REVIEW

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Gene regulation by steroid hormones: Vertebrates and insects

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Steroid hormone receptors, transcription control, zinc finger, ecdysteroid, puffing, insect molting and metamorphosis, *Drosophila melanogaster*, *Manduca sexta*, *Galleria mellonella*

Abstract. In both vertebrates and insects, steroid hormones are known to control diverse physiological events from reproduction to growth and development, primarily by affecting the expression of specific genes. Despite the vast evolutionary distance between arthropods and vertebrates, the molecular mechanisms by which steroids act to regulate genes appear to be conserved. Our understanding of these principles has been advanced significantly during the past few years, owing to the molecular characterisation of genes which encode steroid hormone receptors. This article provides a general survey of the superfamily of nuclear hormone receptors and their mode of action and summarizes the recent knowledge on members of this superfamily discovered in *Drosophila* and other insects.

INTRODUCTION

The molting and metamorphosis of insects are initiated and coordinated by the steroid hormone 20-hydroxyecdysone and related ecdysteroids. Like other steroid hormones, ecdysteroids act directly on specific genes by effecting their transcription. In fact, ecdysteroids were the first hormones shown to act at the level of genome. The classic experiments of Clever & Karlson (1960) showed that two specific puffs known to indicate RNA synthesis appeared in the polytene chromosomes within 15 minutes of the addition of ecdysone to isolated salivary glands of the midge *Chironomus tentans*, independent of protein synthesis (Clever, 1964). This effect was studied in great detail in *Drosophila melanogaster* by Ashburner and culminated in a model for ecdysteroid action (Ashburner et al., 1974). In this model the hormone combined with its receptor binds to the target DNA to induce directly the "early puff genes" and to repress the "late puff genes". The protein products encoded by the early genes then activate the late genes while inhibiting their own synthesis. In support of this model, three genes from early puffs have been cloned and shown to encode multiple proteins that contain DNA-binding motifs of various types and therefore are likely transcription factors (reviewed by Andres & Thummel, 1992). Ecdysteroid-induced expression of these genes in a variety of tissues and findings of homologous genes in other insects suggest that the ecdysteroid regulatory cascade is not confined to the *Drosophila* salivary glands.

Steroid and thyroid hormones, vitamin D_3 and retinoic acid all act to activate or repress gene expression through specific nuclear receptor proteins (Evans, 1988). These receptors comprise a specialized family of transcription factors that contain a DNA-binding domain

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of two zinc finger motifs, the atom of zinc being coordinated by two pairs of cysteine residues. DNA binding usually depends on the presence of the hormonal ligand which binds to the C-terminal region of the same receptor molecule. Some receptors, however, appear to bind to the target DNA of a gene and suppress its transcription when unliganded, whereas the hormone-receptor complex will activate the same gene. Cloning of the ecdysone receptor gene from *Drosophila* (Koelle et al., 1991) has provided strong evidence that both vertebrate and invertebrate steroid hormones act through the same molecular mechanism.

STEROID HORMONE RECEPTOR SUPERFAMILY

Many important cell regulators exert their effects at the level of transcriptional activation or repression of specific genes via nuclear receptor proteins. These include the steroid hormones (estrogens, progestins, androgens, glucocorticoids, mineralocorticoids, ecdysteroids), vitamin D_3, thyroid hormones, and retinoids. Steroid and thyroid hormones are involved in the control of many physiological events including development, growth, differentiation, reproduction, metabolism, ion transport and behavior, retinoic acid has been implicated in vertebrate morphogenesis. Despite their diverse chemical nature, all of the above mentioned compounds act through a very similar molecular mechanism.

Control of gene expression

The cascade of differential gene expression is generally accepted as a mechanism underlying cell differentiation, embryonic pattern formation and many other processes. In most cases, the primary control of gene expression lies at the level of transcription. In eukaryotes, transcription initiation is effected by RNA polymerase II and a group of general initiation factors that participate in the assembly of a preinitiation complex. Promoter elements that are necessary for initiation by the general factors include the TATA box, initiators and other DNA sequences close to the transcription start site (Roeder, 1991). Expression of most eukaryotic genes is further regulated by gene-specific factors that usually bind to distal control elements, known as enhancers (see Ptashne, 1988 for a review). Promoters and enhancers are composed of short regulatory DNA sequences (cis-elements). The trans-acting elements, on the other hand, are represented by a wide spectrum of nuclear proteins (transcription factors) that specifically interact with their target cis-elements as well as with other components of the transcriptional machinery (Mitchell & Tjian, 1989). Transcription factors often act as dimers (e.g. CREB and Fos-Jun families; Busch & Sassone-Corsi, 1990). The ability of some transcription factors to form heterodimers composed of heterologous molecules greatly increases the potential diversity and specificity of transcriptional regulation (Lamb & McKnight, 1991). In other words, combinations of only a limited number of transcription factors and short cis-elements lead to enormously diversified responses, necessary for cells to differentiate.

Nuclear hormone receptors probably enhance gene transcription by facilitating the formation of a stable preinitiation complex (Tsai et al., 1991). Studies on the mouse mammary tumor virus (MMTV) have suggested that steroid receptors may also facilitate transcription by a specific displacement or modification of the nucleosome structure, making the DNA accessible to general transcription factors (see Hager & Archer, 1991 for a review). It has been shown that nuclear hormone receptors can also inhibit gene expression. For example, glucocorticoid receptor represses transcription of the gene encoding the

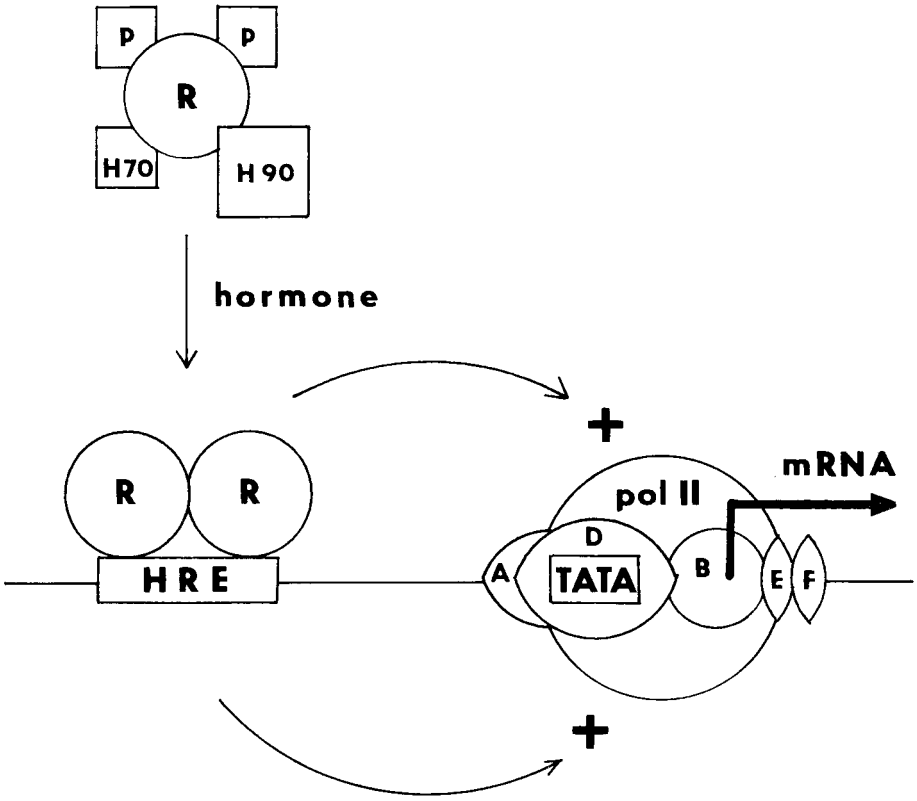

Fig. 1. Model of steroid receptor action. Receptor, activated by the steroid, forms a dimer and binds to its cognate DNA sequence to facilitate transcription. R – receptor; H90 and H70 – heat shock proteins 90 and 70 kDa; P – other protein components; HRE – hormone response element. A, B, D, E/F and pol II denote the general transcription factors TFIIA, TFIIB, TFIID (the TATA-binding protein), TFIIE/F and RNA polymerase II, respectively.

