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The Drosophila *EcR* Gene Encodes an Ecdysone Receptor, a New Member of the Steroid Receptor Superfamily

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Summary

The steroid hormone ecdysone triggers coordinate changes in Drosophila tissue development that result in metamorphosis. To advance our understanding of the genetic regulatory hierarchies controlling this tissue response, we have isolated and characterized a gene, *EcR*, for a new steroid receptor homolog and have shown that it encodes an ecdysone receptor. First, EcR protein binds active ecdysteroids and is antigenically indistinguishable from the ecdysone-binding protein previously observed in extracts of Drosophila cell lines and tissues. Second, EcR protein binds DNA with high specificity at ecdysone response elements. Third, ecdysone-responsive cultured cells express EcR, whereas ecdysone-resistant cells derived from them are deficient in EcR. Expression of *EcR* in such resistant cells by transfection restores their ability to respond to the hormone. As expected, EcR is nuclear and found in all ecdysone target tissues examined. Furthermore, the *EcR* gene is expressed at each developmental stage marked by a pulse of ecdysone.

Introduction

In Drosophila melanogaster, metamorphosis to the adult fly is triggered by a pulse of the steroid hormone 20-hydroxyecdysone that occurs at the end of larval life (reviewed by Richards, 1981a). Most, if not all, tissues are ecdysone targets at this stage of development. In one of these targets (the larval salivary gland), the transcriptional activity of more than 100 ecdysone-regulated genes can be monitored by the size of the polytene chromosome puff at the locus of the respective gene. Ashburner and his colleagues proposed a model for this puff response that was based on their studies of isolated glands exposed to ecdysone under a variety of conditions (Ashburner et al., 1974; Ashburner and Richards, 1976). In this model, ecdysone, complexed with a hypothesized receptor protein, differentially regulates the transcription of two classes of target genes: a small class of "early" genes that are directly induced by the complex, and a large class of "late" genes that are repressed by the complex. Transcription factors encoded by early genes then produce two kinds of secondary response to the hormone by counteracting each of the above ecdysone receptor functions. Thus, at least one of these factors is assumed to repress early gene transcription, while one or more factors induce late gene transcription.

Tests of the Ashburner model at the molecular level have focused on the early genes and their proteins. Of the half-dozen early gene loci defined by the puffing studies, three have been analyzed at this level: the *E75* gene at the 75B puff site (Segraves, 1988; Feigl et al., 1989; Segraves and Hogness, 1990), *E74* at the 74EF site (Burtis, 1985; Janknecht et al., 1989; Burtis et al., 1990; Thummel et al., 1990), and a member of the *Broad-Complex* at the 2B5 site (Chao and Guild, 1986; Belyaeva et al., 1987; Kiss et al., 1988; Galceran et al., 1990; DiBello et al., 1991). The results of these analyses meet several expectations of the model. Not only is the transcription of each gene induced by ecdysone in a primary response and subsequently repressed in a secondary response, but these genes encode DNA-binding proteins with properties indicative of transcription factors. For example, the amino acid sequences of the E75 and the E74 proteins indicate that they belong, respectively, to the steroid receptor and *ets* proto-oncogene superfamilies, while the proteins encoded by the gene at 2B5 contain arrays of the Cys_2His_2 zinc-finger motif (references above). The recent observations that E74 and E75 proteins bind to both classes of ecdysone-inducible puff sites provide further support for the proposition that the early gene proteins mediate the secondary ecdysone response of early gene repression and of late gene induction (E74 [Urness and Thummel, 1990]; E75 [R. J. Hill, W. A. S., W. S. T., E. McAvoy, and D. S. H., unpublished data]).

These studies also provided the basis for extending the Ashburner model beyond its original confines of the larval salivary gland to the other ecdysone target tissues involved in the coordinated process of metamorphosis (Burtis et al., 1990; Thummel et al., 1990). Thus, early gene transcription is induced by the late larval pulse of ecdysone not only in the salivary gland but in many other target tissues, including both the imaginal tissues that metamorphose to adult structures and the strictly larval tissues that, like the salivary glands, are programmed for cell death ending in tissue histolysis. If genetic regulatory hierarchies akin to that postulated in the Ashburner model are operative in each of these target tissues, the question arises as to when the tissue specificity of the ecdysone response is first introduced into the hierarchy: at the level of the ecdysone receptor gene, of the early genes, or of the late genes. Presumably, the same question arises at the other stages of development that are similarly marked by a pulse of ecdysone, during which early gene transcription is also observed (Segraves, 1988; Thummel et al., 1990). Clearly, the identification and characterization of the ecdysone receptor gene and its products are a prereq-

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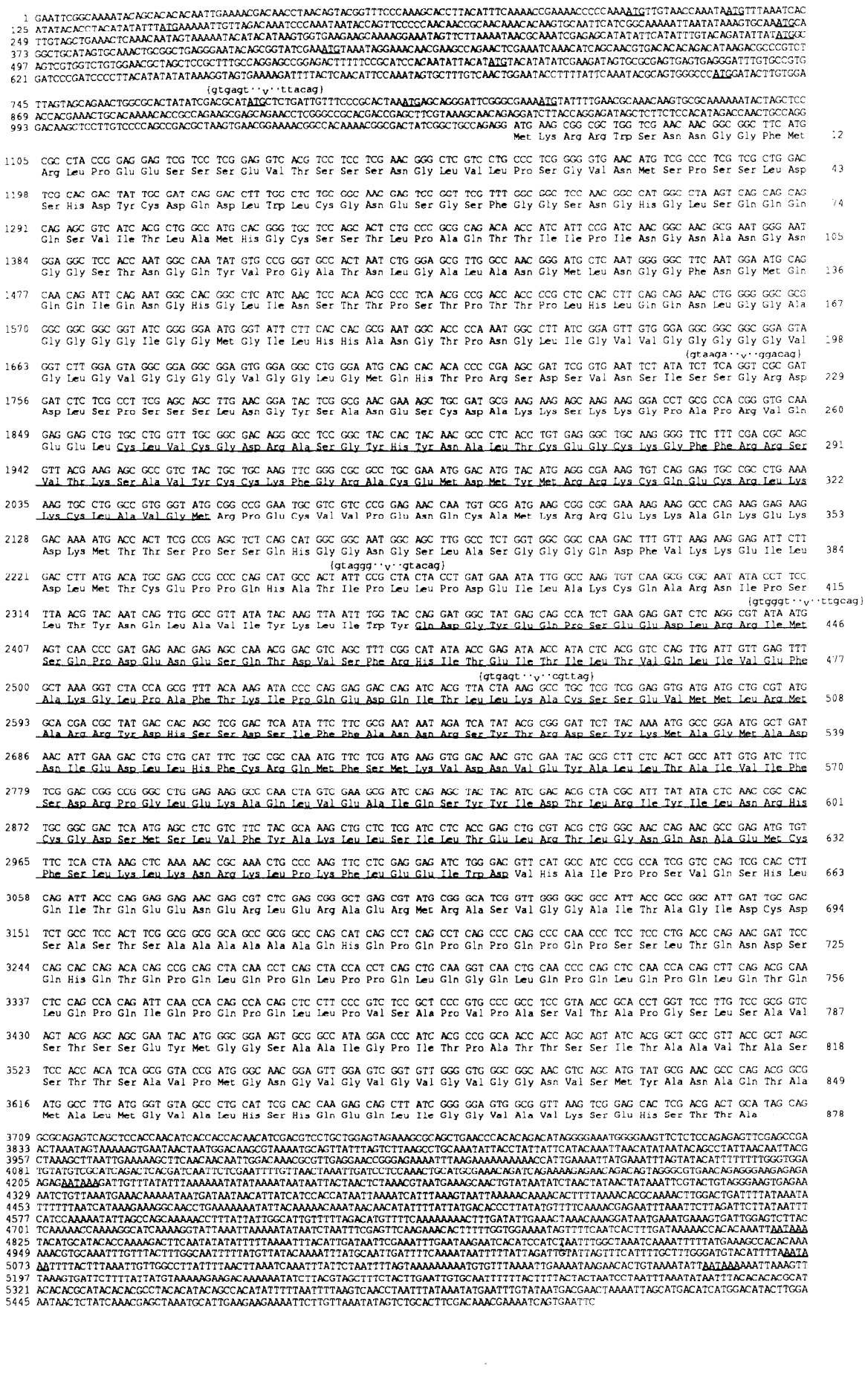

uisite for answering this question and for further definition of the ecdysone response.

Proteins with the biochemical properties expected of an ecdysone receptor have been detected in crude Drosophila extracts by two different assays. Proteins with high affinity for ecdysone have been identified in extracts of a number of Drosophila tissues and in the Kc cell line (Cherbas et al., 1988, and references therein). In addition, a DNA-binding activity specific for an ecdysone response element (EcRE) has been detected in extracts of Schneider 1 cells (Riddihough and Pelham, 1987). This EcRE sequence is a potential ecdysone receptor-binding site, since it can confer ecdysone inducibility on a heterologous promoter. These ecdysone- and DNA-binding activities are likely to reside in the same protein (Cherbas et al., 1991).

In recent years, genes encoding receptors for the vertebrate steroid hormones, thyroid hormone, and retinoic acid have been cloned. These receptors form a family of homologous, hormone-regulated transcription factors, known as the steroid receptor superfamily (for reviews see Evans, 1988; Green and Chambon, 1988). In Drosophila, a number of genes encoding proteins with some sequence similarity to the steroid receptor superfamily members have been identified (Nauber et al., 1988; Oro et al., 1988; Rothe et al., 1989; Henrich et al., 1990; Mlodzik et al., 1990; Oro et al., 1990; Pignoni et al., 1990; Segraves and Hogness, 1990; Shea et al., 1990; Koelle et al., 1991; Lavorgna et al., 1991). One of these is, of course, the ecdysone-inducible early gene *E75*. Neither *E75* nor any of the other Drosophila genes, however, have been shown to encode a receptor for ecdysone or any other known hormone.

In an effort to identify an ecdysone receptor gene, we have searched for additional members of the steroid receptor superfamily in Drosophila (Koelle et al., 1991). In this paper we characterize a gene identified in this screen and show that it encodes a protein with all of the characteristics expected of an ecdysone receptor. Consequently, we have named this gene *EcR* and the protein it encodes, EcR.

