both at the level of the liver through suppression of CYP7A to reduce synthesis and at the level of the intestine through activation of IBABP to increase recycling of bile acids (reviewed in Ref. 286).

V. Orphans in Search of a Home

While functional studies of nuclear receptors in the absence of ligands are intrinsically more limited in scope and pharmacological relevance, extensive usage of modern molecular, biochemical, and genetic tools have allowed investigators to obtain a first glance of the biological functions associated with many unliganded orphan nuclear receptors. Below is a brief summary of current knowledge on the molecular mechanisms and functions associated with these putative receptors.

A. HNF4: diabetes and possible regulation by acyl-coenzyme A (CoA) thioesters

HNF4 (hepatocyte nuclear factor 4) represents one of two orphan nuclear receptors not associated with RXR for which

an association with a putative ligand has been proposed. HNF4α was initially identified as a transcription factor required for liver-specific gene expression (287). To date, three genes encoding HNF4 subtypes have been identified in vertebrates: two in human and rodents (HNF4α and -γ) (287–289) and one in *Xenopus* (HNF4β) (290). Each gene product differs significantly in its expression pattern and transactivation potential (288, 289, 291). HNF4α is expressed at high levels in liver, kidney, intestine, and pancreas and at low levels in the testis (287, 291, 292). HNF4γ transcripts are not expressed in liver but can be found at low levels in the kidney, intestine, and pancreas (291). During development, HNF4α is expressed in primary endoderm at 4.5 days postcoitus (d.p.c.) and in visceral endoderm between 5.5 d.p.c. and 8.5 d.p.c. (293). Hepatic expression of HNF4α is detected in the liver primordia by 8.5 d.p.c. and during all subsequent stages of development. HNF4α expression in other tissues begins at 9.5 d.p.c. in the gut and at 10.5 d.p.c. in the developing pancreas and the mesonephric tubules (293, 294). HNF4 subtypes bind as homodimers to DR-1 HREs (295) and regulate the expression of genes involved in cholesterol, and xenobiotic and amino acid metabolism, as well as all aspects of carbohydrate and lipid metabolism and various liver specific-genes (201, 292, 296–322).

HNF4α is a constitutive inducer of gene expression that interacts with steroid hormone coactivators and p300 (323), suggesting that its activity may be regulated by an endogenous ligand present in most cell types (287). Recently, long-chain fatty acyl-CoA thioesters have been shown to modulate gene activation by binding directly to HNF4α (324). Long-chain fatty acyl-CoA thioesters are amphiphilic molecules that play important roles in the regulation of energy metabolism through direct interaction with a variety of cellular enzymes (reviewed in Ref. 325). High intracellular fatty acyl-CoA concentrations, which sometime result from prolonged fasting or diabetes mellitus, have been shown to inhibit the activity of glycolytic enzymes resulting in fatty acid oxidation replacing glycolysis as the primary cellular energy source (325, 326). The observation that long-chain fatty acyl-CoA thioesters could also regulate the transcription of genes implicated in these metabolic pathways is of considerable interest. However, long-chain fatty acyl-CoA thioesters displayed marked differences in their ability to regulate HNF4α transcriptional activity: poly- and monounsaturated acyl-CoAs inhibited the constitutive activity of HNF4α, while different saturated acyl-CoAs activated HNF4α over its normal activity (palmitoyl-CoA) or inhibited (stearoyl-CoA) it. In addition, treatments with long-chain fatty acyl-CoA thioesters never resulted in more than a 2-fold change in HNF4α activity measured in transcriptional assay (324). Since a mixture of long-chain fatty acyl-CoA thioesters may have mutually antagonistic effects on HNF4α function, it may be difficult to demonstrate the importance of these ligands as modulators of HNF4α *in vivo*. Another caveat to this observation is the previous finding that long-chain fatty acyl-CoA thioesters may also regulate gene expression by interfering with T_3R signaling (327). Nonetheless, if confirmed by additional physiological studies, the identification of long-chain fatty acyl-CoA thioesters as natural HNF4α ligands could lead to the development of more specific synthetic HNF4α ligands, which could be used to differentiate the effects of these compounds mediated by HNF4α from those mediated by direct enzyme inhibition.

Insights into HNF4α function *in vivo* have come from both population and reverse genetics. Recently, a locus linked to maturity-onset diabetes of the young (MODY1) has been associated with mutations in the human HNF4α gene (328–331). The association between MODY1 and HNF4α is probably specific to this form of diabetes, as HNF4α mutations have not been identified so far in patients with other forms of noninsulin-dependent diabetes mellitus (332). Furthermore, disruption of the HNF4 binding site in the HNF1 promoter has been identified in an Italian family with MODY, providing an unusual example of patients whose disease state likely results from a combined impairment of HNF4α and HNF1 function (333). Of particular interest, HNF4α mutations identified in MODY1 patients can alter the cellular localization of HNF4α or reduce its activity in transcriptional assays, providing strong support for a direct link between reduced HNF4α function and the MODY phenotype (331, 334, 335). On the other hand, gene targeting experiments have not been informative with regard to possible HNF4α functions in liver development or metabolic control in adult animals. Ablation of the *Hnf4α* gene results in apoptosis of embryonic ectoderm at 6.5 d.p.c, followed by abnormal mesoderm differentiation and embryonic death (336). However, *Hnf4α* ablation in either ES cells or 8.5 d.p.c. embryos is associated with significantly reduced expression of glycolytic enzymes as well as glucose and fatty acid transport proteins (334).