common α-subunit of gonadotropic hormones (Akerblom et al., 1988). Several models of negative regulation have been suggested, including displacement or masking of the positively acting transcription factors by the receptor, competition of two factors for a common cis- element or negative protein-protein interactions such as formation of inactive receptor heterodimers (Akerblom & Mellon, 1991). Hormone response elements (HRE) to which receptors bind are regarded as enhancers as they function both upstream or downstream of the transcriptional start site in either orientation. Since HREs are often located far from the transcriptional start site, their interaction with the preinitiation complex in vivo may be mediated by a third protein and by looping of DNA. The regulatory regions of steroid hormone-responsive genes usually consist of HREs and multiple general or specific cis-elements whose cooperation in a combinatorial fashion ensures the tissue- and stage-specific cell responses to identical regulators (Beato, 1989).

A two-step mechanism (Fig. 1) has been proposed for the steroid hormone receptor action in vivo (reviewed by Jensen, 1991). According to this model, a native receptor is maintained in an inactive form, being associated with certain protein(s) (e.g. the 90 kDa heat shock protein) that obscure important receptor functions in the absence of appropriate ligand. In the first step, steroid transforms the receptor to its active state, possibly by displacing the inactivating protein. A conformational change then enables receptor to dimerize and bind to its cognate DNA in order to affect transcription (Tsai et al., 1991). Usually, there are only 10^3 to 10^4 receptor molecules per cell that must scan the immense genome for these short target DNA sequences. Whereas some receptors (glucocorticoid) appear to translocate from cytosol to the nucleus upon activation, others (progesterone or estrogen receptors) are mostly found in the nucleus. Thyroid hormone receptor (and likely some others) reside permanently in the nucleus and are capable of binding DNA in the absence of ligand (Damm et al., 1989). The thyroid hormone receptor does not associate with hsp90 (Dalman et al., 1990) and probably acts to repress gene transcription when unliganded.

Transcription factors

Transcription factors are usually composed of several functional domains that enable their multiple protein-protein and protein-DNA interactions (Latchman, 1990) and often contain short sequences of basic amino acids that function as signals for nuclear localization. There are a few types of protein domains known to bind DNA that are well conserved in eukaryotes. First, the helix-turn-helix motif should be mentioned as it constitutes the core of the homeobox genes, originally demonstrated to play a critical role in *Drosophila* embryonic development (see Hayashi & Scott, 1990 for a review). Second, related to the homeodomain is the POU domain described in mammalian octamer-binding (Oct1, Oct2) and pituitary-specific (Pit1), and in nematode (Unc86) proteins. Third, various domains of basic amino acids bind DNA, usually being associated with motifs responsible for dimerization. These include the leucine zipper (C/EBP, CREB, oncoproteins Fos and Jun), helix-loop-helix or both (oncoprotein Myc). A similar but distinct DNA-binding domain is shared by a group of ETS proto-oncogene-related putative transcription factors.

Finally, there are two types of zinc finger domains present in numerous DNA-binding proteins. The finger-like structure is kept together by two pairs of cysteine or histidine residues, which coordinate a single atom of zinc. These fingers make direct contacts with the major grooves of DNA (Evans & Hollenberg, 1988). In the first type, zinc is chelated by cysteine and histidine pairs (C_2H_2) and the fingers are arranged in multiple copies. The first zinc finger motif was identified by Miller et al. (1985) in the TFIIIA transcription factor of *Xenopus*, controlling ribosomal gene transcription by RNA polymerase III. Some of the gap segmentation (kruppel, hunchback) and dorso-ventral polarity (snail) genes of *Drosophila* encode C_2H_2 zinc fingers, implying an important role of zinc finger proteins in embryonic development (reviewed by St. Johnston & Nüsslein-Volhard, 1992). The *Drosophila* broad-complex (BR-C) genes (DiBello et al., 1991) also encode pairs of C_2H_2 zinc fingers. The second similar but unrelated (Amero et al., 1992) and functionally distinct type of zinc finger domain is characterized by only two nonrepetitive fingers, in each of which two pairs of cysteine residues (C_2C_2) coordinate the zinc atom (Schwabe & Rhodes,

1991). Besides numerous vertebrate and insect proteins whose roles are as yet unknown, all of the steroid, thyroid and retinoid receptors possess two C_2C_2 zinc finger motifs.

Functional domains of nuclear hormone receptors

Nuclear hormone receptors comprise a specialized type of transcription factors whose activity depends on hormonal ligands. The first steroid receptor genes were identified for the human glucocorticoid (Hollenberg et al., 1985) and estrogen (Green et al., 1986) receptors. During the late 1980s, receptor genes for steroid, thyroid, and retinoid regulators known in vertebrates were cloned and shown to belong to one multigene family (Evans, 1988). The total number of known nuclear receptors currently stands at around 40 (Laudet et al., 1992). Many of them, however, are regarded as orphan receptors whose ligands are unknown. Most receptor genes were cloned from vertebrates and *Drosophila*, but related genes have been reported from organisms as distant as nematodes (*Caenorhabditis elegans*) and a slime mold *Dictyostelium discoideum* (cited in Laudet et al., 1992).

Functional analyses of the receptor genes provided a critical advance in our understanding of hormonal control of transcription (Green & Chambon, 1988). Analyses of regulatory regions of numerous genes known to be directly activated or repressed by hormones revealed target response elements with which the nuclear receptors interact (Martinez & Wahli, 1991). The ability of receptor proteins to activate transcription in vivo is usually studied using transfection assays. Two expression vectors, one carrying the receptor cDNA, the second containing a reporter gene with a response element for the putative transcription factor are introduced into suitable host cells whose genome lacks both the receptor and the reporter genes. In such assays, activities of novel or modified receptors, their putative ligands as well as their response elements can be tested.

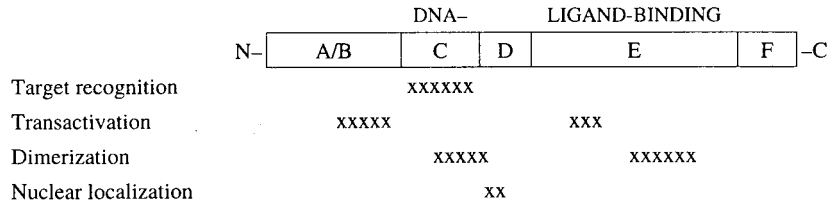

Fig. 2. Functional domain organization shared by the members of the steroid hormone receptor superfamily. Localization of the major receptor functions along a receptor molecule is shown.