Results

The *EcR* Gene Is a Member of the Steroid Receptor Superfamily

The *EcR* gene was identified in a screen of the Drosophila genome for members of the steroid receptor superfamily (Segraves, 1988; Koelle et al., 1991). In this screen, the gene for the previously identified Drosophila steroid receptor homolog, *E75*, provided the hybridization probe, which consisted of the cDNA sequence encoding the putative DNA-binding domain and adjacent amino acids. Under low stringency conditions this probe detected the *EcR* gene on genomic Southern blots, and was used to isolate *EcR* clones from a Drosophila genomic library. By in situ hybridization to polytene chromosomes, the *EcR* gene mapped to the cytological location 42A (Segraves, 1988; Koelle et al., 1991). Following preliminary Northern blot analysis, the genomic clones were used to screen a cDNA library prepared from third instar tissues treated with ecdysone and cycloheximide. This allowed the isolation of a large number of cDNA clones since the *EcR* mRNA has a peak of expression in late third instar after the rise in ecdysone titer (see below). The insert sizes of 20 cDNA clones were determined, and the longest (clone *EcR*-17) was selected for nucleotide sequencing.

Figure 1 shows a 5534 bp cDNA sequence, of which nucleotides 1–5194 are from the *EcR*-17 clone, while nucleotides 5195–5534 are from a shorter cDNA (clone *EcR*-9) that was subsequently sequenced because of its longer 3′ tail. The predominant *EcR* transcript is 6 kb (see Figure 8A), which approximates the length of this cDNA sequence plus a poly(A) tail, indicating that this cDNA sequence is nearly full length. The sequence contains an 878 codon AUG-initiated open reading frame (ORF) that encodes a new steroid receptor protein, as discussed below. This ORF shows an excellent match to Drosophila codon usage (O'Connell and Rosbash, 1984). A second AUG occurs as the 12th codon of this ORF. Neither AUG is found in a context with a strong match to the general eukaryotic (Kozak, 1984) or Drosophila (Cavener, 1987) translation start consensus sequences. The long ORF is preceded by a 1068 bp AT-rich leader containing 11 short AUG-initiated ORFs, the longest of which is 25 codons. A similarly long and complex leader sequence has been observed in the Drosophila *E74A* mRNA (Burtis et al., 1990). The long *EcR* ORF is followed by an 1831 bp AT-rich 3′ untranslated region. Neither of the sequenced cDNA clones contains a poly(A) tract at its 3′ end.

Comparison of the predicted EcR protein sequence with the sequence data base and with individual members of the steroid receptor superfamily shows that EcR shares with these proteins the two conserved domains (underlined in Figure 1) characteristic of steroid receptor superfamily members (Evans, 1988; Green and Chambon, 1988). We refer to the more N-terminal domain as the C region, or DNA-binding domain, and to the more C-terminal domain as the E region, or hormone-binding domain, according to the nomenclature of Krust et al. (1986).

Figures 2A and 2C present alignments of the C and

Figure 1. Composite Sequence of *EcR* cDNAs

Numbers at the left refer to the nucleotide sequence; those on the right refer to the amino acid sequence of the EcR protein. Nucleotides 1–5194 are the sequence of the *EcR*-17 cDNA, while nucleotides 5195–5534 derive from the *EcR*-9 cDNA. The underlined sequences in the 5′ and 3′ untranslated regions refer, respectively, to ATG codons and AATAAA consensus polyadenylation/cleavage signals. Positions of the introns and the donor and acceptor splice sequences are indicated above the cDNA sequence in lowercase type. The amino acid sequences homologous to the conserved DNA-binding (C region) and hormone-binding (E region) domains of the steroid receptor superfamily are underlined. Like a number of Drosophila regulatory proteins, the predicted EcR protein contains some simple repetitive sequences (Scott and Carroll, 1987). Amino acids 165–176 and 187–211 are glycine-rich regions encoded by a DNA sequence similar to the PEN repeat (Haynes et al., 1987). Amino acids 700–705 are an $(Ala)_5$ repeat. Amino acids 706–781 are a Gln/Pro-rich region in which, with a few exceptions, Gln is found as every other amino acid.

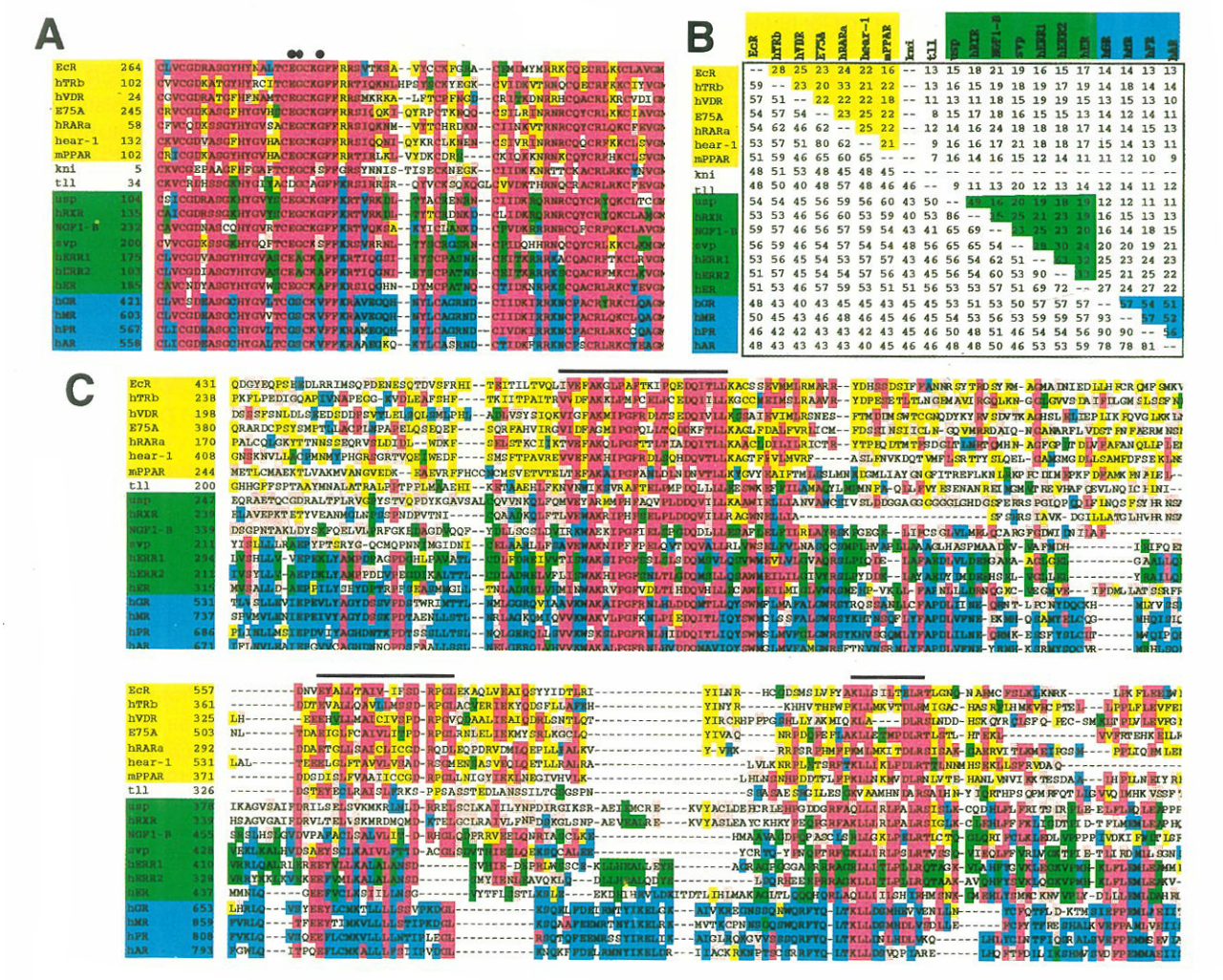

Figure 2. Sequence Comparison of the Conserved C and E Regions of EcR and Some Representative Members of the Steroid Receptor Superfamily

(A) C region alignment. Numbers at the left indicate the amino acid positions within the individual receptors; dashes indicate gaps introduced to obtain maximal alignment. Black dots indicate three positions important in determining the DNA-binding specificity of this domain (see text).

(B) Computed percent identities among the C region sequences (lower left) and among the E region sequences (upper right).

(C) E region alignment. Black lines indicate the three most highly conserved stretches within this domain. The kni sequence shows no significant E region similarity and is therefore omitted from this comparison. The colors in this figure emphasize the division of these receptor homologs into three subfamilies, shown in yellow, green, and blue. Within any one column of the alignments, identical amino acids are shown in the same color. Magenta is used for amino acids conserved across more than one subfamily. Sequences shown are from: EcR (this work); hTRβ (hTRb in figure) (Weinberger et al., 1986); hVDR (Baker et al., 1988); E75A, Drosophila ecdysone–inducible protein encoded at 75B (Segraves and Hogness, 1990); hRARα (hRARa in figure) (Giguere et al., 1987; Petkovich et al., 1987); hear-1, human erbA-related protein (Miyajima et al., 1989); mPPAR (Isseman and Greeen, 1990); kni, Drosophila *knirps* protein (Nauber et al., 1988); tll, Drosophila *tailless* protein (Pignoni et al., 1990); usp, Drosophila *ultraspiracle* protein (Oro et al., 1990); hRXR, human retinoid receptor (Mangelsdorf et al., 1990); NGF1-B, rat nerve growth factor–induced protein (Milbrandt, 1988); svp, Drosophila *seven-up* protein (Mlodzik et al., 1990); hERR1 and hERR2, human estrogen–related receptors 1 and 2 (Giguere et al., 1988); hER, human estrogen receptor (Green et al., 1986; Greene et al., 1986); hGR, human glucocorticoid receptor (Hollenberg et al., 1986); hMR, human mineralocorticoid receptor (Arriza et al., 1987); hPR, human progesterone receptor (Misrahi et al., 1987); hAR, human androgen receptor (Lubahn et al., 1988).

E regions, respectively, of EcR with the corresponding regions from representative members of the steroid receptor superfamily. Comparison of E region sequences allows division of the receptors into subfamilies (Petkovich et al., 1987), the members of any one subfamily being more related to each other than to those in other subfamilies. In Figure 2, the receptors are shown divided into three subfamilies, with EcR falling into a subfamily with the human thyroid hormone receptor β (hTRβ), human vitamin D receptor (hVDR), E75, human retinoic acid receptor α (hRARα), hear-1, and mouse peroxisome proliferator–activated receptor (mPPAR).