B. FTZ-F1 (fushi tarazu-factor 1): steroidogenesis and sexual development

FTZ-F1α was initially characterized as an adrenal gland-specific factor (SF-1) able to bind to conserved AGGTCA consensus motif in the proximal promoter regions of steroid hydroxylases *CYP11A*, *CYP11B2*, and *CYP21* genes, suggesting that this factor was a member of the nuclear receptor superfamily (337). FTZ-F1 was eventually cloned from an adrenal gland cDNA library based on its homology to the RXRβ DBD (338). The *FTZ-F1* gene generates several distinct isoforms through alternative splicing and promoter usage (339–341). A second closely related gene, *FTZ-Fβ*, has also been identified and may be an important regulator of the α-fetoprotein locus and the CYP7A gene (342–344). During development, FTZ-F1α expression is first detected at 9.0 d.p.c. in the urogenital ridge (345). At 10–10.5 dpc, FTZ-F1α expression is associated with the precursors of adrenal steroidogenic tissue and gonadal steroid-producing cells. FTZ-F1α expression is also detected in the ventromedial hypothalamic nucleus (VMH) after 11.5 d.p.c. and in the pituitary gland after 13.5 d.p.c. Pituitary FTZ-F1α expression precedes the onset of FSH expression in gonadotropes, suggesting that FTZ-F1α might either directly regulate FSH gene transcription or regulate gonadotrope differentiation (346). In adult mice, FTZ-F1α expression is highest in steroid-secreting cells of the adrenal gland and gonads; lower level expression is present in the spleen and pituitary gonadotropes (340). FTZ-F1α is a monomeric receptor that binds to

HREs with the consensus sequence TCAAGGTCA (see Ref. 108). FTZ-F1α target genes include steroidogenic enzymes (reviewed in Ref. 337), Müllerian inhibiting substance (MIS) and its receptor (347, 348), the pituitary glycoprotein α-subunit (349), the LH β-subunit (350), the ACTH receptor (351), the steroidogenic acute regulatory protein (StAR) (352–354), oxytocin (355), and the orphan nuclear receptor DAX-1 (356, 357), all supporting an important role for FTZ-F1α in steroid metabolism and sexual differentiation.

FTZ-F1α usually constitutively activates gene expression, and its activity is regulated by phosphorylation: *in vitro*, protein kinase A-induced phosphorylation of FTZ-F1α reduces the receptor's DNA-binding affinity, while *in vivo*, FTZ-F1α phosphorylation may regulate cAMP-dependent gene induction (358–360). In addition, phosphorylation of the AF-1 domain (located in the hinge region) leads to increased SF-1 transcriptional activity via direct recruitment of the coactivator GRIP-1 (361). These observations suggest a way by which peptide hormones such as ACTH could regulate steroid synthesis via ligand-independent activation of SF-1. However, recent studies have shown that certain oxysterols, distinct from those regulating the activity of LXRα, increase FTZ-F1α transcriptional activity (362). The oxysterol 25-hydroxy-cholesterol is the most efficacious FTZ-F1α inducer (EC_{50} 5 μM), while 26-hydroxy-cholesterol (EC_{50} 5 μM), 27-hydroxy-cholesterol (EC_{50} 5 μM), and 21-hydroxy-pregnenolone (EC_{50} 11 μM) are less efficient FTZ-F1α activators, and the potent LXR activator 22(R)-hydroxycholesterol does not alter FTZ-F1α activity. Although oxysterol treatment results in increased FTZ-F1α activity, direct binding has not yet been demonstrated. In addition, oxysterol stimulation of FTZ-F1α activity has not been observed in all cell types (353), suggesting that further metabolism of these compounds may be required for the synthesis of the natural high-affinity FTZ-F1α ligand.

