MINIREVIEW

The Steroid Receptor Superfamily: More Excitement Predicted for the Future

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The overall molecular pathway for the action of steroid hormones has been known for the past 15 yr (1–9). Within the past 5 yr, the cDNAs for all major steroid hormones have been cloned and sequenced (10, 11). As might have been expected, they were related in structure and led to the definition of an even larger family of putative regulatory proteins. This superfamily of regulatory molecules was shown to include also the receptors for thyroid hormone (T_3) vitamin D_3 and retinoic acid. Perhaps more surprising was the inclusion of oncogenes such as v-erb A in this family. The latter is one of the two genes which comprise an oncogenic cassette in the avian myeloblastosis virus and appears to be a mutated form of a cellular gene coding for one of the multiple receptors for thyroid hormone.

A summary of selected members of the steroid receptor superfamily is shown in Fig. 1. The schematic presentation is based on primary amino acid sequence and reveals three major regions of conserved amino acids (I–III) set against the glucocorticoid receptor as a reference. Regions II and III are located within the C-terminal or hormone binding domain of the molecule. Regions II and III are considered to be functionally important due to their conservation but it is unclear whether they participate directly in ligand binding, protein-protein structural interactions, or transcriptional activation. It is likely that the receptor makes multiple contacts with its activating ligand in this domain. Mutational analyses of this region have revealed that practically the entire C-terminal domain must remain intact for high affinity ligand binding.

Region I, a highly conserved 66 amino acid sequence, is located within the interior of the molecule and comprises the DNA-binding domain. Of special note are nine conserved cysteines, eight of which are thought to form two zinc fingers, each containing one molecule of zinc (10–12). A schematic representation of this region is shown in Fig. 2. The zinc finger structure was observed initially in the amphibian transcription factor TF IIIA, but was of a different sub-type, employing two cysteines and two histidines to coordinately bind one zinc ion (type I, Fig. 2). The steroid receptor superfamily can be distinguished from other transcription factors by its subtype (type II, Fig. 2). This region of the molecule contains the amino acids which serve to recognize specifically the cognate steroid response elements (SREs) located in genomic DNA adjacent to target genes. This concept was substantiated by domain swapping experiments which revealed the conversion of a glucocorticoid receptor into an activator of genes containing an estrogen response element (ERE) when its zinc finger domain was replaced with the identical region of the estrogen receptor (12). It has been suggested that the first finger contains primary information for sequence specificity of binding while the second finger stabilizes binding of receptor to its DNA response element. Mutational analyses have implicated amino acids at the bases and at the tips of both fingers as important for the function of specific gene activation.

In a series of recent experiments, we have learned more about the role of specific amino acids in the DNA binding domains of receptors (13). The first eight, of nine, cysteines are required for function in this region since mutations abolish all activity. More recently, a series of elegant and more subtle mutational studies have allowed a greater definition of the region of the cysteine-rich domain that appears to be in direct contact with the DNA nucleotides (13–16). Changing the Glu-Gly sequence between the second pair of residues in the first zinc finger of the estrogen receptor to the Gly-Ser present in the identical region of the glucocorticoid receptor creates a receptor that no longer activates an ERE receptor but has some stimulatory activity on a GRE reporter. Conversely changing the Gly-Ser of the glucocorticoid receptor to a Glu-Gly as found in the estrogen receptor creates a hybrid protein which now activates predominantly ERE-containing reporter genes. Evidence implicates the amino acids immediately following the second pair of cysteines in the first zinc finger as playing some additional role in sequence specific binding.

Artificially generated or naturally occurring mutations in the second finger give rise to inactive receptors which bind poorly to DNA (13). The precise role of the second zinc-finger is less evident but is thought to stabilize the binding to DNA via interactions with the phosphate backbone of the SRE or perhaps more likely by protein-