Studies with truncated, chimeric or mutated receptor molecules revealed several functional regions (A, B, C, D, E, and F; Kumar et al., 1986) that are characteristic of all members of the steroid/thyroid receptor superfamily (Fig. 2). The two major receptor functions, DNA and ligand binding, are localized in regions C and E, respectively, which show the highest degree of conservation amongst the superfamily members. Green & Chambon (1987) demonstrated that a chimeric estrogen receptor containing the C region of the glucocorticoid receptor will activate a glucocorticoid-responsive gene in HeLa cells in the presence of estradiol. About 66–68 amino acids in length, the C region involves two C_2C_2 zinc finger motifs, responsible for the sequence-specific DNA binding (Green et al., 1988). Deletion experiments (e.g. Kumar et al., 1986) have shown that the approximately

250 amino acids long E region is required for high affinity (K_d 10^{-9} to 10^{-10} M) ligand binding. The E region is also important for dimerization of certain types of receptors, particularly those that form heterodimers (thyroid hormone, retinoic acid, retinoid X receptors; Marks et al., 1992; Laudet et al., 1992). Another function of the E domain, at least in some receptors, is interaction with the 90 kDa heat shock protein, which precludes receptor activation in the absence of steroid. Between the DNA- and ligand-binding domains there is region D, often referred to as a hinge, of variable length. There is usually a short sequence of basic amino acids adjacent to the C-terminal zinc finger motif that may act as a nuclear localization signal (Picard & Yamamoto, 1987). Recently, the N-terminal portion of the hinge was shown to contain a helix important for DNA binding by some mammalian (Wilson et al., 1992; Lee et al., 1993) and insect (Ueda et al., 1992) receptors. The D region also contributes to receptor dimerization.

Transactivation occurs via interaction of the receptor molecule with other transcription factors that leads to gene expression. Two transactivation domains were found in some (e.g. glucocorticoid and estrogen) receptors, in the N-terminal A/B region and in the hormone-binding domain. Estrogen receptor deprived of the latter transactivation domain revealed a substantially reduced ability to activate gene expression; deletion of the A/B region had a similar effect (Kumar et al., 1987). In either case, the activation varied widely depending on the recipient cell type, indicating the importance of cell-specific transcription factors (Tora et al., 1989).

Although exceptions have been noted (Ueda et al., 1992; Lee et al., 1993), nuclear hormone receptors bind their response elements as dimers. Whereas some receptors (e.g. estrogen; Kumar & Chambon, 1988) were shown to form homodimers, heterodimer formation appears to be critical for both the DNA binding and transactivation by a subclass of receptors including thyroid hormone, vitamin D_3 and retinoic acid receptors. Two regions located within the DNA- and ligand-binding domains, respectively, appear to be important for the dimerization. A heterodimer of the human thyroid hormone receptor with the human retinoic acid receptor RARα was shown to cooperatively bind DNA and to differentially activate gene expression depending on the thyroid response element in the reporter promoter (Glass et al., 1989). It has been noted that certain receptors require auxiliary factors contained in nuclear extracts to bind their target DNA efficiently. Such a coregulator was identified (Yu et al., 1991) as a product of the retinoid X receptor ß gene, closely related to the human retinoid X receptor (RXRα; Mangelsdorf et al., 1990). Whereas all-trans retinoic acid activates retinoic acid receptors (RAR), its 9-cis stereoisomer is the high-affinity ligand of RXR (Heyman et al., 1992). RXRα has been shown to dimerize with thyroid hormone, retinoic acid and vitamin D_3 receptors, enhancing both their specific binding to DNA and their transcriptional activity (Zhang et al., 1992; Kliewer et al., 1992). Importantly, the different combinations of receptor monomers induce transcription from different hormone response elements with varying activity (reviewed by Laudet & Stehelin, 1992). For example, the RXR-RAR heterodimer binds to a palindromic response element in preference to a direct repeat element through which the RXR homodimer otherwise activates gene transcription. Heterodimeric complexes were also shown to behave differently in response to ligands. The affinity of the thyroid hormone receptor homodimer but not that of its RXR complex to DNA is reduced by triiodothyronine (Yen et al., 1992). The product of *Drosophila* ultraspiracle (usp) gene, a

homolog of RXRα (Oro et al., 1990), can be substituted for RXRα in the cooperative DNA binding shown with thyroid hormone, retinoic acid and vitamin D_3 receptors (Yao et al., 1992). Receptor heterodimer formation thus appears to be a widespread mechanism underlying the specificity and flexibility of the hormonal response.

Orphan receptors for which ligands have not yet been identified may also modulate the activity of hormone receptors by heterodimer formation. In both vertebrates and insects a number of such orphan receptors are known. Moreover, there are multiple forms of many receptors that are either encoded by distinct genes (thyroid hormone receptors α and ß; retinoic acid receptors α, ß and γ) or that arise from alternate promoters and/or by differential mRNA splicing. It has been demonstrated that multiple receptor isoforms have different spatial and temporal expression, suggesting that differential expression of receptor types is important for developmental regulation in various tissues. For example, Dolle et al. (1989) have found differential distribution of the three retinoic acid receptor forms α, ß and γ during mouse limb development that may help retinoic acid specify the positional information.

Multiple receptor isoforms, some of which may be less active, might also play an important role in gene suppression by competing for a common DNA binding site, ligand or for some trans-acting factor required for the activation. Alternatively, receptor monomers lacking essential functions can inhibit gene expression by forming inactive heterodimers. For example, a product of the thyroid hormone receptor gene α generated by alternative splicing of 3' exons (c-erbAα-2), which is capable of interaction with DNA but does not bind thyroid hormone, probably acts as a natural negative regulator of the thyroid hormone action (reviewed by Chin, 1991). Thus, within one cell, the expression of a single receptor gene may yield mutually antagonistic proteins. The avian erythroblastosis virus (v-erbA) oncoprotein, a homolog of thyroid hormone receptor that has lost the ability to bind the hormone, functions as a constitutive transcription repressor (Damm et al., 1989).

Receptor – DNA interactions

Analyses of the regulatory regions of numerous genes whose transcription is directly activated by steroid hormone receptors have revealed short (15–25 bp) sequences, called hormone response elements (HRE), to which hormone-receptor complexes bind. Hormone responsive genes often possess multiple HREs that cooperatively bind receptors to enhance transcription (Martinez & Wahli, 1991). Most HREs represent inverted repeat (or palindromic) structures, with the two half-sites of the palindrome being separated by up to 3 base pairs. However, some receptors (heterodimers of RXR with retinoic acid, thyroid hormone and vitamin D_3 receptors) preferetially bind to direct repeat half-sites separated by spacers of various lengths (Laudet & Stehelin, 1992).

Sequence comparisons of hormone response elements from different hormone-responsive genes and functional studies with mutated HREs using transfection assays have led to identification of consensus response elements for steroid, thyroid and retinoic acid receptors (reviewed by Martinez & Wahli, 1991). Despite the original expectation that each receptor would recognize its specific target element, the different HREs display a high structural similarity that suggests their common origin (Martinez et al., 1991). For example, the glucocorticoid, progesterone, mineralocorticoid and androgen receptors all

bind to the same response element, which can be converted into an estrogen response element (ERE) by swapping of two nucleotides. Deletion of 2 bp from the spacer between the two half-palindromes makes the ERE responsive to ecdysteroid (Fig. 3). Thyroid hormone receptor can bind to the same ERE, and is capable of gene activation if the entire spacer is deleted (Glass et al., 1988). Thyroid hormone and retinoic acid receptors were also shown to act through a common response element (Umesono et al., 1988).

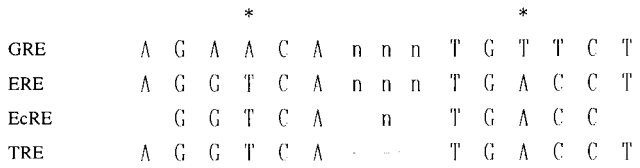

Fig. 3. Hormone response elements (HRE). The DNA binding sites of the glucocorticoid (GRE), estrogen (ERE), ecdysteroid (EcRE), and thyroid hormone (TRE) receptors are compared. Asterisks indicate the nucleotides critical for recognition by the glucocorticoid versus estrogen receptor subfamilies. n denotes any base.

