Steroid hormone receptor homologs in development

ANTHONY E. ORO¹, KAZUHIKO UMESONO¹ and RONALD M. EVANS¹

¹Howard Hughes Medical Institute, Salk Institute for Biological Sciences, La Jolla, California 92038-9216, USA
²Department of Biological Sciences, University of California, San Diego, La Jolla, California 92093, USA

Summary

The steroid/thyroid receptor superfamily are ligand-dependent transcription factors which consist of distinct functional domains required for transcriptional control of a network of genes. Members of this superfamily are beginning to be studied for their contribution to embryogenesis. Two human receptors for the vertebrate morphogen retinoic acid have been isolated and further characterized on model promoters. Moreover, the presence of homologs of these receptors in Drosophila reveals that members of this superfamily predate the divergence of the vertebrates and invertebrates. One locus is knirps-related (knrl), whose product is closely related to that of the gap segmentation gene knirps (knf). knrl is one of the most diverged steroid receptor-like molecules and displays a spatially restricted blastoderm pattern.

Key words: steroid hormone, thyroid receptor, transcriptional control, Drosophila, knirps.

I. Development as a problem of differential gene regulation

In the 1930s T. H. Morgan theorized that the process of development occurred by the differential expression of small invisible entities called genes and that expression of different sets of these genes gives rise to differences in the adult organism (Morgan, 1934). Indeed differential gene expression does appear to play an important part in normal development as many classes of transcriptional regulators have been recently isolated and shown to play important roles in embryogenesis (Dressier and Gruss, 1988). One of them is the steroid/thyroid receptor superfamily of proteins. These proteins are receptors for small hydrophobic molecules which as nuclear hormone-receptor complexes mediate their effects. The biologically active ligands play roles in both homeostasis in the adult and development, as illustrated in Table 1. For example, the adrenal steroids widely influence energy metabolism, controlling glycogen, muscle, and mineral metabolism as well as mediating behavioral responses to perceived stress. They have widespread effects on the immune and nervous systems and influence the determination of neural crest fate. The sex steroids provoke the development and determination of body sexual dimorphisms including the reproductive organs embryonically, the central nervous system perinatally, and reproductive behavior in the adult. Aberrant production of these hormones have been associated with a broad spectrum of clinical disease. Further, both thyroid and steroid hormones appear to be important in metamorphosis. Thyroidectomy inhibits tadpole maturation to a frog, but addition of thyroxine to its drinking water induces all of the changes into a terrestrial adult (Schwind, 1933). Similarly, ecdysteroids are required for insect metamorphosis, allowing the various molts into adulthood (Ashburner, 1971).

An initial insight into how small, relatively simple molecules elicit such a diversity of complex responses was provided by the identification of steroid and thyroid hormone receptors with radiolabeled ligands in the early 1970s (Jensen and DeSombre, 1972). After the addition of hormone, the receptor apparently underwent a conformational change such that it associated with high affinity binding sites in chromatin and induced or repressed a limited number of genes (Ivaric and O’Farrell, 1978). This type of experiment led to the idea that these receptors controlled a specific network of genes in order to facilitate homeostasis. Purification and biochemical characterization of the glucocorticoid receptor was accompanied by the identification of a variety of glucocorticoid responsive genes (Yamamoto, 1985; Ringold, 1985). Each gene contained a short cis-acting sequence (about 20 bp) in the promoter region which was required for hormone-dependent activation of transcription (called a hormone responsive element or HRE, Scheidereit et al. 1983; Chandler et al. 1983; Karina/ et al. 1984). Selectivity of gene expression is achieved by synthesis of the cognate ligand, restricted expression of a receptor in specific cells and tissues, the presence of an HRE in the promoter of a particular gene, and the presence of other transcription factors required for that promoter to function at normal levels.

II. Functional domains

The cloning of the human glucocorticoid receptor
Deletions in this cysteine-rich, zinc finger region destroy all function. Second, the carboxyl terminus increases both activities (Oro et al. 1988b). Effects of mutations can be rapidly assayed for their transcriptional effects in the presence or absence of hormone (Figure 1).