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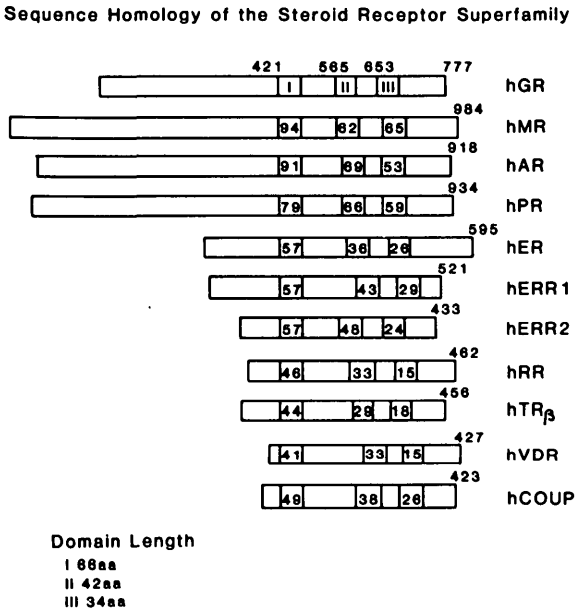

Fig. 1. Sequence Homology of the Steroid Receptor Superfamily

Linear representations are shown (*top to bottom*) for human receptors for glucocorticoid (hGR), mineralocorticoid (hMR), androgen (hAR), progesterone (hPR), estrogen (hER), estrogen-related receptors (hERR1, hERR2), retinoic acid (hRR), thyroid hormone (hTRB), vitamin D (hVDR) and the orphan receptor (COUP, chicken ovalbumin upstream promoter). Conserved region I (DNA-binding site) and regions II and III (hormone binding region) are shown for all members, using hGR as a reference point.

protein interactions which stabilize dimer formation at the SRE. Mutations in the zinc finger tips can inhibit receptor function also. In fact, two unrelated families whose children displayed vitamin D_3 resistant rickets were shown to each harbor a single amino acid substitution in this region of the receptor for vitamin D_3 (17). The mutation in one family was at the tip of the first zinc finger and the mutation in the other family was at the tip of the second finger. It is clear that the three dimensional structure of these proteins must be determined if we are to understand the mechanism by which receptors interact with their cognate DNA response elements.

Deletion mutants of the rabbit progesterone receptor were used to identify two major mechanisms of its nuclear localization. A putative signal sequence, homologous to that of the SV40 large T antigen, was localized around amino acids 638-642. When amino acids 638-642 were deleted, the receptor became cytoplasmic but could be shifted into the nucleus by the addition of hormone (or anti-hormone). In the absence of ligand, the receptor was transferred into the nucleus as a monomer. After administration of hormone (or anti-hormone) a cytoplasmic monomer was transferred into the nucleus through interaction with a nuclear monomer. These interactions were thought to occur through the steroid binding domains of both monomers. Additional, recent information has been published on a domain of the receptor thought to be involved in nuclear translocation (50). Crystallization and diffractional analysis should generate a great deal of needed information on the conformation of this region in the absence and presence of DNA.

The N-terminal region of the steroid receptors is poorly conserved in both length and amino acid sequence. It is considered to be a transcriptional modulation domain. It contains information which enhances transcriptional stimulatory potency and also contains sequences which allow preferential activation of certain genes (18, 19). These functions are thought not to be transmitted via conserved runs of specific amino acids but rather by aggregate charge and higher order structure. Deletion of this domain reduces gene activation potential in glucocorticoid and progesterone receptors by 50–85% after reintroduction to animal cells. It is clear, however, that sequences in the carboxy terminal ligand-binding domain participate also in transcriptional regulation by most receptors.

The existence of functional domains in steroid receptors was deduced first by biochemical analyses of receptors; protease degradation led to elucidation of domains for antibodies, DNA, and ligands (50). These domains correspond to the N-terminal, zinc-finger (interior), and C-terminal domains of receptor molecules and have been verified by mutational analyses of the receptor cDNAs. They correspond also to the three main functions that were ascribed previously to receptors: 1) modulation of target gene transcription; 2) specific interaction with target gene regulatory sequences in DNA; and 3) specific and high affinity ligand binding.

The precise mechanism by which steroid hormone receptors regulate gene expression is unknown but the four main reactions appear to be: 1) ligand induced allosteroic activation of the receptor, 2) specific binding to SREs, 3) stable complex formation at these DNA enhancer sites, and 4) recruitment of transcription factors and RNA polymerase to initiate transcription of target genes.