The high degree of HRE conservation raises the question of how receptors can distinguish among similar or identical response elements to specifically activate gene expression. There are two major sites of specificity within a hormone response element. First, the two nucleotides at symmetric positions in the palindrome (Fig. 3) determine whether a

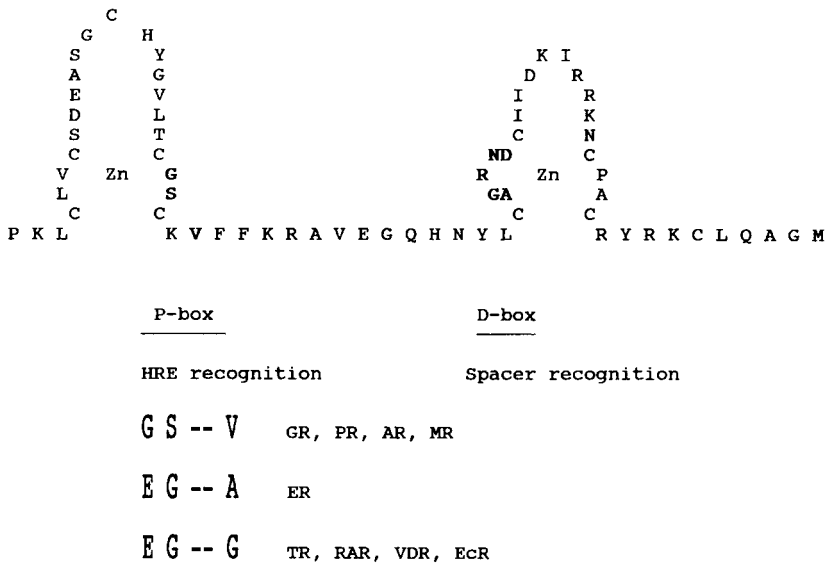

Fig. 4. The glucocorticoid receptor DNA-binding domain composed of two zinc finger motifs. Amino acid residues that are critical for the sequence-specific DNA binding (P-box) and dimerization (D-box) are shown in bold type. P-boxes of the glucocorticoid (GR), progesterone (PR), androgen (AR), mineralocorticoid (MR), estrogen (ER), thyroid hormone (TR), retinoic acid (RAR), vitamin D_3 (VDR) and ecdysone (EcR) receptors are compared.

given HRE will function as glucocorticoid or estrogen response element. Second, the spacing between the two palindrome halves further specifies the receptor type since estrogen, ecdysteroid and thyroid hormone or retinoic acid receptors require spacers of different lengths. However, this still does not provide for the hormone-specific gene activation through identical HREs. Activities of different receptors (e.g. glucocorticoid and progesterone) have been shown to reveal a different correlation with increasing distances between HREs and from other DNA sequences, suggesting that these receptors require different stereospecific arrangement for cooperative DNA binding and for efficient interaction with other trans-acting elements (see Beato et al., 1991; Muller et al., 1991 for reviews).

The palindromic nature of HREs reflects the fact that nuclear hormone receptors bind to DNA as dimers. It has been shown by deletion studies that both zinc finger motifs of the DNA-binding domain are essential for DNA binding, but the N-terminal finger is responsible for the specific DNA target recognition (Green et al., 1988). Mader et al. (1989) have further demonstrated that the ability of the estrogen receptor to distinguish between ERE and GRE resides in three amino acid positions within the N-terminal zinc finger motif of the estrogen receptor, including the glutamic acid (E) and glycine (G) between the second pair of cysteine (C) residues and alanine (A), following the invariant lysine (K). Similarly, conversion of glycine and serine (S) within the corresponding positions of the glucocorticoid receptor into E and G, respectively, is sufficient to confer an estrogen response element specificity on the glucocorticoid receptor (Danielsen et al., 1989). Thus the steroid hormone receptor superfamily can be divided into two major subclasses based on this short amino acid region, which is often referred to as a P-box (proximal). Whereas glucocorticoid, progesterone, mineralocorticoid and androgen receptors possess a **CGSCKV** sequence, the estrogen receptor is characterized by **CEGCKA** residues. Thyroid hormone, vitamin D_3, retinoic acid and ecdysone receptors differ from the estrogen receptor in that they have glycine in the last position (Fig. 4). Importantly, the same subfamilies can be discriminated by comparing the respective hormone response elements (Martinez & Wahli, 1991), suggesting that the amino acids which determine the HRE specificity directly interact with the specific base pairs of HREs. Many orphan receptors whose functions are yet unknown have the same amino acids at the critical positions and therefore should be capable of binding to the same HREs as their related receptors. Extensive sequence comparisons of receptors known in vertebrates and insects (Laudet et al., 1992) have suggested that the divergence of receptors to subfamilies preceded the divergence of arthropod and vertebrate lineages.

The finding of Danielsen et al. (1989) that a chimeric glucocorticoid receptor carrying portions of the interfinger or the C-terminal zinc finger of the estrogen receptor can activate transcription from both GRE and ERE, has indicated that these regions of the DNA-binding domain also prevent nonspecific HRE recognition. The second zinc finger motif has been further implicated in receptor dimerization and in the discrimination of the length of spacers separating the HRE half-sites. More specifically, the five amino acids between the first pair of the invariant cysteines (D-box) appear to specify the HRE spacer length (Fig. 4). Umesono & Evans (1989) have shown that a receptor recognizing a palindrome separated by 3 bp will recognize the same HRE in the absence of the spacer if these

five amino acids are replaced by the D-box of the thyroid hormone receptor whose response element has no spacer.

With the aid of NMR spectroscopy, the three-dimensional structures of the zinc finger-DNA complex have been solved for glucocorticoid (Härd et al., 1990) and estrogen (Schwabe et al., 1990) receptors. In accordance with the biological data discussed above, both models show that each receptor monomer makes a direct contact with a HRE half-site within the DNA major groove via the N-terminal zinc finger motif, whereas the C-terminal finger mediates the receptor dimerization.

ECDYSTEROID CONTROL OF GENE EXPRESSION

Growth of insect larvae is punctuated by a series of molts, necessary to allow expansion of the hardened cuticle as the larva becomes larger. These molts are initiated by surges of the steroid hormone ecdysone, which is converted into an active form 20-hydroxyecdysone (20E) by the fat body and some other organs. The intermolt periods of each larval instar usually begin with low ecdysteroid titer, which transiently elevates towards the end of instar (Sehnal, 1989). Whether a larval molt or metamorphosis will take place is determined by the presence or absence of juvenile hormone (JH) at the time of ecdysone release (reviewed by Riddiford, 1985). In the last instar of lepidopteran and some other insect larvae, one or more minor ecdysone pulses in the absence of JH, usually coincident with the cessation of feeding, are important for the commitment (or genetic reprogramming) of larval tissues to the pupal development. Synthesis and release of ecdysone and juvenile hormones are affected by neuropeptide factors secreted by specialized cells in the CNS, transported via nerves, and released into the blood from neurohemal organs such as the corpora cardiaca. Prothoracicotropic hormone (PTTH) stimulates synthesis and release of ecdysteroids from the prothoracic gland, while allatotropins and allatostatins control JH secretion from the corpora allata.

Puffing of salivary gland chromosomes and ecdysteroid action

Like other steroid hormones, ecdysteroids exert their effects primarily at the level of transcriptional activation or repression of specific genes. Cloning of the ecdysone receptor and a number ecdysteroid-responsive genes have shown that the ecdysteroid mode of action fits well into the general model proposed for other steroid hormones.