Gene knockout experiments have provided strong evidence for a direct role for FTZ-F1α in regulating mammalian sexual development as well as the differentiation of steroidogenic tissues (363–366). FTZ-F1α null mutants are viable at birth, but die during the first 8 days of life due to adrenocortical insufficiency. As suggested by FTZ-F1α expression studies, gonadal development is also dramatically affected in the null mutant embryos. In that respect, the FTZ-F1α knockout phenotype is comparable to the phenotype of patients affected by X-linked adrenal hypoplasia congenita (AHC). This syndrome, which results from mutations within the nuclear orphan receptor DAX-1 locus (discussed in more detail below), is characterized by adrenal hypoplasia, often associated with reduced serum gonadotropin levels and abnormal gonadal development (367, 368). FTZ-F1α null mice also display abnormal hypothalamic and pituitary development. Finally, isoforms of the Wilms' tumor 1 (WT-1) gene have been shown to markedly increase FTZ-F1α transactivation of the MIS promoter through a direct interaction with FTZ-F1α (369). WT-1 gene mutations are commonly associated with male genital ambiguity or male pseudohermaphroditism, suggesting that WT-1 may regulate FTZ-F1α activity during mammalian sexual differentiation (369).

C. Rev-Erb: singular members of the superfamily

The two members of the Rev-Erb family are best known for their unusual features. First, the original member of this family, the Rev-Erbα gene, was so named because it is encoded on the opposite strand of the T_3Rα (370, 371). The Rev-Erbβ gene, simultaneously cloned by several groups by homology screening (110, 372–375), is also closely linked to the T_3Rβ gene but apparently not encoded by an overlapping locus. Second, Rev-Erbs can bind DNA with affinity as both monomers and homodimers (110, 373, 376–378). The Rev-Erb monomeric consensus site is GAATGTAGGTCA in which the T at position −1 and A at −4 relative to the AGGTCA are essential for high-affinity binding (110, 376). The Rev-Erb homodimeric site is a DR-2, but unlike RAR-RXR, Rev-Erb binding requires the monomeric 5′-flanking A/T-rich sequence located upstream of the first half-site (377). x-Ray structure analysis of the Rev-Erbα DBD bound to a DR-2 HRE confirmed the importance of the role played by the CTE in the recognition of 5′-flanking A/T-rich sequence by nuclear receptors and revealed a additional role for the CTE in establishing productive protein-protein contacts in the homodimer complex (37). Third, the Rev-Erb LBDs lack an AF-2 domain, and this feature may be linked to the observation that Rev-Erbs are constitutive repressors of gene transcription (110, 372, 373, 377, 378). Transcriptional repression by Rev-Erbs is mediated through direct interactions with the nuclear receptor corepressors N-CoR, SMRT (silencing mediator for RAR and thyroid hormone receptor), and SUN-CoR (379–383). Stoichiometric studies have led to the suggestion that Rev-Erbα can repress transcription only via DR-2 HREs, but not from monomeric sites, as binding of N-CoR appears to require two receptor carboxyl termini (382). However, both Rev-Erbα and -β were shown to repress basal transcription via monomeric sites contained within the natural regulatory sequences of the ApoA-1 and N-Myc genes, respectively (384, 385). Repression on monomeric sites may occur through a passive mechanism, such as competition for positive transcription factors or for components of the basal transcriptional machinery, or through an active mechanism independent of N-CoR.

Little is known about the potential developmental and physiological function of Rev-Erbα and -β. Studies using the C_2C_{12} myoblasts differentiation model have indirectly implicated Rev-Erbα and -β as negative regulators of myogenesis (386, 387). However, the combined observations that both putative receptors are widely expressed during development and in adult tissues (110, 372–374, 388) and that Rev-Erbβ is a strong repressor of *N-Myc* expression (384) suggest that these two proteins may play a more general role in the control of cell proliferation and organ physiology. Rev-Erbα expression in liver has been shown to be stimulated by fibrates via PPARα, suggesting that Rev-Erbα could also play a role in lipid metabolism (389). Finally, it is interesting to note that the Rev-Erb homolog in *C. elegans*, referred to as SEX-1, determines sex in nematodes by repressing the transcription of the sex determining gene *xol-1* (390). Given the tissue distribution of Rev-Erb transcripts and their chromosomal localization, it is unlikely that the two Rev-Erbs are implicated in sex determination in mammals.

However, in contrast to Rev-Erbs, SEX-1 possesses an AF-2 domain, suggesting that the ancestral Rev-Erb gene product may have been responsive to a ligand. Based on these observations, it may be possible to find a ligand for members of the Rev-Erb family.

D. ROR: neuronal development and T cell selection

The ROR family contains three genes, RORα (109, 342, 391), -β (111), and -γ (392–394). The human RORα gene encodes at least four distinct isoforms (RORα1, -2, -3, -4), which differ solely in their N-terminal domain (109, 111). The RORα1 and -4 isoforms have also been isolated from mouse brain and muscle cDNA libraries (391, 395). A thymus-specific isoform of RORγ containing a truncated N-terminal domain has also been identified (396). The three ROR proteins are closely related to each other both in their DBD and LBD, although RORγ is evolutionarily more distant. The mouse RORα gene is ubiquitously expressed. However, higher levels of expression have been observed in the Purkinje cells of the cerebellum, retina, lens, spleen, skeletal muscle, and testis (110, 391, 395, 397–399). The RORβ gene is abundantly expressed in the retina, brain, pineal gland, and spleen (111, 400–403). The RORγ transcripts can be detected at high levels in skeletal muscle and thymus, but are also present at low levels in most tissues studied (392, 393, 396).