The C region is a 66–68 amino acid domain that has been shown to function as a zinc finger–like DNA-binding domain in vertebrate receptors (Green and Chambon, 1987; Freedman et al., 1988). This domain has also been implicated in receptor dimerization (Kumar and Chambon, 1988). As shown in Figure 2A, all 19 C region residues that are absolutely conserved in the other receptor homologs are also conserved in EcR, including the nine invariant

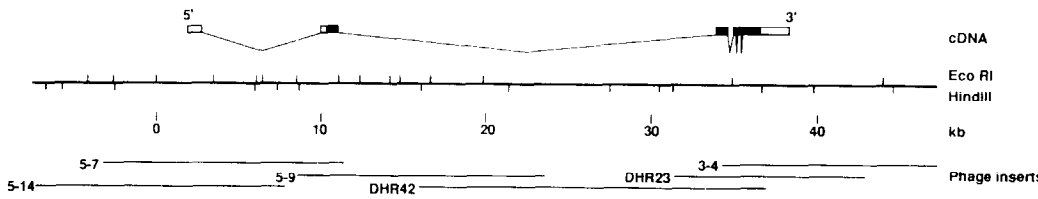

Figure 3. Genomic Organization of *EcR*

Exons mapped by comparison of cDNA and genomic clones are shown at the top. The ORF is indicated by the solid region. EcoRI and HindIII restriction sites in the genomic DNA are indicated by ticks on the line above the scale, which has its zero point located at an arbitrarily chosen EcoRI site. Genomic phage inserts are shown at the bottom. Because the 5′ and 3′ ends of the *EcR* transcripts have not been determined, there may be additional noncoding sequences at the ends of this transcription unit.

Cys residues, eight of which coordinate two zinc ions (Härd et al., 1990). Figure 2B shows that the Drosophila C region sequences are not more closely related to each other than they are to C regions from the vertebrate receptor homologs; indeed, the EcR C region is most similar to that of hTRβ. Studies on the glucocorticoid receptor, estrogen receptor, and thyroid hormone receptor have identified three C region residues (indicated by dots in Figure 2A) that are critical determinants of the differential DNA-binding specificity of these receptors (Danielsen et al., 1989; Mader et al., 1989; Umesono and Evans, 1989). The Drosophila proteins EcR and E75A, as well as hRARα, hTRβ, and hVDR, all have identical amino acids at these three positions. These proteins may therefore all have related DNA-binding specificities, as has already been shown for hVDR, hRARα, and hTRβ (Schüle et al., 1990; Umesono et al., 1988). Indeed, the EcRE consensus sequence is similar to the response elements for vitamin D, retinoic acid, and thyroid hormone (Cherbas et al., 1991).

The E region is a ~225 amino acid domain that functions as a hormone-binding domain in vertebrate receptors (Evans, 1988; Green and Chambon, 1988). This domain has also been implicated in receptor dimerization (Kumar and Chambon, 1988; Guiochon-Mantel et al., 1989; Glass et al., 1989), hormone-dependent nuclear localization of the glucocorticoid receptor (Picard and Yamamoto, 1987), and binding of the glucocorticoid receptor to the 90 kd heat shock protein (Pratt et al., 1988). Figure 2C shows an alignment of the EcR E region with those of other receptor homologs. The three relatively highly conserved stretches within the region (Wang et al., 1989; Segraves and Hogness, 1990) are overlined; each contains a cluster of residues conserved in all or most of the receptor sequences. EcR shows strong similarity to the other proteins in these stretches, and a lower similarity outside of them. The presence of these conserved features suggests that the EcR protein functions as a hormone receptor, and distinguishes it from the Drosophila knirps (Nauber et al., 1988), knirps-related (Oro et al., 1988), and egon (Rothe et al., 1989) proteins, which show similarity to other superfamily members in the C region but not the E region. The EcR E region is most similar to that of hTRβ, but this level of similarity is lower than those found among E regions of many other receptors (Figure 2B). Thus, within the steroid receptor superfamily, EcR is not especially closely related to any previously cloned receptor.

EcR Genomic Structure

We have isolated a set of overlapping genomic clones for the *EcR* gene, and have mapped *EcR* exons by comparing the cDNA and genomic clones by Southern blotting, restriction mapping, and finally sequencing of the genomic exon boundaries. The deduced gene structure is shown in Figure 3, and the splice junctions are indicated on the cDNA sequence in Figure 1. These splice junctions all conform to the splice donor and acceptor consensus sequences (Mount, 1982). The *EcR* cDNA sequence shown in Figure 1 is split into six exons spread over 36 kb of genomic DNA, with the ORF beginning in the second exon and ending in the sixth.

The exon/intron organization for that part of the *EcR* gene encoding the C region differs from that typically found in steroid receptor superfamily genes. Typically, the C region is encoded by two exons, one for each of the zinc finger–like motifs (Segraves and Hogness, 1990, and references therein). However, the Drosophila genes *EcR* and *DHR3* (Koelle et al., 1991) encode the C region in a single exon, and the Drosophila *ultraspiracle* (Oro et al., 1990) and *tailless* (Pignoni et al., 1990) genes may lack introns altogether. Another generally conserved characteristic of steroid receptor genes is a splice donor site normally located in the fourth codon past the conserved Met codon at the end of the C region (Met-329 in Figure 1). *EcR*, along with the intronless *ultraspiracle* and *tailless* genes, also lacks this feature.

EcR Protein Binds Ecdysone

A Schneider 2, or S2, Drosophila cell line overexpressing the EcR protein was constructed to produce EcR protein for ecdysone-binding studies. This Mt/EcR/Hy cell line contains a stably integrated plasmid (described in Experimental Procedures) in which the EcR protein-coding sequences are linked to the metal-inducible Drosophila metallothionein promoter (Bunch et al., 1988). The Mt/Hy cell line is a related control cell line that lacks the *EcR* coding sequences.

Whole cell extracts prepared from copper-induced cultures of both the Mt/EcR/Hy and Mt/Hy cell lines were assayed for ecdysone-binding activity using the high affinity ecdysone analog [^{125}I]iodoponasterone A (Cherbas et al., 1988). As shown in Figure 4A, specific (saturable) ecdysone binding was elevated 7-fold in the Mt/EcR/Hy extract compared with the Mt/Hy control. Expression of EcR

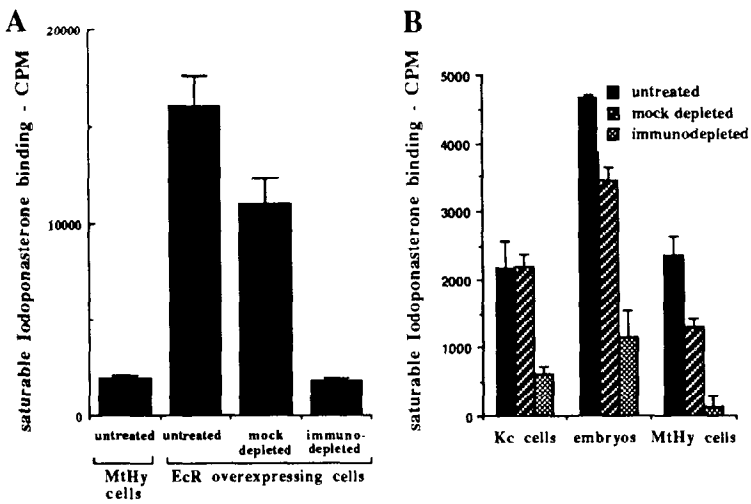

Figure 4. Ecdysone-Binding Assays on EcR Overexpressor and EcR-Depleted Extracts

(A) Binding assays for the high affinity ecdysone analog [^{125}I]iodoponasterone A were performed on extracts of control (Mt/Hy) or EcR-overexpressing (Mt/EcR/Hy) cells. The EcR overexpressor extract was mock depleted with preimmune serum or immunodepleted with anti-EcR antibodies, and the treated extracts were assayed for hormone-binding activity.

(B) Extracts from Drosophila Kc cells, embryos, or the Mt/Hy S2 cell line were assayed for ecdysone-binding activity. Extracts were either untreated, mock depleted with preimmune serum, or immunodepleted with anti-EcR antibodies.

Error bars in this and subsequent figures are standard deviations for $N \geq 2$.

from the *actin 5C* promoter in transient transfection experiments gave a similar increase in ecdysone binding (data not shown).

To determine whether the induced ecdysone-binding activity required the EcR protein, it was depleted from the Mt/EcR/Hy extract by immunoprecipitation using an affinity-purified anti-EcR polyclonal antibody (see Experimental Procedures). As a control, the extract was mock depleted with preimmune serum. Figure 4A shows the results obtained when the treated extracts were then assayed for ecdysone-binding activity. Comparison of the immunodepleted extracts with the mock-depleted extracts showed that most of the binding activity was removed by the anti-EcR antibody treatment, indicating that the induced ecdysone-binding activity is associated with the EcR protein. (The phrase ". . . activity is associated with the EcR protein . . ." used here and below means that the activity results from the EcR protein alone or from a protein complex containing EcR.)

The question arises as to whether the previously characterized endogenous ecdysone-binding activity in Drosophila cell lines (Cherbas et al., 1988) and tissues (Osterbur and Yund, 1982; Yund et al., 1978) is due to the EcR protein. To answer this question, extracts from embryos and two cell lines were immunodepleted or mock depleted as described above and assayed for ecdysone-binding activity (Figure 4B). Again, comparison of these treated extracts showed that the majority of the endogenous binding activity was removed in each case by treatment with the anti-EcR antibody. The amount of binding activity depleted from the extracts was dependent on the number of rounds of immunoprecipitation used for the depletion (data not shown). The anti-EcR antibody may therefore have failed to deplete all of the binding activities, owing to its inefficiency at immunoprecipitation. Using an antibody raised against a different EcR fusion protein, it has been possible to immunoprecipitate all detectable high affinity iodoponasterone-binding material from extracts of Kc cells (A. Mintzas and P. C., unpublished data). We conclude that most, if not all, of the endogenous binding activity in embryo and cell line extracts is associated with EcR or an antigenically related protein.