Both activation and repression by hGR share some common features. First, both processes demonstrate a requirement for the DNA-binding domain and reflect the fact that positive and negative regulation are DNA sequence-specific. Deletions in this cysteine-rich, zinc finger region destroy all function. Second, the carboxyl terminal deletions show that activation and repression require an intact ligand binding domain and the presence of hormone. Consistent with previous results for activation (Godowski et al. 1987), removal or replacement of this region by heterologous sequences leads to hormone independence for both processes.

In contrast, several experiments provide criteria that distinguish positive and negative regulatory effects of the hGR. First, the amino terminal domain that contains a potent activator sequence (Tau 1) is not necessary for trans-repression. The Tau domain can be placed on heterologous DNA-binding domains such as Gal4 or moved to other parts of the receptor and still maintain function (Hollenberg and Evans, 1988). These facts substantiate the duality of receptor function and highlight the observation that deletion of Tau 1 engenders a more potent repressor. Second, heterologous proteins such as β-galactosidase can functionally replace the hGR carboxyl terminus only in repression. Removal of the carboxyl terminus results in a receptor variant with greatly reduced repression and activation activity (Figure 1). The addition of a β-galactosidase moiety selectively increases repression and not activation activity, while addition of a mineralocorticoid receptor carboxyl terminus increases both activities (Oro et al. 1988b). Given the lack of amino acid identity or similar charge distribution between the hGR, hMR and β-gal, one plausible model is that hGR represses through its carboxyl terminus simply by steric hindrance but requires specific sequences for activation activity.

III. A superfamily of proteins

Analysis of the amino acid sequence of the hGR revealed a segment with striking relatedness to the viral oncogene erbA (Weinberger et al. 1985). Two groups initiated the characterization of the erbA proto-oncogene product which led to its identification as the thyroid hormone receptor (Weinberger et al. 1986; Sap et al. 1986). Although steroid and thyroid hormones are not structurally or biosynthetically related, the existence of a common structure for their receptors supports the proposal that there is a large superfamily of genes whose products are ligand-responsive transcription factors. Apparently it is the analogous action of the hormones that is reflected in the homologous structure of their receptors. An extension of this proposal predicts that other small, hydrophobic molecules may interact with structurally related intracellular receptors in order to modulate the expression of specific networks of genes. Molecular cloning studies support this proposal as the receptors for many of the molecules listed in Table 1 including estrogen, progesterone, aldosterone, and vitamin D have been isolated (For review see Evans, 1988).

<table>
<thead>
<tr>
<th>Hormone</th>
<th>Cellular source</th>
<th>Principal adult action</th>
<th>Known developmental action</th>
</tr>
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<tbody>
<tr>
<td>Glucocorticoid</td>
<td>Adrenal</td>
<td>Carbohydrate synthesis Stress behaviour</td>
<td>Influence neural crest fate</td>
</tr>
<tr>
<td>Mineralocorticoid</td>
<td>Adrenal</td>
<td>Salt and water balance</td>
<td>?</td>
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<tr>
<td>Estrogens</td>
<td>Gonads, Adrenal</td>
<td>Sexual reproduction Behaviour and physiology</td>
<td>Reproductive organ development and neural development</td>
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<td>Androgens</td>
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<td>Progesterone</td>
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<td>Thyroxine-1,25(OH)2</td>
<td>Kidney</td>
<td>Calcium, phosphate balance</td>
<td>Bone development</td>
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<td>Cholecalciferol</td>
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<td>Retinoids</td>
<td>Liver, Intestine</td>
<td>Structural elements of vision</td>
<td>Positional information</td>
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<td>Ecdysone</td>
<td>Prothoracic gland, Ovary</td>
<td>Oogenesis</td>
<td>Insect molting and metamorphosis</td>
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<td>Juvenile hormone</td>
<td>Corpus Allatum</td>
<td>Oogenesis</td>
<td>Inhibition of insect maturation</td>
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iv. Steroid hormone receptor homologs in development