The polytene chromosomes of the salivary glands of higher Diptera have proven to be a unique tool to study the hormonal control of development. In these chromosomes, many genes are directly visible as large puffs when active. Puffs appear and regress in a precise temporal sequence. Clever & Karlson (1960) showed that injection of ecdysteroids into late third instar larvae of the midge, *Chironomus tentans*, caused a rapid appearance of two new puffs independent of protein synthesis. These "early puffs" were followed by a second series of puffs, whose induction was prevented by the addition of protein synthesis inhibitors (Clever, 1964). This was the first demonstration in any organism that a hormone could directly affect gene activity.

Using *Drosophila melanogaster*, Ashburner (1972a; 1974) showed that a small number of early puffs appeared within 15–30 min of exposure to 20E in the absence or presence of protein synthesis inhibitors, followed several hours later by a second set of over 100 late puffs that required protein synthesis. This puffing sequence mimicked that normally seen

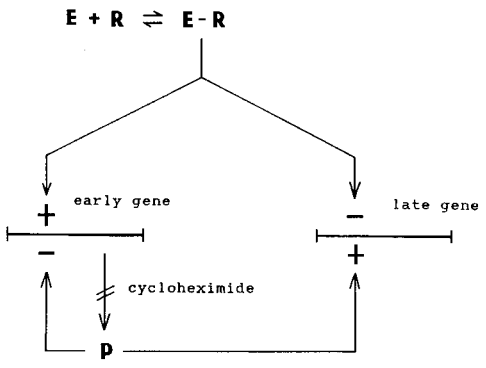

Fig. 5. The Ashburner model of ecdysteroid regulation of the early and late larval puffs in the salivary glands of *Drosophila*. E – ecdysteroid; R – receptor; P – protein products of early puffs.

in the salivary glands prior to pupariation. Importantly, the early puffs regressed spontaneously after a certain period of exposure to 20E and this regression did not occur when protein synthesis was blocked. In contrast, many late puffs expanded prematurely when the hormone was withdrawn. Whereas the early puffs were activated by 20E in a dose-dependent manner, the late puffs displayed a threshold response, indicating an indirect induction by the hormone (Ashburner, 1973). The above findings led Ashburner et al. (1974) to postulate a model of ecdysteroid action (Fig. 5), in which the ecdysone-receptor complex acts directly on the target DNA to activate transcription of specific early puff genes and to prevent the premature expression of the late puff genes. Protein products of the early genes then promote (derepress) transcription of the late genes and inhibit their own synthesis. Thus the products of early puffs can be generally regarded as regulatory proteins (transcription factors) directing expression of other, tissue- and/or stage-specific structural proteins (Ashburner, 1990). The model does not include a special puff class described by Ashburner as "early late" puffs (e.g. 78C, 62E), whose appearance follows closely after 20E addition but requires protein synthesis.

Of the six major early puffs, three have been analyzed at the molecular level and shown to contain genes encoding proteins that possess DNA-binding domains of various types, consistent with their presumed regulatory functions (see Thummel, 1990; Andres & Thummel, 1992 for reviews). The Broad-Complex (BR-C) gene within the 2B5 locus encodes numerous proteins composed of a common core region and one of the three alternate pairs of C_2H_2 zinc finger motifs that may bind to different DNA sites (DiBello et al., 1991). One of the BR-C products is required for transcription of six genes in the 71E late puff (Guay & Guild, 1991). The 74EF locus encompasses 3 transcriptional units of the E74 gene that belong to the ETS proto-oncogene family (Burtis et al., 1990). It has been shown that one of the products of the E74 gene binds to numerous early and late puffs including 74EF itself (Urness & Thummel, 1990) and that the repression of E74 synthesis depends on early gene activity (Thummel et al., 1990). Finally, the E75 gene at the 75B position gives rise to at least three transcripts encoding members of the steroid hormone receptor superfamily (Feigl et al., 1989; Segraves & Hogness, 1990; Karim & Thummel, 1992). The E75A protein is localized to many early and late puff loci including 75B itself (Hill et al., 1993).

In support of the Ashburner model, BR-C, E74 and E75 transcription can be directly induced by 20E (Thummel et al., 1990; Segraves & Hogness, 1990; Karim & Thummel, 1992). These genes are expressed in a wide variety of ecdysteroid target tissues and

mutations of them are typically lethal during late larval, prepupal and pupal development, indicating that they play critical roles in metamorphosis (Segraves & Richards, 1990). In depth studies on the E74 gene (Thummel et al., 1990; Boyd et al., 1991; Karim & Thummel, 1991) led to an extension of the Ashburner model called the tissue coordination model, according to which the ecdysteroid regulatory hierarchy is not limited to the late third *Drosophila* salivary glands, but applies to other tissues and developmental stages as well. The tissue- and stage-specific response to the hormone is proposed to be determined by different combinations of the early regulatory proteins and their multiple isoforms. Recent finding of three alternative ecdysone receptor isoforms and their differential spatial distribution in *Drosophila* prepupae (Talbot et al., 1993) introduces further complexity to the model and suggests that the type of receptor molecule itself might also specify the character of response in a given cell.

Ecdysteroid receptor and related *Drosophila* genes

In *Drosophila*, 10 members of the steroid hormone receptor gene superfamily have been characterized to date (Table 1) that encode proteins possessing both the DNA- and ligand-binding domains. However, only the ecdysone receptor (EcR) can presently be called a receptor as the ligand specificities of all the remaining nine proteins are yet unknown. In contrast to this number, only two types of insect hormones are known that can be expected to act via nuclear receptors, ecdysteroids and the sesquiterpenoid juvenile hormones. Since there are only very distant homologies between EcR and other putative insect receptors (Koelle et al., 1991), it is unlikely that some of the orphans would bind 20E or another ecdysteroid. Initially it seemed promising that the E75 protein might be a JH receptor but its temporal expression practically precludes this possibility. Transfection studies indicated that E75 is not a receptor of either 20E or JH (W.A. Segraves, pers. commun.). Retinoic acid, a regulator of morphogenetic events in vertebrates, is another conceivable ligand as its diterpenoid structure resembles that of JH. Although retinoic acid and some other retinoids had a weak JH activity in *Manduca sexta* (Palli et al., 1991), there is no evidence of vitamin A requirements for *Drosophila* development (Oro et al., 1990). The major argument supporting the idea that there are specific ligands for the insect orphan receptors derives from the structural similarity shared by their ligand-binding domains (Segraves, 1991). This would mean that there are either unknown steroid-like regulators in insects, or that these receptors interact with some other types of molecules. The fact that both insects and vertebrates possess many orphan receptors (Laudet et al., 1992) suggests that the missing ligands may be small intracellular regulators or second messengers rather than true hormones. In such a case, receptor activation would occur only in specific cell types. Alternatively, the orphans may be ligand-independent transcription factors or dimerization partners for EcR.

ECDYSONE RECEPTOR (EcR). The EcR gene at the cytological position 42A was originally cloned by homology with the E75 cDNA and shown to restore hormone responsiveness in ecdysteroid-resistant Schneider 2 (S2) cells (Koelle et al., 1991). The EcR protein was shown to bind [^{125}I] labeled ponasterone A (an active molting hormone analog) and to specifically recognize a previously discovered ecdysteroid response element (EcRE). Transfection of the EcR gene into the *Drosophila* S2 cells caused 210-fold induction of a reporter gene carrying multiple EcRE copies in response to 20E (Koelle et al., 1991).

However, transfection of EcR failed to confer ecdysteroid responsiveness to a mammalian cell line CV1, indicating that some necessary auxiliary factor was lacking in these heterologous cells. Yao et al. (1992) and Thomas et al. (1993) have found that EcR must dimerize with another *Drosophila* receptor protein, ultraspiracle (USP), in order to produce a transcriptionally active ecdysone receptor. Indeed, EcR and USP were colocalized by specific antibodies in ecdysteroid-responsive puffs on the salivary gland chromosomes (Yao et al., 1993).