EcR Protein Binds Specifically to EcRE DNA

A 23 bp sequence from the promoter of the ecdysone-inducible *hsp27* promoter has been shown to function as an EcRE in that it can confer 20-fold ecdysone inducibility on an ecdysone-nonresponsive promoter (Riddihough and Pelham, 1987). Figure 5A shows that multiple copies of this sequence can act as a very strong EcRE. A cassette of seven copies of a 30 bp sequence from *hsp27*, which includes the 23 bp EcRE sequence, was cloned just upstream of the TATA box of the Drosophila *Adh* distal promoter. When transfected into Drosophila S2 cells, this promoter construct was 1200-fold ecdysone inducible, whereas the *Adh* promoter lacking the EcRE cassette showed no response to the hormone.

We tested the ability of the EcR protein to specifically bind the EcRE cassette in vitro. Plasmid DNA containing the EcRE cassette was restriction digested, end labeled, and mixed with an extract from the EcR overexpressing cell line, Mt/EcR/Hy. The same plasmid without the EcRE cassette was used as a control. The EcR–DNA complexes were then immunoprecipitated using the affinity-purified anti-EcR polyclonal antibody, and the precipitate was analyzed on agarose gels. As shown in Figure 5B, the DNA fragment containing the EcRE cassette was specifically and efficiently precipitated. Similar results were obtained when any of three different anti-EcR monoclonal antibodies were substituted for the polyclonal antibody (data not shown). Given that the fragment was not precipitated appreciably in the absence of anti-EcR antibody, or when the EcRE cassette was deleted, the precipitation of the fragment evidently resulted from the binding of the EcR protein to the 30 bp *hsp27* sequence. Under the conditions used, EcR protein has approximately 850-fold preference for binding to this 30 bp EcRE sequence versus average vector sequences (see Experimental Procedures). There was no effect of ecdysone on DNA binding either in this assay or in a gel shift assay similar to that shown in Figure

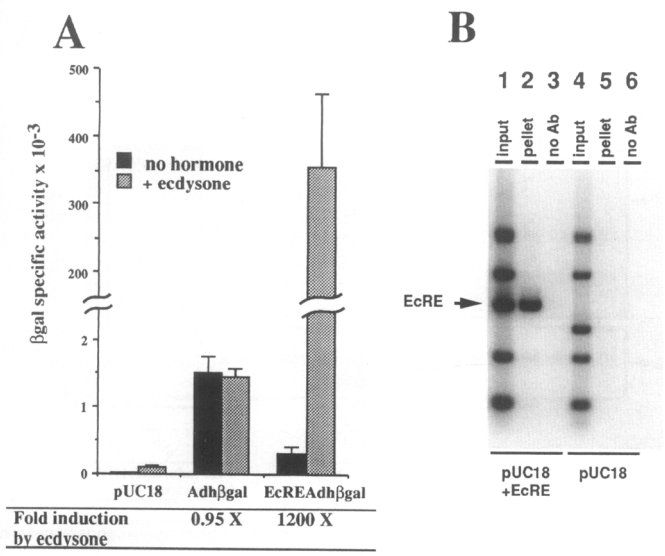

Figure 5. The EcR Protein Binds to an EcRE

(A) Characterization of an EcRE. S2 cells were transfected with plasmid DNAs and either left untreated or treated with ecdysone. Cell extracts were then assayed for β-gal activity. The plasmid DNA used for each transfection is given on the abscissa, and the β-gal activity ratio between hormone-treated and untreated cells is indicated below it. Control cells transfected with pUC18 DNA showed only a low level of endogenous β-gal activity. β-Gal activity from the Adh/βgal plasmid, which expresses β-gal from the Drosophila distal *Adh* promoter, was unchanged by hormone treatment. Expression from the EcRE/Adh/βgal plasmid, which is identical to Adh/βgal except that it has seven EcREs inserted upstream of the *Adh* promoter, is induced 1200-fold by ecdysone.

(B) DNA binding by the EcR protein. Restriction fragments from the control plasmid pUC18 (lanes 4–6), or from pUC18 containing a cassette of seven EcREs (lanes 1–3), were tested for their ability to specifically bind the EcR protein. Lanes 1 and 4 contain 0.1 fmol of each of the fragments tested. For lanes 2 and 5, 0.2 fmol of DNA fragments was mixed with an extract from EcR-overexpressing cells and immunoprecipitated with an anti-EcR antibody. Lanes 3 and 6 show controls in which the antibody was left out. The arrow points to the fragment in lanes 1–3 that contains the cassette of seven EcREs.

6C (data not shown). The immunoprecipitation DNA-binding assay has also been used to identify specific EcR-binding sites in the ecdysone-induced *E74* gene—binding sites that also confer ecdysone inducibility on the *Adh* promoter in the transfection assay described above (W. S. T. and D. S. H., unpublished data).

EcR Confers Ecdysone Responsiveness to Ecdysone-Resistant Cells

The commonly used Kc and S2 Drosophila cell lines are ecdysone responsive, and extracts of these cells contain a high affinity ecdysone-binding activity (Cherbas et al., 1980, 1988, and this work). Ecdysone arrests the growth of these cells. Kc cells capable of growth in the presence of ecdysone, however, can be produced by continuously exposing a Kc cell culture to ecdysone over a period of weeks. The resulting cells are deficient in both ecdysone-binding activity and ecdysone responsiveness. The "ecdysone-resistant" state of these cells is then maintained even after the cells are switched to ecdysone-free medium for several months, although reversion may eventually occur (Stevens and O'Connor, 1982). Ecdysone-resistant lines can similarly be produced from S2 cells, which are more easily transfected with DNA than Kc cells. However, both S2 and Kc ecdysone-resistant lines retain a low but significant level of ecdysone responsiveness, as measured by the level of inducibility of an ecdysone response reporter plasmid in these cells. The level of this responsiveness varies over a period of months during culture of the cells in the continuous presence of ecdysone (data not shown). By monitoring S2 ecdysone-resistant cell cultures periodically, we were able to obtain cells severely deficient in ecdysone responsiveness for use in the experiments described below.

Immunoblot analysis shows that the abundance of EcR protein is greatly reduced in the ecdysone-resistant S2 cells compared with the ecdysone-sensitive S2 cells from which they were derived (Figure 6A). Extracts of the resistant cells also lack a measurable level of ecdysone-binding activity (Figure 6B). In addition, the resistant cell extracts show much less EcR DNA-binding activity than S2 extracts. This is shown in Figure 6C, where a gel shift assay with a labeled EcRE DNA probe and an S2 cell extract reveals a single specifically retarded complex (Figure 6C, EcR complex, lane 2) that is competed by excess unlabeled EcRE oligonucleotide (lane 5), but not by a control oligonucleotide (lane 4). The complex was destroyed by an anti-EcR antibody (lane 7), but not by a control antibody (lane 6), and it was more abundant when an extract of EcR-overexpressing cells was used (compare lanes 8 and 2). The complex therefore results from the binding of EcR, or a protein aggregate containing EcR, to the EcRE DNA. When an extract of ecdysone-resistant S2 cells was used, no EcR complex was detected in the assay (compare lanes 3 and 2).

Ecdysone responsiveness in Drosophila tissue culture cells can be measured by the hormonal induction of β-galactosidase (β-gal) expression from the "reporter" plasmid pEcRE/Adh/βgal (see Figure 5A). The ecdysone-resistant S2 cells transfected with this reporter plasmid alone showed a greatly reduced response to the hormone (Figure 7A) when compared with the ecdysone-sensitive parent cell line that was similarly transfected (Figure 7B). However, when the resistant cells were cotransfected with an *EcR* expression plasmid and the reporter plasmid, the inducibility of the reporter increased from 10-fold in the absence of EcR overexpression to 210-fold in its presence (Figure 7A)—a level essentially the same as the 200-fold inducibility observed in cotransfected sensitive cells (Figure 7B). Evidently, resistant cells can be rescued to a high

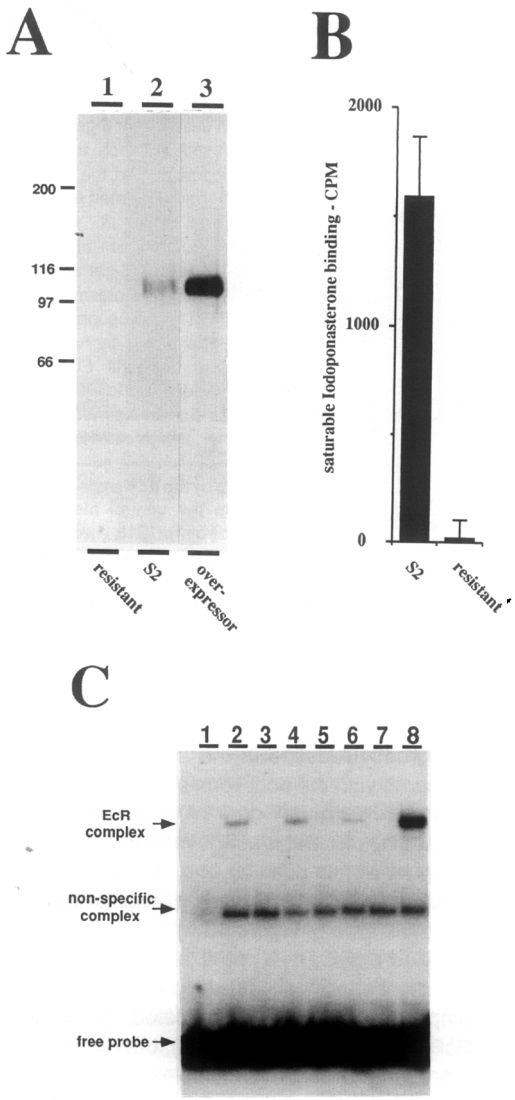

Figure 6. Ecdysone-Resistant Cells Are Deficient in EcR Protein

(A) Western analysis of ecdysone-resistant cells (lane 1), the ecdysone-sensitive parent cell line S2 (lane 2), and cells transfected with the EcR expression plasmid pAct/EcR (lane 3). Five-fold less total protein was loaded in lane 3 than in lanes 1 and 2. The blot was probed with the anti-EcR monoclonal antibody AD4.4. The mobilities of molecular weight standards, and their sizes in kilodaltons, are indicated on the left.

(B) Ecdysone-resistant cells are deficient in ecdysone-binding activity. Binding assays for the ecdysone analog [^{125}I]iodoponasterone A were performed on extracts of ecdysone-sensitive S2 cells and on the ecdysone-resistant cells derived from them.