Structure/function analysis of the RORα isoforms show them to have unique DNA-binding properties (109, 113, 118). RORα isoforms bind DNA as monomers to HREs composed of a 6-bp A/T-rich sequence immediately preceding a half-site core motif AGGTCA. The ROR DBD is bipartite, and the two DBD subdomains bind to the same face of the DNA helix. By analogy with steroid hormone receptors (404), the first conserved first zinc finger module contacts the major groove of the AGGTCA element. The CTE interacts with the adjacent minor groove and makes specific contacts with the 5′-A/T-rich element of the RORE, making contacts likely to be analogous to those observed for Rev-Erbα binding to the DR-2 HRE as a homodimer (37). The close similarity in DNA binding properties between ROR and Rev-erb is exemplified by the observation that substitution of only four amino acid residues within the RORα DBD to those present in Rev-Erbα is sufficient to confer RORα with the ability to form homodimer complexes on a DR-2 element (405). This study clearly demonstrated that only a few changes are required for a receptor to acquire novel DNA binding characteristics such as conversion from monomeric to homodimeric DNA binding, thus providing a simple mechanism for receptor evolution. In a manner somewhat unique to the ROR family, the distinct RORα isoforms display different binding specificities despite sharing the same DBD. It has been shown that the distinct amino-terminal domains encoded in the human RORα1 and 2 isoforms cause slight structural changes that fine tune the interactions between the CTE and the 5′ A/T-rich sequences. The distinct contacts between the CTE and the amino-terminal domains result in the observed different binding specificities between each RORα isoform (118).

ROR binding sites have been found in the regulatory regions of numerous genes (109, 114, 211). However, direct transcriptional regulation by RORs has been demonstrated only for γF-crystallin (406), N-myc (384), laminin B1 (407), ApoA-1 (408), Purkinje cell protein 2 (PCP2) (409), and prosaposin (410). Given the broad physiological functions covered by potential ROR target genes, it is difficult to assign precise roles to these receptors on that basis. On the other hand, genetic studies have revealed that both RORα and -β play a role in the development of the central nervous system, and remarkably, both receptor genes are associated with previously described genetic lesions in mice. The RORα locus on chromosome 9 (411) is disrupted in *staggerer* mice (395, 412), whereas mutation of the RORβ locus by homologous recombination leads to *vacillans* phenotype in mice (413). *Staggerer* mice show tremor, body imbalance, and hypotonia as well as small size and die shortly after weaning (414). The cerebellar cortex of *staggerer* mice exhibits a cell-autonomous defect of the Purkinje cells, and failure of synaptic contact between Purkinje cell dendrites and granule cell parallel fibers leads to granule cell loss (415, 416). Although the *staggerer* mice express a truncated RORα protein, complete disruption of the RORα locus by gene targeting experiments mimics the cerebellar defects of *staggerer* (417, 418). Since the premature death of RORα mutant mice does not correlate with the well studied cerebellar defects, it is expected that a more complete investigation of the phenotypic abnormalities present in these mice will reveal additional roles for RORα in development and physiology. In this regard, *staggerer* mice bred in a C57BL/6 background and given special care (mashed food and maintained at 25 C) can be kept alive for up to 12 weeks. Under these conditions, *staggerer* mice fed a high-fat diet develop severe atherosclerosis and hypoalphalipoproteinemia, suggesting that RORα could regulate plasma HDL level and susceptibility to atherosclerosis (419). The phenotype associated with disruption of the RORβ locus in mice includes juvenile ataxia, duck-like gait, circadian activity deviations, retinal degeneration, and delayed onset of male fertility (413). This phenotype is reminiscent of the abnormalities observed in *vacillans*, a spontaneous mouse mutant first described more than 40 yr ago and now believed to be extinct (420). Taken together, these results suggest that at least two members of the ROR family (α and β) are important regulators of cell survival in the central nervous system, and identification of target genes will be crucial in furthering our understanding of ROR cellular functions. While the phenotype of the RORγ null mice has not been reported to date, cell-based studies suggest that RORγ may play a role in thymocyte development (396). The apparent role of RORγ in the thymus is to suppress the expression of the Fas ligand and interleukin-2 secretion in immature CD4+/CD8+ thymocytes during the process of negative and positive selection.

Finally, a major controversy has surrounded the claim that melatonin and a specific class of TZDs active in suppressing inflammation could act as ROR ligands (421–424). Unfortunately, experiments demonstrating that melatonin is a ROR ligand could not be replicated in a number of laboratories (211, 406, 425), and the current consensus among investigators in the field is that this claim should be ignored until further studies prove otherwise.