Although it is widely believed that differential regulation of gene expression is the critical level at which development is controlled, this does not provide a conceptual framework for how specific processes like spatial organization or pattern formation are achieved. Many mechanisms have been proposed to explain patterning in different organisms, but one long-standing theory is that certain patterns are formed through the establishment of a gradient of a diffusible substance or morphogen (Crick, 1975). One example of a morphogen is the Drosophila bicoid protein, which acts as a diffusible concentration gradient to establish anterior polarity of the embryo (Driever and Nusslein-Volhard, 1988). Another example is the vitamin A-related metabolite, retinoic acid (RA). Work by numerous laboratories over the last several years has indicated that the RA manifests morphogenic properties in vertebrates (Maden, 1982). Evidence from work on the developing chick limb bud suggested that RA was produced in a gradient with its highest concentration posteriorly at the zone of polarizing activity. Recently, RA was directly shown to be present in the chick limb bud in a 2.5-fold concentration gradient across the limb (Thaller and Eichele, 1987), supporting its morphogenetic role. Moreover, reversal of the gradient by the addition of exogenous RA resulted in duplication of limb structures such as the digits (Tickle et al. 1975). One important question to be answered is how a shallow RA gradient can be transmitted into different cell fates.

Two retinoic acid receptors have been identified that are members of the steroid hormone receptor superfamily. The identification of the retinoic acid receptor α
Fig. 2. The human retinoic acid receptor activates through a thyroid hormone response element. (A) Construction of the chimaeric receptor hGRG. The hGRnx and hRARnx are mutated hGR and hRARa, respectively, with common NotI and XhoI sites in the cDNAs. The amino acid numbers represent the possible domain boundaries in the receptor proteins. The ligand binding domains are indicated by their cognate hormones, DNA-binding domains by ‘DNA’. The chimaeric receptors were created by exchanging the DNA-binding domains at the NotI/XhoI sites. (B) Transactivation of a T3 responsive reporter by the chimaeric receptor for the homologous region from the hGR, a thyroid hormone receptor (Umesono et al. 1988). This result was predicted based upon the structural relatedness of the DNA-binding domains between the RAR and the thyroid hormone receptor. Although the biological significance has not yet been established, the two distinct receptor systems may indeed regulate an overlapping set of genes. While an authentic RAR response element has not been characterized, another apparent target of regulation for the retinoic acid receptors is the β receptor gene itself. From RNA analysis of hepatoma cells, the expression of the β receptor appears to increase in a cycloheximide-independent manner while the expression of the α receptor remains constant in response to RA (deThe et al. 1989). One interpretation of these data is that the β receptor is autoregulated, perhaps to amplify the expression of the set of genes it regulates. How this amplification plays a role in the establishment of positional information and to which genes the receptor transmits the information are questions being actively pursued.

Retinoic acid has a clear role within the developing organism in the establishment of positional information. However, other small molecule ligands might exist that act in distinct developmental paradigms to establish positional information or determine cell fate, and which might act via a steroid hormone receptor-like molecule. Moreover, the need for a well-characterized embryological and genetic system with which to analyze the function of these molecules pointed to the Drosophila melanogaster system for study.

To identify homologs of the vertebrate steroid receptors, a Southern blot of Drosophila genomic DNA was probed with a cDNA fragment encoding the hRARα DNA-binding domain (Giguere et al. 1987; Petkovich et al. 1987) Under conditions of reduced hybridization stringency, six distinct EcoRI bands ranging in size from 2 kb to greater than 12 kb were detected. Screening of a Drosophila genomic library using the same probe and hybridization conditions resulted in the isolation of three distinct single copy gene loci (Oro et al. 1988a).

One class of inserts mapped on the third chromosome approach, the receptor was shown to bind retinoic acid with high affinity (Brand et al. 1988; Benbrook et al. 1988). The structure of the two receptors are very similar (90% in the ligand-binding domain), but the β form of the receptor transactivates at a slightly lower concentration of RA (Brand et al. 1988). Moreover, RNA from the two receptor genes have different tissue distributions. RNA from the α form is expressed in hematopoietic cell lines while RNA from the β receptor has a more complex distribution, being highest in the brain, kidney and prostate (deThe et al. 1989).