TABLE 1. *Drosophila* members of the steroid hormone receptor superfamily. The numbers in the right two columns indicate per cent amino acid identities in the DNA- and ligand-binding domains. Only such vertebrate proteins are listed that reveal identities > 60% in the DNA-binding region. References for all *Drosophila* receptors are given in text. References for other insect homologs are: [a]Imhof et al. (1993); [b]Tzertzinis et al. (unpublished); [c]Segraves & Woldin (1993); [d]Jindra et al. (1994b); [e]Palli et al. (1992); [f]Jindra et al. (1994a) and [g]Ueda & Hirose (1990). [h]human retinoid X receptor α (Mangelsdorf et al., 1990); [i]human erb-A-related protein 1 (Miyajima et al., 1989); [j]human RZRα protein (Becker-Andre et al., 1993); [k]mouse long terminal repeat-binding protein (Tsukiyama et al., 1992); [l]chicken ovalbumin upstream promoter transcription factor (Wang et al., 1989); [m]rat hepatocyte nuclear factor 4 (Sladek et al., 1990). [n]The authors used only a selected portion of the domain for comparison.

Receptor	Functions or properties	Ligand	P-box	Multiple forms or mRNAs reported	Homologs reported in other insects	Closest vertebrate relative	% amino acid identity	
							DNA	Ligand
EcR	Ecdysone receptor, expressed in most tissues Directly induced by 20E	20E	EGCKG	Yes	*Chironomus*[a]			
USP	Formation of functional 20E receptor heterodimer with EcR Abdominal segmentation, larval development, eye morphogenesis	?	EGCKG	No	*Bombyx*[b]	RXRα[h]	86	49
E75	75B early puff product Directly induced by 20E in most tissues	?	EGCKG	Yes	*Manduca*[c] *Galleria*[d]	ear-1[i]	79	35
DHR3	Product of the 46F puff Directly induced by 20E?	?	EGCKG	Yes	*Manduca*[e] *Galleria*[f]	RZRα[j]	76	32
E78	78C early late puff product Full induction by 20E requires protein synthesis	?	EGCKG	Yes	No	ear-1	74	35
FTZ-F1	Embryonic pattern formation Activator of the ftz segmentation gene and Adh gene	?	ESCKG	Yes	*Bombyx*[g]	ELP[k]	88	56[n]
DHR39	Expressed mainly in embryonic brain and ventral CNS Coregulator of Adh and ftz?	?	ESCKG	Yes	No	ELP	62	17
SVP	Embryonic CNS development Cell fate determination in compound eye morphogenesis	?	EGCKS	Yes	No	COUP-TF[l]	94	93
TLL	Embryonic pattern formation of terminal structures	?	DGCAG	No	No			
HNF-4(D)	Embryonic midgut, fat body and malpighian tubule organogenesis	?	DGCKG	Yes	No	HNF-4(R)[m]	90	67

While the EcR/USP heterodimer displayed an enhanced EcRE binding and transcriptional activity, EcR alone proved incapable of both. Moreover, USP could substitute for its vertebrate counterpart RXRα (retinoid X receptor) in that it combined with the heterodimerizing vertebrate receptors, increasing their DNA binding activities. These studies have demonstrated that both vertebrates and insects share a common mechanism of hormonal regulation through a promiscuous receptor partner. Unexpectedly, RXRα failed to substitute for USP in either DNA binding or transactivation in the CV1 cells in response to 20E (Yao et al., 1993). However, both the DNA binding and transactivation by the EcR/RXR complex were observed in the presence of a potent ecdysteroid agonist muristerone A (Thomas et al., 1993; Yao et al., 1993). The differential response of the EcR heterodimers to 20E vs. muristerone A indicates that not only the dimerizing partner but also the interaction with ligand may determine the properties of a receptor. These data also suggest that there may be other partners for EcR in *Drosophila* that could produce receptors with diverse ligand specificities.

Talbot et al. (1993) have recently shown that the EcR gene spans over 73 kb and encodes at least three proteins [EcR-A, EcR-B1 (previously known as EcR) and EcR-B2] that have identical DNA- and ligand-binding domains but differ in their N-terminal A/B regions. All three EcR isoforms are functional ecdysone receptors. Monoclonal antibodies directed against the unique A/B domains of EcR-A and EcR-B1 revealed a differential distribution of the two isoforms in cell nuclei of various tissues in *Drosophila* prepupae. Interestingly, the ratio of EcR-A vs. EcR-B1 expression reflected the character of tissues. For example, EcR-B1 predominated in strictly larval tissues (fat body, muscle, midgut, salivary gland, epidermis), whereas large discs and rings of imaginal cells (e.g. wing discs) revealed a reciprocal isoform expression. Small clusters of imaginal cells (midgut islands, epidermal histoblast nests) had high levels of EcR-B1, while EcR-A was not detected (Talbot et al., 1993). The EcR isoforms have also been implicated in cell fate determination of neurons in the *Drosophila* CNS during larval-adult transition. At the onset of metamorphosis, the neurons to be remodeled for an adult function initially express EcR-B1 and later EcR-A, while those programmed to perish after adult eclosion express high levels of EcR-A (Robinow et al., 1993, Truman at al., 1994).

Developmental studies (Karim & Thummel, 1992; Andres et al., 1993; Talbot et al., 1993) have shown that EcR transcripts are present in all *Drosophila* stages and that higher mRNA levels coincide with increased ecdysteroid titer. EcR transcription can be induced by 20E in cultured *Drosophila* tissues (Karim & Thummel, 1992). Compared to other ecdysteroid primary response transcripts, EcR (together with E74B and some BR-C mRNAs) is sensitive to lower 20E doses and appears earlier both after the hormone addition in vitro and in third instar larvae (Karim & Thummel, 1992; Andres et al., 1993). Although USP is an integral component of the active ecdysone receptor, its expression appears to be constant and largely independent of ecdysteroid titer throughout development (Andres et al., 1993). It is therefore regulation of EcR expression that is thought to determine the level of functional receptor in response to ecdysteroid.

OTHER MEMBERS OF THE NUCLEAR RECEPTOR SUPERFAMILY. USP, also called CF1 or 2C, maps to the cytological position 2C (Henrich et al., 1990; Shea et al., 1990; Oro et al., 1990). The USP protein is most closely related to the human retinoid X receptor α (RXRα; Mangelsdorf et al., 1990). USP, however, is not a retinoid receptor as it fails to respond to

either all-trans or 9-cis retinoic acid, the natural RXRα ligand (Heyman et al., 1992). The functional similarity between USP and RXRα in modulating functions of other receptor molecules by heterodimer formation has been discussed above. USP is essential for embryonic abdominal segmentation, eye morphogenesis and in multiple tissues throughout development (Oro et al., 1992).

The 75B early puff gene E75 is structurally similar to EcR in that it encodes three isoforms designated E75A, E75B and E75C that arise by alternative splicing of unique 5' exons to a common set of 3' exons (Feigl et al., 1989; Segraves & Hogness, 1990; Karim & Thummel, 1992). Containing both the DNA-binding domain composed of two C_2C_2 zinc fingers and a ligand-binding domain, E75A and C belong to the steroid hormone receptor family. The E75B isoform is an exception since it only has the C-terminal zinc finger and thus may not bind DNA. To my knowledge, E75B is the only one-fingered steroid receptor superfamily protein identified to date. However, the biological purpose of this feature is not understood. Sharing the same C-terminal domains with E75A and C, E75B might form inactive heterodimers with these or other (EcR) receptor proteins, incapable of binding DNA. Although the E75A protein binds to numerous early and late puff loci (Hill et al., 1993), the particular genes whose expression it regulates remain to be identified. The E75 transcripts can be directly induced by 20E in cultured *Drosophila* organs (Karim & Thummel, 1992) and salivary glands (Segraves & Hogness, 1990). Consistent with the Ashburner model (Fig. 5), the presence of cycloheximide does not block E75 transcription but prevents its decline. Recently, homologs of E75 were cloned from *Manduca sexta* (Segraves & Woldin, 1993) and *Galleria mellonella* (Jindra et al., 1994b) and shown to share many structural features with the *Drosophila* gene including the existence of the one-fingered B isoform. In *Galleria* isolated larval abdomens, the E75A mRNA was induced within 20 min by 20E injection.