E. TR2: the testis receptors

The two members of the TR2 family were so named because of their high levels of expression in the testis (426–431). TR2s bind DNA as homodimers or heterodimers between the two subtypes with broad specificity to HREs composed of direct repeats of core half-sites separated from 1 to 5 bp (432–439). Transfected TR2s function as repressors of gene transcription on various promoters (434, 440–442), although these putative receptors possess an AF-2 domain and could act as activators in the presence of a ligand. Their roles in development and physiology remain to be elucidated.

F. TLX: forebrain development

Vertebrate TLX has been identified based on its relatedness to the *Drosophila* gene *tailless* (443). TLX displays a unique DNA-binding property: due to the substitution of a conserved lysine residue in the P-box of the DBD, TLX monomers or homodimers show a marked preference for the consensus half-site AAGTCA in which the conserved guanine at position 2 is replaced by an adenine (443). TLX is predominantly expressed in the developing forebrain (443, 444), and disruption of *tlx* by gene targeting in mice results in impaired development of a subset of forebrain-derived structures, including the olfactory, infrarhinal and entorhinal cortex, amygdala, and dante gyrus (445). Both male and female animals displayed abnormal aggressive behavior, and female mice failed to nurse their offspring. As observed for the RORs, it is likely that TLX is required for the proliferation and/or survival of specific neuronal cells.

G. COUP-TF: neurogenesis, angiogenesis, and heart development

Members of the COUP-TF (chicken ovalbumin upstream promoter transcription factor) family are, with the exception of the liganded PPARs, the most extensively studied orphan nuclear receptors. Since the COUP-TF family has been the subject of a recent review in this journal (446), only salient and novel features will be described here. COUP-TFα owed its name to the fact that it was initially identified as a transcription factor required for expression of the chicken ovalbumin gene (447). COUP-TFβ was simultaneously cloned by homology screening (448) and characterized as a factor regulating expression of the ApoA-I gene (449) while the more distant COUP-TFγ was identified by low-stringency screening of cDNA libraries (450). During murine development, COUP-TFs are preferentially expressed in the central nervous system (451–455) and in mesenchyme, particularly in organs whose development depends on interactions between the mesenchyme and other epithelial layers (451, 452). In adult animals, COUP-TFs are widely expressed but at reduced levels (449, 450, 456, 457). Expression of COUP-TFα has also been reported in specific types of adrenal tumors (458, 459).

COUP-TFs exist in solution as homodimers and bind DNA to a wide variety of HREs composed of direct, inverted, and everted repeats of the AGGTCA core motif (447, 460, 461). COUP-TFs can also form heterodimeric complexes with RXR on DNA (460, 462, 463) and NGFI-Bα in solution (464). COUP-TFs are potent transcriptional repressors that antagonize transcriptional activation mediated by nuclear receptors PPAR (465), HNF-4 (301), RXR (462), and ER (466, 467) as well as RAR, VDR, and T_3R (468). Proposed mechanisms for COUP-TF-mediated repression include both passive and active mechanisms: competition for binding sites, competition for RXR and formation of inactive receptor-receptor complexes, and active repression mediated by amino- and carboxyl-terminal repression domains and interactions with corepressors (see Ref. 446 for references and Refs. 469 and 470). Given the wide range of HREs recognized by COUP-TFs *in vitro*, it is not surprising that these receptors have been identified as potential regulators of the expression of a large number of genes (298, 300, 302, 303, 305, 309, 310, 314–316, 318, 357, 449, 452, 462, 463, 465, 466, 471–498). However, a direct role for COUP-TFs in the regulation of these potential target genes has not yet been demonstrated *in vivo*.

Expression of COUP-TFs is regulated by two important morphogenetic signals involved in neuronal development, atRA and sonic hedgehog (451, 499, 500), giving additional support to the hypothesis that COUP-TFs play essential functions in neurogenesis. Indeed, gene targeting experiments have recently demonstrated that COUP-TFα plays a crucial role in the development of the peripheral nervous system (501). COUP-TFα null mice have difficulties in suckling and swallowing and die shortly after birth apparently from starvation and dehydration. This phenotype appears to result, in large part, from a defective morphogenesis of the glossopharyngeal ganglion and nerve, which innervate the pharynx and the root of the tongue. On the other hand, COUP-TFβ null mutants die *in utero* around 10 d.p.c. due to defects in angiogenesis, vascular remodeling, and heart development (502). Since embryonic expression of COUP-TFβ localizes in the mesoderm, these results suggest that COUP-TFβ may be required to sustain the necessary cross-talk between endothelial cells and the surrounding mesenchymal cells for correct development of the vascular system and heart.

H. ERR: placenta development and control of lipid metabolism

The ERR family contains three closely related members. ERRα and -β were the first orphan nuclear receptors identified during a search for genes related to the estrogen receptors (2). ERRα was subsequently identified as a mammalian protein that bound to the SV40 major late promoter and repressed its activity, implicating ERRα in regulation of the early-to-late switch of SV40 gene expression (503). The third member of the family, ERRγ, was recently isolated during a search for the gene responsible for Usher syndrome located on chromosome 1q41 (504). However, analysis of the locus has shown that *ESRRG* is not the *USH2a* gene (505).