It has been proposed that certain of the unoccupied receptors may exist in association with cellular heat shock proteins (*e.g.* hsp90) in a complex which is unable to bind DNA (20–23). This is most notable for glucocorticoid, progesterone, and estrogen receptors, but appears not to be the case for vitamin D and thyroid hormone receptors. The prevalent scenario suggests that ligand binding causes dissociation of attendant heat shock proteins so that DNA interactions can now occur. It appears that dissociation of heat shock proteins, however, is not a simple activation switch since certain steroid analogs which have little or no effect on transcription will nonetheless cause dissociation of heat shock proteins and allow receptors to bind to DNA. Nevertheless, the evidence is strong that hsp90 and other heat shock proteins must be stripped from receptor before they can bind DNA *in vitro*.

Target genes for steroid receptors possess short (~15 base pairs) *cis*-elements located usually within

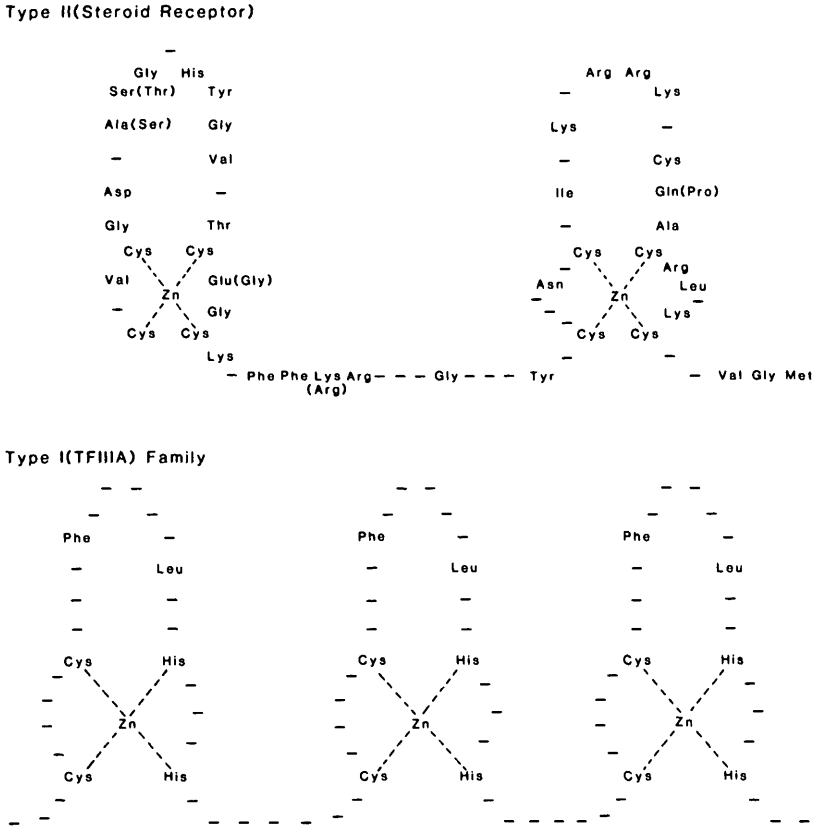

Fig. 2. Theoretical Structures for Type II Zinc Fingers (Steroid Receptor Superfamily) and Type I Zinc Fingers (TFIIIA Related Transcription Factors)

Conserved amino acids are named and possible cysteine bonding to zinc atoms are designated.

their 5′-flanking regions which confer hormonal regulation upon receptor binding (24–27). Single base changes within these SREs can alter receptor binding and destroy hormone response (27, 28). Surprisingly, as little as two base changes in an SRE can convert a glucocorticoid responsive gene to an estrogen responsive gene by weakening the binding of glucocorticoid receptor while enhancing the affinity of estrogen receptor for the element.

Although there are certain conserved bases among all SREs, the consensus sequences of the elements vary for different receptors. Surprisingly, the glucocorticoid and progesterone receptors usually bind to the identical DNA element (GRE/PRE). Since the physiology of glucocorticoid and progestin actions is often quite different, it appears that the specific cellular effects of these two hormones are determined by differential expression of their respective receptors. This conclusion has been substantiated by introduction of progesterone receptor (via an expression vector) into rat hepatoma cells which contain only glucocorticoid receptor and observing that the glucocorticoid-responsive genes are now inducible by progesterone (56).