The DHR3 (*Drosophila* hormone receptor 3; Koelle et al., 1992) gene at the 46F locus encodes at least three transcripts that are abundant during embryonic, late larval, prepupal and pupal development. The 46F puff appears at the time of pupariation, somewhat later than the early puffs but before the prepupal puffs and apparently belongs to a special puff class (Ashburner, 1972b; Walker & Ashburner, 1981). The response of DHR3 to 20E has not been examined in *Drosophila*. A similar gene (MHR3) was found in *Manduca sexta* in a search for insect homologs of the human retinoic acid receptor (Palli et al., 1991). In cultured *Manduca* larval epidermis, MHR3 was induced by 20E with maximal mRNA levels reached after 6 hr of exposure (Palli et al., 1992). This induction was largely, but not entirely, independent of protein synthesis, indicating that MHR3 is an ecdysteroid primary response gene that requires some protein synthesis for full expression. Transcription of a homologous *Galleria* gene GHR3 can be induced by 20E injection in isolated larval abdomens within 1–3 hr (Jindra et al., 1994a).

Another recently discovered receptor gene E78 (Stone & Thummel, 1993) originates from the "early late" puff locus 78C. This puff forms relatively early after 20E addition and (like 46F) does not prematurely expand if the hormone is washed off. Its appearance however requires protein synthesis. The E78 gene encodes 2 isoforms E78A and E78B that share 3' exons but have different 5' exons. The E78B transcript is of particular interest since it lacks both exons encoding the two zinc fingers of the DNA-binding domain. Moreover, E78B but not E78A transcription can be directly induced in cultured

Drosophila organs, although the mRNA level is reduced in the presence of cycloheximide. E78B is similar to MHR3 in that it also requires protein synthesis for full induction (Palli et al., 1992), and different from early genes (E75, E74) that become overinduced if protein synthesis is blocked. Unlike the early genes, E78 is expressed in a highly stage-specific manner. E78A mRNA appears briefly in developing adults, E78B is expressed during puparium formation and pupal development. Stone & Thummel (1993) propose that the E78B "fingerless" protein might serve as an ecdysteroid-induced repressor of early gene expression, perhaps by formation of an inactive heterodimer with another receptor.

The FTZ-F1 gene (Lavorgna et al., 1991) maps to the 75CD mid-prepupal puff. Recently, FTZ-F1 has been shown to encode two proteins αFTZ-F1 and ßFTZ-F1 that differ in their N-termini (Lavorgna et al., 1993). αFTZ-F1 is expressed in early embryos and early during the third larval instar; ßFTZ-F1 appears in late embryos and briefly in mid-prepupae, coincident with puffing at the 75CD locus (Andres et al., 1993). The FTZ-F1 gene had been previously implicated in embryonic segmentation and the αFTZ-F1 protein was shown to activate the fushi tarazu (ftz) pair rule gene expression. Besides the ftz response element, αFTZ-F1 also binds to the alcohol dehydrogenase (Adh) distal promoter. The role of ßFTZ-F1 in prepupae is unknown, but a polyclonal antibody raised against this protein stained as many as 166 loci (incl. 75B, 74EF and 75CD) on the salivary chromosomes (Lavorgna et al., 1993). The closest vertebrate relative of FTZ-F1 is the mouse ELP orphan receptor that binds to the long terminal repeat of Moloney leukemia virus to repress its transcription (Tsukiayma et al., 1992).

Another related *Drosophila* gene has been independently cloned by two groups and named FTZ-F1ß (Ohno & Petkovich, 1992) and DHR39 (Ayer et al., 1993). The latter designation is more appropriate since this gene maps to the 39B-C locus and therefore is distinct from FTZ-F1. An interesting feature of DHR39 is that it binds to the same 12 bp regulatory element of Adh as FTZ-F1, but it acts as a repressor of this gene. Moreover, a natural DHR39 isoform that lacks a portion of the E region important for dimerization has no effect on Adh transcripiton (Ayer et al., 1993). DHR39 also binds to the ftz promoter, suggesting that DHR39 might coregulate ftz expression, perhaps as an FTZ-F1 antagonist. DHR39 is expressed throughout development, but in the late embryo it is particularly abundant in the brain and ventral CNS.

Sevenup (svp; Mlodzik et al., 1990) is another *Drosophila* gene that has an apparent vertebrate counterpart, the COUP transcription factor (Wang et al., 1989). The SVP expression is important for embryonic CNS development and has been shown to determine the cell fate of photoreceptor neurons during the compound eye morphogenesis. SVP is expressed in four of the eight photoreceptor precursor cells; lack of SVP transforms these cells to a functionally and morphologically distinct R7 cell type (Mlodzik et al., 1990). Recently two members of the SVP/COUP subfamily have been found in zebrafish (*Brachydanio rerio*) and shown to be expressed primarily in the embryonic CNS and eye primordia, suggesting conserved roles in insects and vertebrates (Fjose et al., 1993).

Tailless (tll; Pignoni et al., 1990) is a zygotic terminal gene required for the determination of anterior and posterior domains in the early embryo. TLL is strongly expressed in the developing brain and peripheral nervous system.

Expression of the rat hepatocyte nuclear factor [HNF-4(R); Sladek et al., 1990] is mainly restricted to the adult intesine, liver and kidney. Zhong et al. (1993) have found a

homologous HNF-4(D) gene in *Drosophila* that is initially expressed in the embryo from maternally deposited mRNA, and later in the primordia of midgut, fat body and malpighian tubules, i.e. organs that have functions parallel to the mammalian intestine, liver and kidney. HNF-4(D) seems to be critical for organogenesis of the *Drosophila* tissues as they fail to develop and grow properly in HNF-4(D)⁻ mutants (Zhong et al., 1993).

Besides the above listed genes encoding true members of the steroid hormone receptor family, there are three closely related genes in *Drosophila* that encode typical DNA-binding domains but lack homologous ligand-binding domains. These genes have been designated knirps (kni), knirps-related (knrl), and embryonic gonad (egon) (Nauber et al., 1988; Oro et al., 1988; Rothe et al., 1989). Kni is a gap segmentation gene required for the embryonic abdominal pattern formation. The distribution of the KNRL product in the early embryo is similar to that of KNI and expression of both is directed by a posterior determinant gene nanos. EGON expression occurs later in the embryonic development and is restricted to the gonads (Rothe et al., 1989). It is possible that this subfamily of genes evolved by incomplete duplication of an ancestor gene and that KNI, KNRL and EGON might either serve as receptors for structurally unrelated compounds or act as ligand-independent transcription factors (Laudet et al., 1992).

Ecdysteroid primary response genes

Based on the puffing activity in the *Drosophila* salivary gland chromosomes, three direct ecdysteroid effects can be distinguished. The hormone-receptor complex (1) represses the intermolt puffs such as those encompassing the glue genes, (2) induces the early puffs, and (3) prevents premature expression of the late puffs (Fig. 5). Besides that, expression of many genes depends directly or indirectly on ecdysteroid whose activities cannot be visualized on the salivary gland chromosomes. To date, over 50 ecdysteroid-regulated genes have been described using the *Drosophila* salivary glands, imaginal discs, several cell lines, and mutants deficient in ecdysteroid synthesis or release. Functions and responses to ecdysteroid of some of them have been recently reviewed by Riddiford (1993) and Andres et al. (1993). Below, I will only consider genes induced by 20E as a primary response, i.e. independent of protein synthesis.