(C) Ecdysone-resistant cells are deficient in EcRE DNA-binding activity. Gel shift analysis was used to detect complexes of a labeled DNA fragment containing the *hsp27* EcRE with proteins from various extracts: lane 1, free probe; lane 2, S2 cell extract; lane 3, ecdysone-resistant cell extract. Lanes 4–7 show binding reaction using the S2 cell extract, to which the following were added: lane 4, excess unlabeled nonspecific oligonucleotide; lane 5, excess unlabeled *hsp27* EcRE oligonucleotide; lane 6, affinity-purified polyclonal antibody against the Drosophila steroid receptor homolog DHR3 (Koelle et al., 1991); lane 7, similarly prepared antibodies against EcR. Lane 8, binding reaction using an extract of the EcR-overexpressing cell line Mt/EcR/Hy.

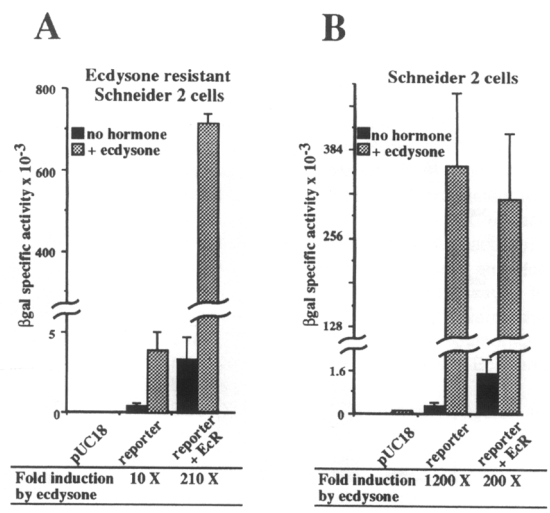

Figure 7. EcR Complements the Ecdysone Nonresponsiveness of Ecdysone-Resistant Cells

(A) Ecdysone-resistant S2 cells were transfected with the control plasmid pUC18, with the ecdysone response reporter plasmid pEcRE/Adh/βgal alone, or with the reporter plasmid plus an *EcR* expression plasmid, pAct/EcR. Transfected cells were left untreated, or treated with ecdysone, and cell extracts were assayed for β-gal activity.

(B) Ecdysone-sensitive S2 cells were subjected to the same treatment as shown for the resistant cells in (A).

level of hormone responsiveness by EcR expression. We note that the 10-fold reporter inducibility observed in resistant cells is significant and therefore suggests a low level of endogenous ecdysone receptor in these cells that was not detected in the Western blot and binding assays of Figure 6. Whether this is due to a difference in sensitivity of the assays or another cause has not been determined.

Ecdysone responsiveness in tissue culture cells can also be monitored by measuring the induction of several endogenous proteins following hormone treatment (Best-Belpomme et al., 1978; Cherbas et al., 1980). We find that the endogenous Drosophila β-gal activity in S2 cells is ecdysone inducible, and that this inducibility is lost in ecdysone-resistant cells but can be restored by expressing EcR in these cells (data not shown).

Taken together, these results indicate that EcR protein is required for the ecdysone responsiveness of Drosophila S2 cells, since cells deficient in ecdysone responsiveness are also deficient in EcR protein, and these cells can be restored to a high level of responsiveness by transfection with an *EcR* expression plasmid.

Temporal Profiles of *EcR* Expression

We have examined the temporal pattern of *EcR* expression by determining the abundance of its mRNA and protein in whole animals during their development. Figure 8A shows the results obtained when a cDNA probe was hybridized to a Northern blot bearing samples of RNA prepared at 3 hr intervals during embryonic development and every 12 hr thereafter until adulthood. A predominant 6 kb transcript was detectable at varying levels of abundance during all of development except at 0–3 hr, when a faint but

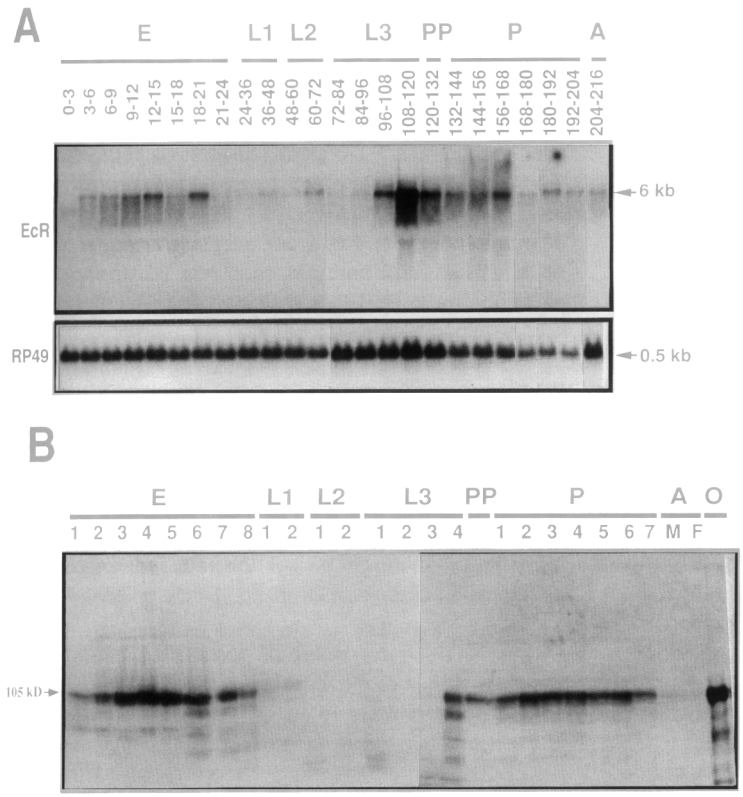

Figure 8. Developmental Profile of *EcR* Expression

(A) Northern analysis. RNA prepared from animals at the indicated developmental stages was fractionated by gel electrophoresis, blotted, and probed with labeled DNA from *EcR* or *RP49*. The *RP49* gene encodes a ribosomal protein (O'Connell and Rosbash, 1984), and is used as a control for loading of RNA. The hours and stages of development are indicated at the top of the figure: E = embryos; L1, L2, L3 = the three larval instars; PP = prepupae; P = pupae; A = adults.

(B) Western analysis. Drosophila protein from the indicated stages of development was electrophoresed, blotted, and probed with the anti-EcR monoclonal antibody DDA2.7. Protein samples were prepared separately from the RNA samples used in (A), but from similar time points, namely, every 3 hr during embryogenesis and every 12 hr thereafter. Within a developmental stage, the time points are labeled 1, 2, 3, etc., instead of in hours after egg laying, to emphasize that these protein samples are distinct from the RNA samples. Adult male and female samples are labeled M and F. Lane O contains an extract of the EcR-overexpressing cell line Mt/EcR/Hy. Total protein was underloaded 1.5- to 3-fold in lanes L2-2, L3-2, and L3-3 relative to the other lanes. Total protein in lane O was underloaded ~15-fold so that the signal intensity would be comparable with those of the other samples.

significant 3.7 kb band was observed. The 6 kb transcript is most abundant in embryos and during the period from late third instar through early pupal development. The most striking feature of its expression pattern is the intense peak of *EcR* mRNA in the 108–120 hr sample, which was prepared from wandering third instar larvae. Larvae at this stage are initiating the ecdysone-induced metamorphic molt.

Figure 8B shows the pattern of EcR protein expression obtained by probing a developmental Western blot with an anti-EcR monoclonal antibody. The major band detected by this antibody corresponds to a protein with an apparent molecular size of 105 kd, somewhat larger than the 94 kd calculated for the 878 amino acid sequence given in Figure 1. Bacterially expressed EcR protein also has an apparent molecular size of 105 kd (data not shown). The antibody also detects some lower molecular weight bands that may represent EcR breakdown products, but have not been characterized. The abundance of the 105 kd band generally follows the *EcR* mRNA, as it is also high during embryogenesis and from late third instar through pupal development. EcR protein is detectable at low levels in first instar larvae and adults. It is not seen during the second and early third instars. However, the fact that low levels of RNA were seen during these periods of development suggests that the protein may indeed be present during these stages, but only at levels below the limit of detection in our immunoblots.

Spatial Expression of EcR Protein

To determine the spatial expression pattern of EcR, we used anti-EcR monoclonal and affinity-purified polyclonal antibodies to stain Drosophila at various stages of development. Figure 9A shows a 13–16 hr embryo in which the EcR protein is seen widely distributed throughout the organism. The protein is also distributed throughout embryos at all other stages that we have examined (data not shown). Figures 9C and 9E–9G show the EcR distribution in a variety of late third instar or prepupal tissues. The anti-EcR antibodies detect a nuclear antigen in imaginal discs (Figure 9C), fat body (Figure 9E), tracheae (Figure 9F), and salivary glands (Figure 9G). In addition, we have seen nuclear staining in all other larval tissues examined, including central nervous system, gut, ring gland, and cells associated with cuticular structures (data not shown).

To determine the intracellular localization of EcR protein in the absence of ecdysone, we stained cells from a line that overexpresses EcR and was cultured in the absence of ecdysone (Figure 9H). The protein is seen predominantly in the nuclei of these cells, indicating that EcR does not require hormone for nuclear localization in these cells. Similarly, the estrogen (King and Greene, 1984) and progesterone (Perrot-Applanat et al., 1985) receptors are also nuclear in the absence of their ligands, whereas the glucocorticoid receptor is cytoplasmic in the absence of hormone and is translocated to the nucleus in the presence of glucocorticoids (Picard and Yamamoto, 1987).