By analogy with steroid receptors, a potential model for transmission of positional information via the morphogen retinoic acid is through the two retinoic acid receptors. Upon ligand binding, the receptors would trigger the activation or repression of specific networks of genes. Key to this model is that the RAR is a sequence-specific transcriptional activator. Interestingly, the RARα was found to activate at high levels through a previously isolated HRE for the thyroid hormone receptor (Umesono et al. 1988). This result was predicted based upon the structural relatedness of the DNA-binding domains between the RAR and the thyroid hormone receptor. Although the biological significance has not yet been established, the two distinct receptor systems may indeed regulate an overlapping set of genes. While an authentic RAR response element has not been characterized, another apparent target of regulation for the retinoic acid receptors is the β receptor gene itself. From RNA analysis of hepatoma cells, the expression of the β receptor appears to increase in a cycloheximide-independent manner while the expression of the α receptor remains constant in response to RA (deThe et al. 1989). One interpretation of these data is that the β receptor is autoregulated, perhaps to amplify the expression of the set of genes it regulates. How this amplification plays a role in the establishment of positional information and to which genes the receptor transmits the information are questions being actively pursued.
at cytologic position 77E, the same location as the previously identified gap segmentation gene kni (Nusslein-Volhard and Wieschaus, 1980). kni mutants had been previously isolated in a genetic screen for zygotic mutants affecting embryonic pattern formation and falls into a small set of genes required for abdominal segmentation (Nusslein-Volhard et al. 1987). Mutational analysis indicates that kni\(^{+}\) activity apparently interacts with maternally derived information (Lehmann and Nusslein-Volhard, 1986).

The genomic and corresponding cDNA clones for the RAR homolog were sequenced and predicted amino acid sequence was found to have a striking similarity to the predicted amino acid sequence of the kni product (Näuber et al. 1988) and thus called knirps-related (knrl) (Oro et al. 1988a). Southern blots of kni mutant and wild-type genomic DNA revealed mutant DNAs XT1 and XT106 removed both kni and knrl loci localizing both genes to cytological positions 77E3–5. Further, kni mutant FC\(^{13}\) contained a two kb deletion in the kni transcription unit while leaving knrl apparently intact (Oro, unpublished). No phenotypic differences in the abdominal region are seen between the kni mutants (R. Lehmann, personal communication), indicating that the kni function is epistatic to the putative knrl function. Loss-of-function alleles for knrl are required before the developmental role of the knrl product can be addressed.

The knrl gene is expressed early in development. Northern blot of stage-specific RNA showed a single RNA species of approximately 3–8 kb expressed at low levels between 0 and 3 h after egg-laying (AEL) and at significantly higher levels in later embryos, larvae and adults. The spatial location of knrl transcripts was assayed by in situ hybridization on sections of 0–2 and 2–4 hour embryos (Figure 3). After egg deposition and until approximately the 8th nuclear division, a weak, spatially uniform distribution of apparently maternal transcript was detected (Figures 3A and 3B). The first apparently zygotic expression is detected at nuclear division 12, when the knrl transcript is localized to a small anteroventral region of the embryo (Figures 3C and 3D), at approximately 80–100% of egg length (EL) on the ventral side (domain I). Expression in this domain intensifies through the cellular blastoderm stage, and two additional circumferential bands of transcript become detectable, centered at approximately 70% EL ventrally (domain II) and 25% EL...
Fig. 4. Comparison of the predicted knr1 product to vertebrate steroid/thyroid hormone receptors. (A) Alignment of the DNA-binding domains of representative members of the superfamily, showing the conserved amino acids and the extensive structural similarity between knr1 and kni. Note that the identity of knr1 and kni extends past the conserved Gly and Met residues of the DNA-binding domain. (B) Overall structural comparison of the predicted protein sequence of knr1 to other members of the steroid/thyroid hormone receptor superfamily. Comparisons of the region marked DNA are to the 66–68 amino acid DNA-binding domains and the region marked Ligand Binding is compared with the amino acids starting at a point 25 amino acids past the conserved Gly and Met residues of the DNA binding domain. The intervening 25 amino acids are highly related between knr1 and kni, but not significantly related to the other vertebrate receptors (indicated by an asterisk). As there is no significant similarity of knr1 to the other receptors in the carboxyterminal region, no specific alignment of these regions is shown. The programs of Devereux et al. 1984 were used for comparison. Numbers indicate amino acids as detailed in Oro et al. 1988; Nauber et al. 1988 Weinberger et al. 1986; Giguere et al. 1987 and Hollenberg et al. 1985 for knr1, km, hTRβ, hRAR, and hGR, respectively.

ventrally (domain III) (Figures 3E and 3F). It is noteworthy that expression in domain II appears significantly more intense ventrally than dorsally.