The early puff genes BR-C, E74, and E75 were already described above. The genomic organization of BR-C, E74, E75, as well as that of another ecdysteroid primary response gene, EcR, is remarkably similar and very complex. Extensive molecular studies have revealed that these genes possess multiple promoters and that they are unusually long (up to 100 kb) for *Drosophila* genes. All of them contain numerous exons, some of which can be alternatively used in different transcripts, separated by vast introns. Thummel (1992) has proposed that the delay caused by synthesis of these long primary transcripts may be significant for the proper timing of gene expression in the regulatory cascade. The E74 gene of *Drosophila* has three transcription units E74A, E74B1 and E74B2 that derive from unique promoters but share a single polyadenylation site (Burtis et al., 1990). Both E74A and B mRNAs are directly induced by ecdysteroid in a variety of tissues (Thummel et al., 1990; Boyd et al., 1991), displaying different temporal distribution. The E74B isoform appears earlier than E74A in the third instar larvae and prepupae and after 20E addition in vitro. This delay is consistent with the difference in the E74A and B primary transcript lengths and a measured transcript elongation rate of 1.1 kb per minute. E74B is also

induced by a 20E concentration 25-fold lower than that required for the initiation of E74A transcription (Karim & Thummel, 1991). Both E74 proteins have a common ETS DNA-binding domain at their C-termini that is 50% identical to that of the human c-ets-2 proto-oncogene. Besides other early and late ecdysteroid-induced puff sites, E74A protein binds to the E74 gene at three elements similar to those recognized by other ETS nuclear proteins in mammals (Urness & Thummel, 1990).

Karim & Thummel (1992) have shown that in cultured *Drosophila* organs, some early transcripts including E74B start to appear at lower 20E doses than others. Thus the early transcripts could be divided into 2 classes based on the threshold 20E concentration. Whereas class I transcripts (E74B, EcR and certain BR-C mRNAs) respond to $2x10^{-9}$ M 20E, class II (E74A, E75A, E75B) requires at least $1x10^{-8}$ M 20E concentration for induction. These data indicate that the category of early genes is not as simple as might be expected from the Ashburner model. Rather, there seems to be a cascade of differential gene activity triggered by the gradually increasing ecdysteroid titer in vivo.

In addition to the genes originating from the early puffs, there are several other genes directly induced by ecdysteroid whose expression is tissue-specific. Since these genes do not seem to encode transcription factors, their protein products might be involved in facilitating the tissue-specific ecdysteroid effects (reviewed by Andres & Thummel, 1992). Three genes (IMP-E1, IMP-E2 and IMP-E3) have been identified that are specifically expressed in the *Drosophila* imaginal discs. These genes can be induced in the imaginal discs cultured with 20E and cycloheximide (Moore et al., 1990; Paine-Saunders, 1990; Natzle, 1993). Expression of the Fbp1 gene (originally known as P1) is restricted to the fat body of late third instar *Drosophila* larvae (Lepesant et al., 1986). Fbp1 can also be induced by 20E in vitro, independent of protein synthesis. Finding of a sequence similar to the ecdysteroid response element within the Fbp1 promoter further indicates direct ecdysteroid induction of this gene (Maschat et al., 1991; Antoniewski et al., 1993). The function of Fbp1 is presently unknown.

Molecular analyses of two other ecdysteroid-induced genes, hsp27 (encoding a small heat shock protein) and Eip28/29, led to the characterization of the ecdysteroid response element (EcRE), to which the EcR protein binds (Fig. 3). Riddihough & Pelham (1987) have shown that a 23 bp sequence within the hsp27 promoter is responsible for the 20E stimulation of hsp27 mRNA synthesis in transfected *Drosophila* S1 cells and was sufficient to confer the 20E responsiveness to a heterologous promoter. Using the hsp27 EcRE in affinity chromatography, Luo et al. (1991) biochemically purified the ecdysone receptor protein.

Although the Eip28/29 gene is expressed in a complex tissue-specific pattern in the third instar larvae, its function is as yet unclear (Cherbas et al., 1990; Andres et al., 1993). In the Kc cell line derived from *Drosophila* embryos, Eip28/29 is induced by 20E within minutes in the presence of cycloheximide. Using a binding assay with Kc cell extracts containing the ecdysone receptor and [^{125}I]-ponasterone A, Cherbas et al. (1991) identified three EcREs in the Eip 28/29 flanking regions, two downstream of the polyadenylation site and one upstream of the Eip28/29 coding region. Each of these elements conferred ecdysteroid responsiveness to a heterologous promoter. Only the two downstream EcREs, however, were required for the 20E induction in Kc cells, whereas the upstream element was not necessary for this induction. Martinez et al. (1991) and Ozyhar et al. (1991) have

shown that the imperfect palindrome with a single base pair spacer 5'-GGTTCAATGCACT-3' within the hsp27 promoter is required for EcR binding. Comparison of the Eip28/29 EcREs with that of hsp27 has suggested a consensus ecdysteroid response element 5'-RG(G/T)TCAnTGA(C/A)CY-3' (Cherbas et al., 1991). Based on electrophoretic mobility shift assays with mutated hsp27 and Fbp1 EcREs, Antoniewski et al. (1993) have recently proposed a revised and slightly more flexible version of the EcRE consensus 5'-RG(G/T)T(C/G)AnTG(C/A)(C/A)(C/t)Y-3'.

DROSOPHILA AND OTHER INSECT MODELS

The recent data reviewed in this article document the major contribution of modern molecular techniques to our understanding of the mechanisms underlying steroid hormone action. Insect models are of growing importance in what has been primarily a vertebrate field. In particular, *Drosophila melanogaster* provides several unique experimental features: (1) with the aid of the salivary gland polytene chromosomes, the relatively small *Drosophila* genome has been mapped in detail unmatched in any other organism, (2) the chromosome puffing is a powerful tool for monitoring gene activity, (3) fast reproduction allows extensive search for mutants and flies deficient in particular functions are already available for studies, and (4) the actual roles of cloned genes can be tested in transformed flies.

The basic scheme of alternation of molt and intermolt periods directed by ecdysteroid pulses seems common to most insects. Recent findings of homologs in other insects of several *Drosophila* genes involved in the ecdysteroid action (Table 1) further suggest the existence of a conserved mechanism, although some differences start to appear as we look more closely at these genes. For example, the E75 genes of lepidopterans do not seem to produce the long primary transcripts proposed to delay the expression of some early proteins in *Drosophila*, indicating that this mechanism may not be a universal one. The *Drosophila* E75 proteins also have much larger terminal domains which are not highly conserved and which, therefore, might interact with transcription factors different from those of Lepidoptera.

However, the small size of *Drosophila* and the lack of morphological markers that would allow convenient staging are major disadvantages. For example, many of the in vitro studies on the *Drosophila* ecdysteroid-induced genes were performed on mixed "mass-isolated organs" from animals staged only by time. Therefore, molecular biology studies on other insects not only have the comparative value. Using the large lepidopteran larvae, one can easily dissect organs such as the epidermis, central nervous system or silk glands from precisely staged individuals and culture them under conditions of controlled hormonal milieu. Ecdysteroid-responsive genes recently cloned from lepidopterans provide us with an arsenal of specific markers essential for studies of hormonally triggered physiological and developmental events.

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