Discussion

The *EcR* Gene Encodes an Ecdysone Receptor

We have shown that the *EcR* gene encodes a protein that

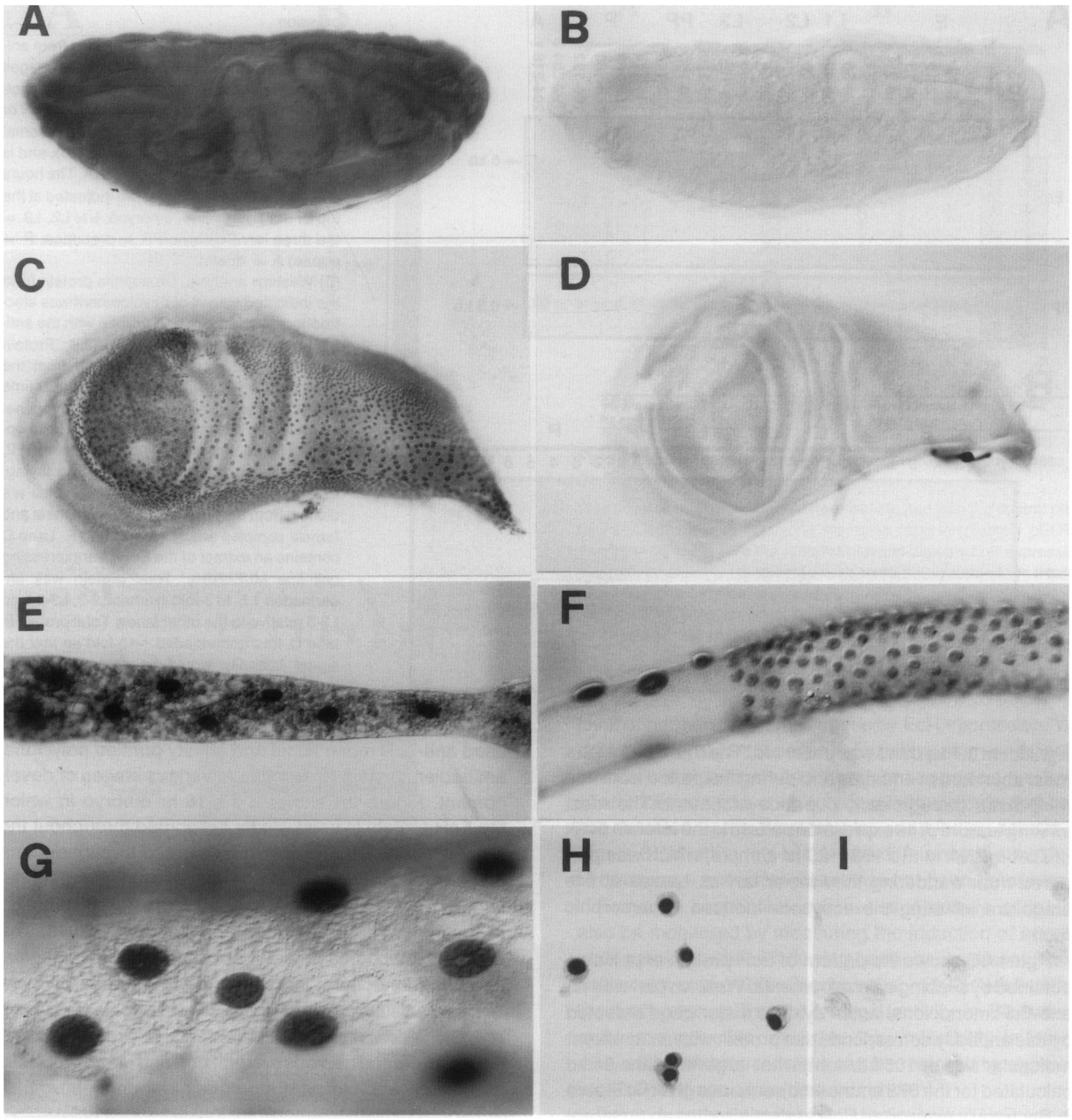

Figure 9. Immunolocalization of EcR Protein

(B) and (D) show embryo and wing disc control stains in which anti-EcR antibody was left out of the staining procedure. The remaining panels show anti-EcR antibody stains. (A) is a 13–16 hr Canton S embryo. Wing imaginal disc (C), fat body (E), and trachea (F) stains were performed on tissues from wandering third instar larvae. In fat body the nuclear stain seen depends on the presence of the anti-EcR antibody; the cytoplasmic staining is present in the negative control stained in the absence of anti-EcR antibody (not shown) and therefore is not indicative of the presence of EcR. The salivary gland (G) is from a prepupa. Stains of cultured cells from the EcR-overexpressing line Mt/EcR/Hy (H) and the control cell line Mt/Hy (I) were performed under conditions that do not detect the low level of endogenous EcR expression in the control cells. Anti-EcR antibodies used were: monoclonal antibody DDA2.7 (A), monoclonal antibody IID9.6 ([C], [E], [F], [H], and [I]), and the affinity-purified rabbit polyclonal antibody (G).

qualifies for full membership in the steroid receptor superfamily. Not only does the EcR protein contain regions homologous to both the DNA- and hormone-binding domains characteristic of this family of ligand-regulated transcription factors, but it also satisfies the functional criteria for membership in this family. Indeed, *EcR* is the only Drosophila gene encoding a protein shown to satisfy both the structural and functional criteria identifying a receptor of this type. While six other Drosophila genes encode proteins containing sequences homologous to both the DNA- and hormone-binding domains, the activating ligands for these proteins have not been identified (*E75* [Segraves, 1988; Segraves and Hogness, 1990], *seven-up* [Mlodzik et al., 1990], *tailless* [Pignoni et al., 1990], *ultraspiracle*

[Shea et al., 1990; Oro et al., 1990], *DHR3* [Koelle et al., 1991], *FTZ-F1* [Lavorgna et al., 1991]).

Our results provide strong evidence that the EcR protein binds the steroid hormone ecdysone and exhibits sequence-specific binding to an EcRE. Furthermore, the ecdysone responsiveness of Drosophila tissue culture cells is strongly correlated with the presence of EcR. In particular, cotransfection experiments with cells lacking EcR and ecdysone responsiveness show that activation of a minimal promoter by ecdysone required both EcR and an EcRE. We therefore conclude that EcR is an ecdysone receptor. However, we note the caveat that because purified EcR protein was not used in the ecdysone and DNA-binding experiments, it is possible that EcR by itself is not sufficient for these binding activities but rather acts in a complex with one or more other proteins. In the same context, we do not know whether the active EcR is the protein represented in Figure 1 or results from posttranslational modification of that protein. Finally, we note that EcR is a receptor that does not appear to require hormone for its DNA-binding activity or for its nuclear localization.

EcR and the Ashburner Model

The Ashburner model was derived from kinetic analyses of the effect of ecdysone on polytene chromosome puffing in salivary glands excised from late third instar larvae just prior to the induction of early puffs (Ashburner et al., 1974). When such glands are exposed to ecdysone, the early puffs are immediately induced whether or not the hormone is accompanied by an inhibitor of protein synthesis. This is also the case for the ecdysone induction of early gene transcription in such glands (Burtis, 1985; Thummel et al., 1990; Segraves, 1988; Segraves and Hogness, 1990). In the model, this induction is independent of protein synthesis because the ecdysone receptor is assumed to be present in the glands prior to their exposure to ecdysone. If the EcR protein is that receptor, it should be present in third instar salivary glands prior to the ecdysone induction of early puffs. This is the case; anti-EcR antibody staining of salivary glands at this stage of development yielded results essentially the same as that shown in Figure 9G for prepupal salivary glands.

In the continued presence of ecdysone, the early puffs regress (and early gene transcription is repressed) and the late puffs appear. Because these events follow early puff (and gene) induction and are eliminated by inhibitors of protein synthesis, they are presumed to represent secondary ecdysone responses mediated by early gene proteins (references above). The concept that one or more of the early proteins represses early gene transcription by antagonizing the inductive effect of the ecdysone–receptor complex has obvious origins in the above experiments. By contrast, the idea that early proteins induce late gene transcription by antagonizing another primary function of the ecdysone–receptor complex has its origins in another kind of experiment. When the excised glands are exposed to ecdysone for a time sufficient for partial early puffing but insufficient for late puffing, and are then washed to remove the ecdysone, the late puffs appear prematurely, while the early puffs exhibit the expected rapid regression (Ashburner et al., 1974). In the model, the premature appearance of the late puffs is accounted for by assuming that the ecdysone–receptor complex represses late gene transcription until one or more early proteins increase to a level sufficient to overcome that repression; in such a system, loss of the repressive element by removal of the ecdysone will lead to premature late gene transcription. If the EcR protein is playing both the inductive and repressive roles of the receptor, it should bind specifically to both the early and late puff loci, an expectation that can be tested by staining polytene chromosomes in late third instar salivary glands with the anti-EcR antibodies. While these studies will be reported elsewhere (W. S. T. and D. S. H., unpublished data), we note here that the large majority of EcR chromosomal binding sites do in fact coincide with early and late puff loci. In addition, EcR DNA-binding sites were detected in association with the cloned *E74* and *E75* early genes.

The recent molecular analyses of the early genes showing that they encode DNA-binding proteins with properties indicative of transcription factors (see Introduction for references) further enhance the proposition that the ecdysone response hierarchy contains two major regulatory motifs, each consisting of a positive-negative regulatory duet. These duets operate simultaneously but are out of phase: the early gene duet starts with positive regulation by an ecdysone–EcR complex and ends with negative regulation by one or more early proteins, while the late gene duet starts with negative regulation by an ecdysone–EcR complex, ending with positive regulation by early gene proteins. As noted in the Introduction, the finding that early gene proteins bind to polytene chromosomal sites corresponding to both early and late puff loci provides further evidence for these duets.

The early gene duet provides a means for shortening the time during which an early gene promoter is active below that which would otherwise be determined by the length of the ecdysone pulse, and, consequently, a means for controlling the burst size of the mRNAs produced per early gene per ecdysone pulse. The late gene duet could provide the mechanism by which different late gene promoters are activated at different times. Imagine, for example, two late gene promoters initially repressed by the same ecdysone–receptor complex and subsequently activated by the same early protein. The concentration of that protein will rise during the initial phase of the ecdysone response, and if the two promoters are so constructed that the concentration of early protein required to overcome the repressed state is less for one than for the other, then one promoter will be activated before the other. This situation is analogous to that in which a spatial concentration gradient of a transcription factor determines where different genes will be activated; the difference is that here the "concentration gradient" is temporal and it determines when different genes will be activated.