Presently, the role of ligand in receptor-DNA interactions at the SRE is unclear. Ligand has been reported to induce small changes in binding kinetics and affinity of purified receptor for SREs (29, 30). None of these reported effects appear great enough to explain the absolute dependence on hormone for gene activation observed in the intact cell. Steroid receptors have the inherent capacity to form dimers in solution (31, 32). Receptors bind to their respective SREs as dimers (33, 34). The SRE is composed of two half-sites, having a dyad axis of symmetry (inverted repeat sequences); each half-site binds one monomer of receptor. Only the dimeric form of receptor binds with an affinity ($K_d \sim 10^{-9}$ M) sufficient to influence transcription. Once bound to an SRE, the receptor dimer can couple with another dimer (or other transcription factor) at an adjacent SRE to create a more stable complex with much higher affinity (K_D approximately 10^{-11} M) (35). It is thought that such stable complexes have a sufficient residence time at the gene to influence transcription significantly. These *in vitro* studies of receptor-DNA interactions correlate well with cellular data which reveal synergistic effects on transcription when two or more regulatory elements are placed in the 5′-flanking region of target genes (36–38).

Cooperation between different proteins at the DNA level has been reported. Such heterodimeric interactions at SREs may be of two types. It has been reported that certain nuclear factors, presumably not receptors,

are required for stable binding of thyroid receptor to its TRE (57). These factors have been partially purified and display a level of tissue specificity. Alternatively, evidence has been reported to support the hypothesis that the human thyroid receptor forms a heterodimer with the retinoic acid receptor at its DNA response element. This heterodimer exhibits interesting transcriptional properties in that coexpression of both receptors in CV-1 cells results in a positive transcriptional effect on promoters containing a palindromic TRE but causes a negative effect on the natural TRE derived from the α-myosin heavy chain gene (58). These results suggest that by forming heterodimers, a greater range of control of target gene expression may be achieved.

Receptor binding to an SRE sequence may have additional combinatorial effects. For instance it could lead to a diminished rate of transcription if the SRE overlapped with the binding site for some other positive regulator of transcription. In this way, receptors have been postulated to exclude a strong positive regulator from binding and promoting transcription of certain genes (39, 40). It is possible also that amino acid domains of receptors could bind and stabilize negative regulatory factors at select target genes. Alternatively, a given gene could be silenced by a strong negative regulator bound to a silencer sequence in its 5′-flanking DNA. Formation of a steroid receptor complex at a nearby SRE could either convert the negative regulator to a positive hybrid complex or exclude the negative regulator from its DNA binding site. Albeit by an indirect mechanism, both types of interactions would lead to induction of transcription at the adjacent gene. Since the control of eucaryotic gene transcription appears to be the result of a multitude of combinations of a finite number of transcription factors, it may be safe to say that any imaginable alternative is likely to be found to exist at some gene locus.

A prime question relative to receptor activation of gene transcription is again the role of the ligand. Mutational studies reveal that most receptors have two regions which participate in transcriptional activation. One is located usually in the N-terminal domain and the other in the C-terminal domain (between regions I and II, Fig. 1) of the receptor. It appears likely that ligand binding induces an allosteric conformation that allows access to these transcription activation regions of receptor to other transcription factors and/or RNA polymerase. These higher order protein-protein interactions are thought to signal the initiation of transcription. Inactive analogs of estrogen such as tamoxifen appear unable to induce the proper allosteric folding of receptor to promote regulation of transcription. The complexities of these reactions make it unlikely that definitive structural information will be forthcoming until receptors are crystallized and studied by x-ray diffraction.

Using crude extracts of amphibian target cell nuclei, addition of estrogen stimulates vitellogenin gene transcription (51). This stimulation has been shown to be hormone-specific and occurs presumably via the estrogen receptor present in the extracts. Recently, selective stimulation of target gene transcription by purified steroid receptor preparations or derivatives has been accomplished *in vitro*. The initial report showed that fragments of the glucocorticoid receptor (region I, Fig. 1) were able to stimulate cell-free transcription of a reporter gene containing multiple GRE elements (41). This result confirmed the conclusion that the DNA binding domain and its surrounding peptides constitute an active site for transcriptional regulation.

In our laboratory, we have carried out reconstitution assays also by using HeLa factor supplements, purified preparations of the native A or B forms of the chicken progesterone receptor and a reporter gene containing an ovalbumin TATA region and PRE elements. Transcriptional stimulation is dependent absolutely on the presence of PRE sequences since deletion or mutation of the PRE prevents the response. Both A and B forms of the progesterone receptor stimulate transcription greater than 25-fold, while internal control genes of adenovirus are unaffected by receptor. Purified bacterial fragments of receptor (region I) are able to stimulate transcription of the target genes also, but only at concentrations 40- to 80-fold higher than the intact (full-length) receptor (41, 42).