ERRα is widely expressed during murine development (116, 506, 507). Expression of embryonic ERRα is first detected at 8.5 d.p.c. in the trophoblast, mesoderm cells of the visceral yolk sac, the primitive heart, and the neural tube. ERRα expression is detected later in development in the brain and spinal cord, pituitary gland, heart, intestinal mucosa, and bone, as well as the premuscular mass of the

limb bud and brown adipose tissue. During late fetal development and early postnatal life, ERRα is most prominently expressed in organs demonstrating a high capacity for fatty acid β-oxidation or activation, suggesting that both ERR isoforms may play a role in regulating energy metabolism (116). ERRβ expression is more restricted during development (508, 509). ERRβ transcripts are first detected in a subset of cells in extraembryonic ectoderm at 5.5-d.p.c. that appear to be a precursor of the chorion, where ERRβ is specifically expressed at 7.5 d.p.c. As fusion of the chorion and ectoplacental cone progresses at 8.5 d.p.c., ERRβ expression is extinguished in all but the free margin of the chorion, while ERRα becomes up-regulated in the remaining trophoblast cells (508). Human ERRγ transcripts were detected at very high levels in fetal brain, and at lower levels in kidney, lung, and liver (504). In adults, the ERRγ transcript is widely expressed and can be observed in brain, lung, bone marrow, adrenal and thyroid glands, trachea, and spinal cord.

ERRs bind as monomers to the extended half-site TNAAGGTCA (115, 116, 510), which is also recognized by FTZ-F1 (see above), and as homodimers to the consensus estrogen-responsive element (509). Therefore, ERR targets potentially include all genes regulated by either FTZ-F1 or by the estrogen receptors. ERRα has been shown to regulate activity of the lactoferrin (511), MCAD (medium-chain acyl CoA dehydrogenase) (116, 512), osteopontin (506), and TRα (507) promoters in cotransfection assays. ERRα generally represses gene transcription in these assays (116), as well as in cell-free systems (115, 503), and has also been shown to antagonize the action of GR via an unknown mechanism (513). The lack of ERR transcriptional activity observed in most transfection experiments may be due to the absence of its cognate ligand. However, it has also been reported that ERRα can display significant constitutive activity under certain conditions (510), and that activity is dependent on a serum compound that is withdrawn by charcoal treatment. This latter observation suggests that a ERR ligand could be present in certain serum preparations but not in others. If this assumption is correct, the active serum could be potentially used to extract and identify the ERR ligand. Although ERRs display significant homology to the estrogen receptors, they do not bind estrogen and its derivatives *in vitro* or respond to them in cotransfection assays (2, 511). However, the crystal structure of the liganded ERα (49) indicates that most amino acid residues shown to be critical for recognition of estradiol are conserved between members of the ER and ERR families, suggesting that ER and ERR ligands should be structurally related.

Examination of ERRβ expression during embryogenesis defined for the first time a subset of extraembryonic ectoderm that subsequently forms the dome of the chorion, suggesting that ERRβ may play a role in early placental development. ERRβ null embryos generated by targeted disruption of the *Estrrb* gene have severely impaired placental formation and die from an apparent lack of nutrients by 10.5 d.p.c. (508). The ERRβ knockout embryos display abnormal chorion development associated with an overabundance of trophoblast giant cells and a severe deficiency of diploid trophoblast. The phenotype can be rescued by aggregation of *Estrrb* mutant embryos with tetraploid wild-type cells that contribute exclusively to extraembryonic tissues. Since the ERRβ phenotype occurs in tissues that do not express the putative receptor during development, the observed phenotype suggests that an inductive signal originating from or modified by the chorion is required for normal trophoblast proliferation and differentiation (508).

The observations that ERRα is expressed in tissues which preferentially metabolize fatty acids and that ERRα can control the expression of MCAD *in vitro* suggest that ERRα may play an important role in regulating cellular energy balance *in vivo* (116, 512). Preliminary phenotypic analysis of ERRα null mice revealed intrauterine growth deficiency and abnormal adult body composition, but otherwise the ERRα null mice develop normally and appear to have normal reproductive function. These mutant mice will therefore provide a model for identifying possible physiological processes regulated by ERRα as well as potential ERRα target genes.