How might an early protein antagonize the regulatory functions of an ecdysone receptor? We consider this question in the context of the E75A early protein because it is a member of the steroid receptor superfamily (Figure 2; Segraves and Hogness, 1990). One model for these antag-

onistic effects is that the two proteins compete for specific binding sites in the chromosomal DNA (Ashburner et al., 1974). Comparison of the DNA-binding domains of EcR and E75A shows that they are identical at positions (indicated by dots in Figure 2A) thought to contact DNA (Härd et al., 1990) and known to determine the differential binding specificities of the glucocorticoid and estrogen receptors (Danielson et al., 1989; Mader et al., 1989; Umesono and Evans, 1989). Thus, E75A and EcR may in fact have similar DNA-binding specificities, allowing them to compete for chromosomal binding sites. An alternative model for antagonism of EcR by E75A derives from the interaction of the vertebrate receptors for retinoic acid (hRARα) and thyroid hormone (hTRβ). hTRβ can form a heterodimer with hRARα, thus altering the DNA-binding specificity of hRARα (Glass et al., 1989). E75 proteins might antagonize EcR by similarly dimerizing with EcR and changing its functional properties.

EcR and the Developmental Diversity of the Ecdysone Response

Diverse responses to ecdysone occur at different developmental stages, and within a stage, in different tissues. For example, the ecdysone-induced cuticle molts that punctuate the first and second larval instars differ dramatically from the massive reorganization of the animal that occurs in the ecdysone-induced metamorphic molt at the end of third instar. During metamorphosis, different tissues undergo opposite types of response to the hormone. Larval tissues are stimulated to histolyse and imaginal tissues to begin their differentiation to adult structures. Even among imaginal tissues there is great diversity in the response. Imaginal discs, which attain their full complement of cells during larval growth, form the exterior structures of the adult head, thorax, and genitalia by an induced change of cell shape and contacts, whereas the abdominal histoblasts consist of small nests of about a dozen cells that are induced to multiply rapidly to fill the exterior abdominal space of the adult.

One scheme by which a single hormone might produce a variety of tissue- and stage-specific responses is through the use of different tissue- and stage-specific ecdysone receptors. There is precedent for this mechanism in vertebrates, where multiple receptors for thyroid hormone and retinoic acid have been identified and shown to have unique developmental and spatial expression patterns (Dollé et al., 1989; de The et al., 1989; Forrest et al., 1990). We are therefore led to ask whether EcR is only one of a family of ecdysone receptors in Drosophila. This question is particularly important to consider because a number of Drosophila steroid receptor homologs besides EcR have been identified without identification of the corresponding ligands, and because there are only two known hormonal candidates for these ligands: ecdysone and juvenile hormone. Current evidence nevertheless suggests that none of these homologs is an ecdysone receptor, as discussed below.

Sequence comparison of EcR and six other known Drosophila steroid receptor homologs for both the C and E regions (*E75*, *seven-up*, *tailless*, *ultraspiracle*, *DHR3*, *FTZ-F1*; references in Discussion, first paragraph) suggests that these proteins are most likely receptors for different hormones. Comparisons of vertebrate receptor sequences reveal a general rule that receptors with nonoverlapping hormone-binding specificities tend to have highly divergent E region (hormone-binding domain) sequences. In the cases where more than one receptor has been identified for a single hormone, these receptors show significant sequence similarity outside of the C and E regions, and at least 75% identity in the E region (Benbrook and Pfahl, 1987; Zelent et al., 1989). Even receptors with distinct but overlapping specificities have substantial similarities in the E region. The human mineralocorticoid receptor (hMR) has a hormone-binding specificity that overlaps those of the human glucocorticoid receptor (hGR) and the human progesterone receptor (hPR); the hMR can bind aldosterone and glucocorticoids with similar affinities, and also progesterone with a significant but lower affinity (Arriza et al., 1987). The E regions in the hGR and hPR are 57% and 56% identical to that in hMR, respectively. Vertebrate receptors with nonoverlapping binding specificities, on the other hand, have E region similarities of only 30% or less. An apparent exception to this rule is the case of hRXRα, which is thought to be a receptor for an unknown retinoid, despite the fact that this receptor shows only a relatively distant similarity to the retinoic acid receptors (Mangelsdorf et al., 1990).

The seven Drosophila receptors are quite divergent by these standards. They show no significant sequence similarity outside of the C and E regions, and the E regions show less than 24% identity. Thus, the expectation based on comparison of vertebrate receptors is that these Drosophila receptors will interact with different hormones. The possibility exists that other receptor genes highly related to *EcR* remain as yet undiscovered in the Drosophila genome, in which case these might encode additional ecdysone receptors. However, we have probed genomic Southern blots with *EcR* cDNAs and have failed to detect any sequences closely related to *EcR*.

Biochemical evidence supports the notion that if different ecdysone receptors exist, they should have similar hormone-binding domains. Scatchard analyses of ecdysone binding have been performed on extracts from a number of Drosophila cell lines and tissues during several stages of development. These studies generally find a single ecdysone-binding activity of approximately the same affinity regardless of the source of protein used (Maroy et al., 1978; Yund et al., 1978; Yund, 1979; Osterbur and Yund, 1982; Sage et al., 1982; Cherbas et al., 1988; but see also Dinan, 1985; Handler and Maroy, 1989). Further evidence supporting this notion is the substantial invariance in relative biological activities of different ecdysone analogs when they are tested in any of several response assays (Cherbas et al., 1980). Thus, the relative affinities of the steroid-binding domains of the functional receptors in these various tissues (salivary glands, imaginal discs, and Kc cells) for a wide range of compounds are indistinguishable, and hence the steroid-binding domains themselves must either be identical or closely related.

The above evidence provides a strong argument that

EcR is the only gene encoding ecdysone receptors in D. melanogaster. This does not mean, however, that multiple ecdysone receptor proteins do not exist in this fly since there are many examples of a single gene encoding a family of similar proteins. Indeed, all the early genes in the ecdysone response hierarchy that have been cloned exhibit a common mechanism for generating such a family of proteins (Burtis et al., 1990; Segraves and Hogness, 1990; DiBello et al., 1991). For present purposes, the best example is the *E75* gene, which encodes a family of proteins that are members of the steroid receptor superfamily (Segraves and Hogness, 1990). These proteins derive from nested transcription units, each with its own promoter but exhibiting a common 3′-terminal region. Consequently, the E75 receptor proteins have unique N-terminal regions and a common C-terminal region. Since this common region contains the putative hormone-binding domain, or E region, each of the E75 proteins is expected to bind and be regulated by the same ligand.

If this common theme for the early genes of the ecdysone response hierarchy was to extend to the *EcR* gene, then a family of ecdysone receptors with identical hormone-binding domains could be generated—an outcome quite compatible with the evidence summarized above. Recent results indicate that this is indeed the case (W. S. T. and D. S. H., unpublished data) and hence that the different tissue- and stage-specific responses to ecdysone may result, at least in part, from different members of an ecdysone receptor family encoded by *EcR*. It also opens the possibility that the inductive and repressive functions of ecdysone receptors postulated for the early and late gene regulatory duets may result from different members of this family.

What Controls *EcR* Expression?

Although the above question is not answered in this paper, we consider it here because the temporal pattern of *EcR* mRNA expression shown in Figure 8A invites speculation that *EcR* transcription may be ecdysone induced. It invites such speculation because the peaks of *EcR* expression seen at mid-embryogenesis (9–15 hr), near the end of each of the three larval instars (36–48 hr; 60–72 hr; 108–120 hr), and during pupal development (156–168 hr) in the figure correspond to five of the six commonly accepted peaks in the ecdysone titer (Richards, 1981b; Handler, 1982). A sixth ecdysone pulse occurs near the end of prepupal development, but the temporal resolution of the sampling is not sufficient to determine if a peak of *EcR* transcription activity occurs here as well. These peaks of *EcR* expression also overlap peaks of *E74A* (Thummel et al., 1990) and *E75A* (Segraves, 1988) mRNA expression that are sensitive detectors of ecdysone. Indeed, a sharp peak of *E74A* mRNA was detected near the end of prepupal development when samples were collected every 2 hr (Thummel et al., 1990); clearly, it would be of interest to determine if *EcR* exhibits a similar peak.

An analogy to the suggested induction of *EcR* by ecdysone can be drawn to the ability of vitamin D, retinoic acid, or estrogen to stimulate expression of their own receptors (Mangelsdorf et al., 1987; Barton and Shapiro, 1988; de The et al., 1989). The estrogen effect occurs at a dose of hormone too low to affect expression of other known estrogen-regulated genes. A similar induction of *EcR* could occur at the end of third instar, when the level of *EcR* mRNA begins to rise at ~96 hr (Figure 8A), several hours prior to the appearance of the ecdysone-induced *E75A* (Segraves, 1988) and *E74A* (Thummel et al., 1990) mRNAs. This early *EcR* induction during the rise in ecdysone titer might occur in response to levels of ecdysone below the threshold required for *E75A* and *E74A* induction. This appears to be the case for *E74B* transcription, which similarly precedes *E74A* transcription in the last half of the third instar (Thummel et al., 1990) and is induced at lower ecdysone concentrations than E74A (Karim and Thummel, 1991).

The peak of *EcR* mRNA near the end of embryogenesis (18–21 hr) does not correspond to a peak in ecdysone titer (Kraminsky et al., 1980; Gietz and Hodgetts, 1985) but does overlap peaks of *E74A* and *E74B* mRNA expression (Thummel et al., 1990) and only slightly precedes a peak of *E75A* mRNA expression (Segraves, 1988). These late embryonic peaks of early gene mRNAs have been taken to indicate that early gene promoters are activated by signals other than ecdysone (Thummel et al., 1990). If this were the case, however, the induction of *EcR* mRNA expression at the same time would be peculiar. Indeed, this *EcR* induction suggests the possibility that the early gene promoters, and perhaps an *EcR* promoter, are activated by an ecdysteroid not detected by Hodgetts and his associates (references above), i.e., an ecdysone other than 20-hydroxyecdysone.

In closing, we note that a comparison of the extremely high level of *EcR* mRNA at the end of the third larval instar (108–120 hr) with the much lower levels during the last half of the first (36–48 hr) and second (60–72 hr) instars nicely illustrates the profound difference between the metamorphic ecdysone response, when virtually every tissue is an ecdysone target, and the larval-to-larval molts, when the known targets represent only a small fraction of the tissues. If the *EcR* mRNA levels at other developmental stages are similarly indicative of tissue participation in the ecdysone response, it should be of considerable interest to determine that participation during embryogenesis and pupal development.

Experimental Procedures

Terminology

In accordance with common usage, we use the term ecdysone as a generic name for compounds with the appropriate biological activity in analogy with the terms estrogen or progestin. Ecdysteroid refers to compounds with structural similarity to the hormone 20-hydroxyecdysone.