Using a series of preincubation and template competition analyses combined with kinetic analyses, we were able to dissect the mechanism of action of cPR in our *in vitro* transcription system. The details of these experiments are reported elsewhere (42) and will be summarized in concept only. In short, the steroid receptor enhances the formation of a rapid start complex by RNA polymerase (52). It appears to do this by enhancing the assembly of a template committed complex of transcription factors (Fig. 3). This stable complex is now

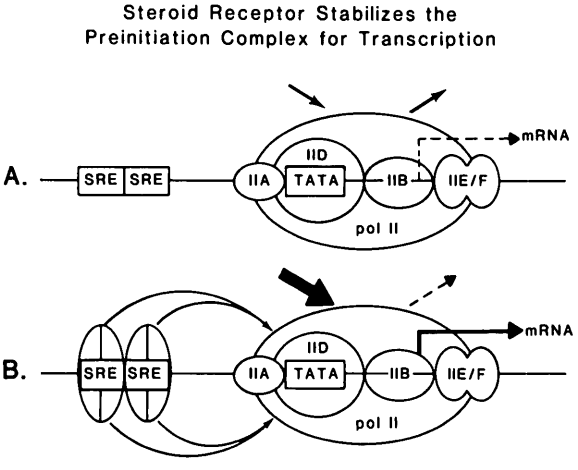

Fig. 3. The Effect of a Steroid Receptor on the Preinitiation Complex Formed at the Proximal Promoter (TATA box)

A reversible association of IID, IIA, IIB, pol II (polymerase) and IIE/F is shown schematically. When all factors are present pol II can initiate RNA synthesis. B, When upstream SREs are occupied by receptor dimers, the resultant tight binding tetramer of receptor exerts a stabilizing effect on proximal promoter factors to generate a committed complex which allows poly II to effect rapid start RNA synthesis. This stabilization by receptor promotes repeated initiations of transcription.

poised for rapid initiation of transcription by RNA polymerase.

Multiple factors must interact at the proximal promoter (TATA box) of a gene to allow initiation of transcription (53). This sequence of events is thought to be dependent on the initial reversible interaction of the TF-IID protein with the TATA-sequence itself. This interaction is subsequently stabilized by sequential binding of TF-IIA, TF-IIB, RNA polymerase and TF-IIE/F but the precise roles of these factors in the initiation complex are only partially known (54). Nevertheless, together they form a three dimensional surface conformation which attracts RNA polymerase to the gene and transcription begins.

In this cell-free system, cPR acts to enhance formation of a stable template committed complex of these transcription factors, thereby promoting formation of the rapid-start complex and stimulating transcriptional initiation. Receptor may do this by facilitating recognition of the promoter by such factors or simply by stabilizing the promoter DNA-protein complex once it is formed. In either case, it is likely that the effect is modulated via local protein-protein interactions between the ligand-activated receptor and the promoter complex. Additional questions to be asked in this context relate to the precise contacts between the receptor amino acids and the surface of individual promoter factors. Also, we must understand whether there is a continuing role for bound ligand in this process. Finally, the continued development of cell-free transcription systems to test function of steroid hormone receptors should permit a precise definition of the transcription activation domains of receptors, the role of hormone in receptor activation, and other interactions of receptor with enhancer and silencer binding proteins in eucaryotic cells.

To my way of thinking, the most fascinating observation to evolve from the cloning of cDNAs for steroid receptors is the unexpected large size of the steroid receptor superfamily of related genes. After elucidation of the receptors for the more traditional members of this family (glucomineralocorticoids, sex steroids, thyroid hormone, vitamin D_3, and retinoic acid), a large number of cloned receptoroids have been discovered. These molecules can be considered to be orphan receptors in search of a function and a ligand. Since they were cloned by cDNA cross-hybridization screening using cDNA probes, we have little clue as to their cellular physiology. A function is implicit, however, since they are expressed in cells as full-processed cytoplasmic mRNAs. The first report of two such molecules was by the Evans laboratory; they were termed ERR-I (estrogen-receptor related) and ERR-2 (43). Their function remains unknown to date. A number of orphan receptor sequences have been published to date. A partial list of those derived from animal cells is included in the schematic drawing shown in Fig. 4. They can be recognized easily by their homology in the DNA binding region and their conservation of type II zinc fingers. I estimate that more than 15 additional related molecules