A comparison of the predicted knr1 protein with other members of the steroid/thyroid receptor superfamily is shown in Figure 4. First, sequence alignment demonstrates greatest similarity with the other receptors in the 67 amino acids of the putative knr1 DNA-binding domain (Figure 4A). Between amino acids 14 and 80 of knr1 there is 85% amino acid identity with the kni product, 49% with the human thyroid receptor, 47% with the human retinoic acid receptor and 43% with the human glucocorticoid receptor. Interestingly, the knr1 and kni DNA-binding domains both contain a glycine in the region linking the two zinc fingers (residues 39 and 30 in knr1 and kni, respectively), at a position which in all other receptors is either an arginine or lysine. This further suggests a common origin for these two genes. Second, amino acid sequence analysis reveals a highly conserved stretch of 30 amino acids immediately following the DNA-binding domain. Between other steroid receptors little or no homology exists (Figure 4), while this region in knr1 or kni is more conserved than the DNA-binding domain. Other transcription factors also contain regions of high conservation outside of the DNA-binding domain, such as the POU domain in homeoproteins (Herr et al. 1988). Perhaps this region plays a novel role in receptor function.

Further, the homology of the predicted knr1 gene product to vertebrate steroid receptors suggests that its function is ligand-dependent. If this is the case, such a ligand might constitute a previously unrecognized small-molecule morphogen, and some of the genes involved in regulating knr1 function might affect the synthesis of the ligand or storage of a ligand precursor, rather than regulating knr1 expression. However, the unrelatedness of the knr1 carboxy terminus to that of
the other receptors makes it difficult to predict a potential ligand. As mentioned above, even distant receptors have particular structural similarities in the carboxyl terminus. Perhaps the knrl product is a constitutive transcriptional regulator, and functions entirely without a ligand. Finger swap experiments similar to those used to identify the retinoic acid receptor may illuminate these differences.

**Conclusion**

Development of an organism is a complex biochemical process. Part of the understanding of the mechanisms has come about from the study of classes of transcription factors such as the steroid/thyroid hormone receptors whose ligands share common biophysical properties and a common mechanism of action in environmentally modulating gene expression and triggering specific networks of genes. The identification of the retinoic acid receptor has allowed the proposal that the morphogenic properties this ligand exerts are mediated through a hormone–hormone receptor complex which regulates a network of genes. Elucidation of proteins that interact with the receptors and their ligand as well as the receptor target genes, their spatial pattern of expression and function will allow new insight into further mechanisms of vertebrate development. Analysis of related receptor systems will identify new paradigms of receptor action. Characterization of one class of *Drosophila* retinoic acid receptor homologs identified a locus highly related in amino acid sequence to the gap segmentation gene knrl with a spatially restricted expression pattern. These two receptors are the most highly diverged members of the gene superfamily; functional analysis may reveal a new class of transcriptional activators. Finally, the characterization of *Drosophila* steroid receptor homologs may reveal receptor systems common to both invertebrates and vertebrates. Although the gross structural features of developing embryos are distinct, common mechanisms using common or related molecules may be uncovered.

The authors would like to acknowledge Mike McKeown, Jon Margolis, Jim Posakony and Charles Zuker for help with the *Drosophila* work and Chris Glass, Vincent Giguere and M. G. Rosenfeld for work on retinoic acid receptor activation. R.M.E. is an investigator for the Howard Hughes Medical Institute and also acknowledges NIH support. A.E.O. is supported by the Medical Scientist Training Program, General Medical Grant PSH GM07198.

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