I. NGFI-B: hypothalamus-pituitary axis (HPA), T cells, and dopaminergic neurons

NGFI-B was initially identified as a factor whose expression was up-regulated in NGF-stimulated PC12 pheochromocytoma cells (514). The NGFI-B family contains three members known under a wide variety of names (see Table 1 for references). NGFI-B and its related family members are highly expressed in the adult nervous system where they are induced as part of the immediate early response to stimuli such as growth factors, membrane depolarization, and seizures (515–521). Their pattern of expression outside the nervous system is broad. In adult rodents, NGFI-Bα is expressed in the adrenal, thyroid, and pituitary glands, as well as the liver, testis, ovary, thymus, muscle, lung, and ventral prostate (514, 516, 522–524). NGFI-Bα expression is up-regulated in T cells undergoing apoptosis (525, 526). NGFI-Bβ is expressed in the adult liver (527) as well as the pituitary gland, thymus, and osteoblasts (524, 528). NGFI-Bγ is expressed at high levels in the pituitary gland and at intermediate or low levels in the adrenal glands, heart, skeletal muscle, thymus, kidney, epididymis, and submandibular glands (529–531). Renal expression of all three members of the family is up-regulated during early stages of antigen-induced glomerulonephritis (532), while hepatic NGFI-Bα and -β expression increases in liver as it regenerates after partial hepatectomy (527).

NGFI-B family members have been shown to bind DNA as monomers, as homodimers, or as heterodimers with RXR. NGFI-Bα binds to monomeric response elements (NBREs) containing the 5′-extended core motif (AAAGGTCA) (106). As discussed above, NGFI-B site specificity is determined by DNA-protein contacts between nucleotides located 5′ to the core motif contained in the NBRE and the CTE (108, 113, 117). Homodimer binding by NGFI-Bα was also observed on the POMC gene promoter (533). The homodimer binding site consists of two inverted NBREs spaced by 6 bp that confer high responsiveness to NGFI-Bα. No ligand has yet been identified for members of the NGFI-B family. However, NGFI-Bα and -β (but not -γ) can bind to DR5 response ele-

ments as heterodimers with RXR and, on these elements, the heterodimer complex is efficiently induced by rexinoids (521, 534). Rexinoids have also been reported to induce transcription of NGFI-Bβ-RXR heterodimers when synthetic reporters containing multiple copies of the monomeric NBRE are used in cotransfection assays (146). In this case, activation by the heterodimer complex occurs in the absence of direct DNA binding by the RXR moiety. The demonstration that rexinoids can activate NGFI-B:RXR heterodimers suggests that rexinoids could enhance the response to growth factors initiated by the rapid induction of expression of these orphan receptors (146, 534). In addition, the activity of NGFI-B family members appears to be regulated by posttranslational modification, which could possibly be induced via ligand-independent pathways triggered by growth factors. NGFI-Bα nuclear localization, DNA-binding affinity, and transcriptional activity can be modulated by phosphorylation of the receptor protein (535–539). As exemplified by the PPAR and LXR families, regulation of the NGFI-B heterodimeric complexes activity by rexinoids and covalent modifications does not exclude the existence of NGFI-B-specific ligands.

In vitro and *in vivo* studies have suggested that NGFI-Bα plays important signaling functions in the HPA and in T cells. NGFI-Bα expression in the paraventricular nucleus and adrenal cortex is induced by stress, and ACTH treatment strongly up-regulates NGFI-Bα and -β expression in the adrenal gland and in Y-1 adrenocortical carcinoma cells (540, 541). In addition, NGFI-Bα was shown to regulate the steroid 21-hydroxylase (CYP21) and steroid 17-hydroxylase (CYP17) gene promoters (493, 540). More recently, the positive action of CRH on the POMC promoter was shown to be modulated via NGFI-Bα and β (533, 542), with the feedback repression of the HPA by glucocorticoids at the level of the pituitary mediated by direct nonproductive GR-NGFI-B interactions (543). However, a role for NGFI-Bα in regulating the functions of the HPA has not yet been demonstrated *in vivo*. NGFI-Bα null mice have no discernible phenotype, display no evidence of adrenal or gonadal dysfunction, and show normal basal and stimulated CYP21 expression levels (544). Since more than one member of the NGFI-B family appear to be involved in regulating the HPA, redundant functions between family members may explain the lack of phenotype in knockout mice. A similar redundant NGFI-B-based mechanism may also function in T cells (545, 546). While it has been shown that the expression of NGFI-Bα is induced in T cell hybridomas or in thymocytes undergoing apoptosis and that blocking its activity by antisense and dominant-negative constructs prevents T cell receptor-mediated apoptosis in T cell hybridomas (525, 526, 547, 548), thymic and peripheral T cell death is unimpaired in NGFI-Bα null mutant mice (549). Unfortunately, mice lacking NGFI-Bβ died soon after birth and do not provide a model in which to study HPA and T cell functions in adult animals. However, NGFI-Bβ mutant mice fail to generate midbrain neurons with a dopaminergic phenotype, and midbrain dopamine precursor cells degenerate as brain development progresses (550, 551). Since loss of midbrain dopaminergic neurons is associated with the etiology of Parkinson's disease, the use of putative NGFI-B ligands or specific rexinoids could provide a novel therapeutic avenue for the treatment of this disease.