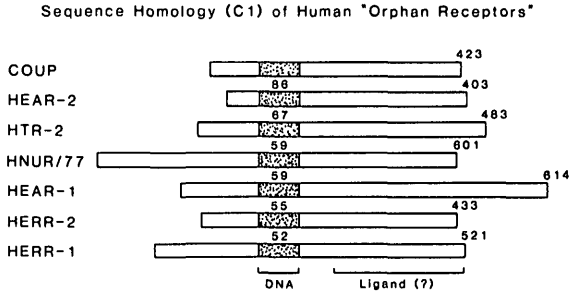

Fig. 4. Sequence Homology and Linear Representations for Selected Human Orphan Receptors

Designations and references are listed elsewhere (10). Homologies in C1 region (DNA-binding) are set against COUP as a reference standard.

may have been cloned in the combined laboratories in our field. Five of these molecules have been cloned from Drosophila (55). Until recently, all of these putative receptors were without designated functions.

A recent discovery in our laboratory has stimulated my conceptual appreciation of these orphan receptors. For the last 2 yr, we have been involved in the purification and cloning of a transcription factor called COUP-TF (chicken ovalbumin upstream promoter-transcription factor). As suggested by the name, this is a high affinity and specific DNA binding protein, which interacts as a dimer with the distal promoter sequence of the ovalbumin gene and promotes initiation of transcription of this gene by RNA polymerase (44). Transcriptional stimulation by COUP-TF requires participation of a second peptide factor termed S300-II, also purified in our laboratory, which interacts with COUP-TF in a protein-protein complex (45). COUP-TF is thought to activate a number of other genes, including the mammalian insulin gene (46), the apolipoprotein gene (VLDL) (47), and the gene coding for proopiomelanocortin (Drouhin, J, personal communication).

When COUP-TF was cloned and its amino acid sequence was derived, we were surprised to find that it was an authentic member of the steroid receptor superfamily (48). It contained a DNA binding domain (region I) comprised of two zinc fingers (type II) (Fig. 1). The amino acid sequence in this region showed identity in 20 of 20 diagnostic amino acids and 11 of 12 conserved amino acids derived as a consensus for the steroid receptor superfamily. Furthermore, significant additional homologies to other members of the family were observed in the ligand-binding (C-terminal) domain. In fact, the sequence of COUP-TF was published recently as one of a group of v-erb A related (receptor related) cDNA clones obtained by cross screening with v-erb A cDNA (49). As might be expected, no function had been assigned and it was destined to sit as a member of the ever-expanding list of receptor related orphans, until it was identified as COUP-TF.

The assignment of COUP-TF to the steroid receptor superfamily of proteins has a number of apparent implications. For the first time, it designates promoter

regulatory proteins as a legitimate subtype in this family. The family had been thought previously to include only enhancer regulatory proteins. Second, it provides information which is useful for understanding the evolution of this family of regulators. Third, it raises the question as to whether COUP-TF, and other promoter activators for that matter, may be ligand-activated gene regulators. This latter query remains to be answered experimentally. Deductive reasoning should permit us to conclude that if one of these orphan receptors now has been adopted for a function, then in time, others will follow suit.

Although direct evidence for regulation of these molecules by ligands does not exist, the conservation of amino acids in the ligand binding region of authentic receptors with those present in the orphan receptors leads me to believe this. A recent study has shown that the Drosophila analog of human COUP-TF, which is called seven-up and regulates retinal cell differentiation, contains a 93% identity with the human protein in the C-terminal region (55). This observation suggests strong conservation of function and implies ligand regulation for both molecules.

Although admittedly speculatory, the question as to whether the orphan receptors have endogenous ligands is clearly the most exciting to be considered. If they do have ligands, I would predict that the ligands are indigenous to the cells of function since transfection into cultured cells shows them to be active in an apparent constitutive manner (unpublished data). My best guess is that many of these putative ligands will be hydrophobic in nature and some may be nutritionally or metabolically derived. In fact, this could be the tip of a new intracrine iceberg wherein a series of yet to be described hormones are discovered to be indigenous to the specific target cells expressing these orphan receptors. If true, the elucidation of a new intracrine regulatory system will inject more than a little interest and excitement into the fields of hormone action and molecular endocrinology. In fact, the impact will be explosive.

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