J. GCNF: one of a kind

GCNF (germ cell nuclear factor) was originally cloned by low-stringency screening (552). GCNF is not closely related to any other nuclear receptor and is therefore isolated in its own family of one. Mouse and human GCNFs are expressed a very high levels in developing germ cells in male and growing oocytes in female animals (553–555). GCNF preferentially binds DNA as a homodimer to DR0 elements and, in the absence of a putative ligand, repress transcription (556, 557). The potential physiological role played by GCNF in gametogenesis is at present unknown.

K. DAX-1: adrenal development and sex determination

The *DAX-1* (dosage-sensitive sex reversal, AHC critical region on the X chromosome, gene 1) gene was identified through a search for gene(s) linked to AHC, a disease affecting the normal development of the adrenal cortex and often associated with hypogonadotropic hypogonadism (367, 368). Analysis of *DAX-1* showed it to encode an atypical orphan nuclear receptor possessing only the conserved LBD but not the nuclear receptor-like DBD. However, DAX-1 appears to bind DNA via an alternative mechanism: it recognizes DNA hairpin structures (104). It is not clear whether the DAX-1 DNA binding function plays a role in the etiology of AHC since all types of mutations in DAX-1 resulting in AHC localize to the LBD (558).

Consistent with its role in controlling the development of the HPA and in sex determination, DAX-1 is expressed in the hypothalamus, pituitary, adrenal gland, and gonads (559–561). Interestingly, this pattern of expression overlaps with that of FTZ-F1α, an observation that led to the suggestion that both receptors may cooperate in the development of the HPA. In addition, the phenotype of mice lacking FTZ-F1α closely resembles AHC in humans, and a functional binding site for FTZ-F1α was found in the DAX-1 promoter (356, 357). However, while DAX-1 was shown to physically interact with FTZ-F1α, DAX-1 paradoxically inhibits FTZ-F1α-mediated transactivation (562) via recruitment of the nuclear receptor corepressor N-CoR to the DAX-1-FTZ-F1α complex (563). The paradoxical antagonistic activity of DAX-I and FTZ-F1α observed *ex vivo* may simply result from the absence of ligands, which when present *in vivo* at the appropriate time during development could easily transform the repressive complex into a transcriptionally active one. DAX-1 was also shown to antagonize the action of the sex-determining gene *Sry* in a transgenic mouse model (564), although the underlying molecular mechanism remains to be elucidated. Taken together, these data suggest that DAX-1, FTZ-F1α, and SRY may act in overlapping functional complexes to regulate sex determination and gonadal development (reviewed in Ref. 565).

L. SHP: a promiscuous and inhibitory heterodimeric partner

SHP (small heterodimeric partner) was originally cloned in a two-hybrid screen set up to identify potential partners for the orphan receptor CAR (566). As previously observed for DAX-1, SHP lacks a typical nuclear receptor DBD. SHP

heterodimerizes with a wide variety of nuclear receptors including RXR, RAR, TR, PPAR, HNF4, and ER (566, 567), often in a ligand-dependent manner. The interactions between SHP and its partners result in an inhibition of the transcriptional activity of these receptors via two distinct mechanisms: SHP inhibits the DNA binding activity of its partners and directly represses gene transcription via its inherent repressor function located near the amino terminus of the LBD (566, 568). Because SHP preferentially interacts with liganded receptors, it has been suggested that the role of SHP may be to dampen expression of hormone-induced genes (568). However, the SHP LBD contains an AF-2 domain, indicating that SHP has the potential to become an activator under a physiologically relevant context.

VI. Concluding Remarks

During the course of the last decade, the identification and functional characterization of close to 40 vertebrate orphan nuclear receptors have led to the realization that nuclear receptor actions are not limited to basic endocrine systems but probably play a role in the development, maintenance, and physiological functions of all organs. Most importantly, the study of orphan nuclear receptors has led to the discovery of novel hormone response systems. The functional repertoire of orphan nuclear receptor ligands appears to be wide, ranging from morphogens to intracellular regulators of basic metabolism, which may explain why their existence has not previously been uncovered by classical physiological experimentation.

It is clear that despite the vast amount of knowledge accumulated so far in this field of research, much more remains to be elucidated. In particular, most identified ligands and activators can still be regarded as promising leads rather than actual physiological ligands. Likewise, many hypotheses on the putative physiological role of orphan receptors currently based on activation studies in cell culture and other *in vitro* experiments may not stand the test of more rigorous *in vivo* investigations. Nonetheless, these initial studies have demonstrated how important orphan nuclear receptor functions are in development and homeostatic control.

Finally, the most important outcome of these studies is the discovery that orphan nuclear receptors and their ligands have direct links with known diseases such as diabetes, atherosclerosis, and cancer. These findings have opened many new therapeutic avenues for the management of these diseases and demonstrated that orphan nuclear receptors constitute excellent targets for drug development.

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