Isolation and Analysis of cDNA and Genomic Clones

Standard methods for manipulating DNA were as described by Sambrook et al. (1989). Subclones of the original *EcR* genomic clone DHR23 (Segraves, 1988; Koelle et al., 1991) were used to screen a cDNA library prepared from third instar tissues treated with ecdysone and cycloheximide (a gift of Carl Thummel). Of the 270,000 primary plaques screened, 220 positives were detected. The longest cDNA analyzed, *EcR*-17, extended the farthest 5′ and was sequenced in its entirety on both strands. An additional cDNA, *EcR*-9, was found to

extend 300 bp farther 3′ than *EcR*-17, and both strands of this 3′ extension were also sequenced. For sequencing, cDNAs were subcloned into Bluescript vectors (Stratagene), and clones for sequencing were generated by exonuclease III digestion (Henikoff, 1984). Double-stranded plasmids were denatured (Gatermann et al., 1988) and sequenced by the dideoxy chain terminating method (Sanger et al., 1977), using the enzyme Sequenase (US Biochemical).

Additional *EcR* genomic clones were obtained by screening a Canton S genomic library in Charon 4 (Maniatis et al., 1978) with probes from the previously isolated genomic clones and with *EcR* cDNA probes. Genomic clones were restriction mapped and probed with labeled *EcR* cDNAs to localize exon-containing fragments on the genomic restriction map. Genomic DNA surrounding each exon boundary was subcloned and sequenced. Genomic exons were either sequenced entirely or, for the longer exons, were restriction digested and electrophoresed in parallel with cDNA clones to confirm the colinearity of the genomic and cDNA clones.

Plasmid Construction

Plasmid pAdh/βgal was constructed in two steps as follows: the BglII–ScaI fragment of pDD5′-34 (Heberlein et al., 1985), containing nucleotides −34 to +53 of the Drosophila Adh distal promoter, was cloned into pUC18 cut with SacI and BamHI, blunting the SacI end. The resulting plasmid was cut with EcoRI, and the EcoRI fragment of cPβbxd6.2 (Irvine et al., 1991), containing the *Ubx* untranslated leader and AUG, the Escherichia coli β-gal ORF, and the SV40 splice and poly(A) signals, was inserted. This plasmid thus drives expression of E. coli β-gal from a minimal Drosophila Adh promoter.

The ecdysone response reporter plasmid pEcRE/Adh/βgal is identical to pAdh/βgal, except that EcREs are inserted just upstream of the Adh TATA box. For construction of pEcRE/Adh/βgal, the following oligonucleotides were synthesized: 5′-TCGAGAGACAAGGGTTCAATGCACTTGTCCAATG-3′ and 5′-TCGACATTGGACAAGTGCATTGAACCCTTGTCTC-3′. These contain the 23 bp ecdysone response sequence from *hsp27* (Riddihough and Pelham, 1987), plus seven extra flanking nucleotides from *hsp27* and four nucleotides at the 5′ ends to generate SalI-compatible overhangs. These oligonucleotides were kinased, annealed, and ligated into SalI-cut pAdh/βgal. By restriction analysis, seven copies of the oligonucleotide were found inserted in this site.

The substrate for immunoprecipitation DNA binding was pUC18 with seven copies of the EcRE oligonucleotide inserted into the SalI site. This was generated by cloning the EcRE-containing HindIII–XbaI fragment of pEcRE/Adh/βgal into pUC18 cut with HindIII and XbaI.

Plasmid pAct/EcR is a derivative of the expression vector Act/SV40/BS, which is designed to express proteins under the control of the Drosophila actin 5C promoter. This vector was constructed in two steps by inserting the XbaI–EcoRI fragment of cosPneoβ-gal (Irvine et al., 1991), containing the SV40 splice and poly(A) signals, into Bluescript$^+$ KS (Stratagene) cut with SacII and XbaI, blunting the EcoRI and SacII ends. The resulting plasmid was digested with BamHI and ApaI, and the BamHI–EcoRI fragment of pP_{ac} (Krasnow et al., 1989) was inserted, with the ApaI and EcoRI ends being blunted. Plasmid pAct/EcR was then constructed by cloning the FspI–HpaI *EcR* cDNA fragment, containing nucleotides 851–4123 of the sequence in Figure 1, into the EcoRV site of ActSV40BS.

Plasmid pMt/EcR/Hy is a derivative of the expression vector pMt/Hy, which is designed to express proteins under the control of the Drosophila metallothionein (Mt) promoter, and also carries a hygromycin resistance gene, allowing selection of stable cell lines carrying the plasmid. pMt/HyI, a precursor of pMt/Hy, was first constructed by inserting the EcoRI–NotI fragment of pHSX-MT (Kaufman et al., 1989), containing the Drosophila Mt promoter, into BamHI-, NotI-cleaved pcophyg (Rio et al., 1986), filling in the EcoRI and BamHI ends. The 3′ end of the actin 5C gene was then inserted into pMt/HyI in two steps. The SalI fragment of pP_{ac} (Krasnow et al., 1989), containing the actin 5C 3′ end, was first cloned into the Bluescript$^+$ KS (Stratagene) XbaI site, filling in the ends. The XhoI–NotI fragment of the resulting plasmid was then inserted into XhoI-, NotI-cleaved pMt/HyI, to create pMt/Hy. This vector thus carries the metal-inducible Mt promoter followed by a polylinker and the actin 5C 3′ end, and also carries a hygromycin resistance gene for selection of stably transformed cell lines. pMt/EcR/Hy was constructed by cutting pMt/Hy with XhoI and BamHI and inserting the SalI–BamHI fragment of pAct/EcR, thus inserting the *EcR* ORF downstream of the Mt promoter.

Cell Culture and Transfection

S2 cells were cultured and transfected by the calcium phosphate technique as described by Krasnow et al. (1989). Kc cells were cultured in D22 medium as described by Schneider and Blumenthal (1978). Hygromycin-resistant stable cell lines carrying the plasmids pMt/Hy and pMt/EcR/Hy were generated as described by Rio et al. (1986). By immunohistochemical staining, about 60% of copper-treated pMT/EcR/Hy cells were found to overexpress EcR at readily detectable levels. Ecdysone-resistant S2 cells were obtained by growing S2 cells in the presence of 4×10^{-6} M 20-hydroxyecdysone (Sigma). This treatment initially halted growth of S2 cells, but after several weeks the selected cells grew well. Prior to their use in transfection experiments, the resistant cells were passaged at least five times in hormone-free medium, thus diluting the hormone to an ineffective concentration. Cells maintained in hormone-containing medium were exchanged into hormone-free medium and checked for ecdysone responsiveness monthly until a severely response-deficient population was obtained. Nonresponsive ecdysone-resistant cells were observed to revert to a much higher level of responsiveness over a period of months even when constantly maintained in ecdysone-containing medium. The factors that contribute to the generation of a severely hormone response-deficient population of cells are not understood; however, we have produced such populations independently on three occasions, and can keep them constantly available for use by freezing aliquots of the cells during their period of low responsiveness.

Extracts for Hormone- and DNA-Binding Assays

Tissue culture cell extracts used for hormone- and DNA-binding experiments were prepared as follows: cells were grown in spinner flasks to a density of 5×10^6 to 7×10^6 cells/ml. Mt/Hy and Mt/EcR/Hy cells were treated with 0.7 mM $CuSO_4$ for 12–16 hr prior to harvesting to induce expression from the metallothionein promoter. Cells were washed once in EcR 40 buffer (40 mM KCl, 25 mM HEPES [pH 7.0], 10% (v/v) glycerol, 1 mM EDTA, 1 mM dithiothreitol [DTT], and the following cocktail of protease inhibitors: 10 mM $Na_2S_2O_5$, 500 μM PMSF, 1 μM leupeptin, 1 μM pepstatin). All further manipulations were at 4°C. Cells were resuspended in 2% of the original culture volume of EcR buffer, divided into 3 ml aliquots, and sonicated using 30 0.5 s pulses with a probe sonicator (Bronson Sonifier 450), resulting in disruption of ~95% of the cells. After centrifugation at 100,000 × g for 1 hr, 100 μl aliquots of supernatant were frozen in liquid nitrogen and stored at −80°C. The extracts used for Figure 6C were prepared by a slightly modified protocol; the cells were lysed in EcR 400 buffer (containing 400 mM KCl), and the supernatant was exchanged into EcR 40 buffer using a PD-10 column (Pharmacia). Protein concentration was determined by the method of Bradford (1976), with bovine serum albumin as the standard, and was typically 6–11 mg/ml. Embryo extracts were prepared by a similar protocol: 3–6 hr Canton S embryos were dechorionated in 55% commercial bleach for 2 min, washed extensively in 0.7% NaCl, and resuspended using 2 g of embryos per ml of EcR 40 buffer. Embryos were broken with 20 strokes in a Dounce homogenizer using a B pestle, and lysis was completed with the probe sonicator on the same settings as used for the tissue culture cells. The extract was adjusted to 400 mM KCl, centrifuged 1 hr at 100,000 × g, and aliquots of supernatant were frozen. This extract was 13.4 mg/ml protein. Before use in hormone binding, it was diluted 10-fold in 0 mM KCl EcR buffer to bring the final KCl concentration to 40 mM.

Hormone-Binding Assays

For hormone-binding experiments, extracts were first diluted to the following concentrations in EcR buffer: 0.9 mg/ml for Mt/Hy and Mt/EcR/Hy extracts, 3 mg/ml for S2 and S2-resistant cell extracts, 4 mg/ml for the Kc cell extract, and 1.3 mg/ml for the embryo extract. All manipulations were performed on duplicate samples in order to quantify variability in the results. For immunoprecipitation experiments, extracts were immunodepleted, mock depleted, or left untreated. For depletions, 300 μl of diluted extract was incubated for 30 min at 25°C with 3.5 μl of affinity-purified anti-EcR antibody, or with 3.5 μl of preimmune serum for the mock depletion control. Then, 38 μl of 10% Staphylococcus aureus (Pansorbin, Calbiochem) in EcR 40 buffer was added,

and incubation was continued for 15 min at 25°C. After centrifugation for 3 min in a microcentrifuge, the supernatant (depleted extract) was recovered. The immunoprecipitation was repeated except in the case of the embryo extract, which was subjected to only one round of precipitation. The "untreated" extract aliquots were left at 4°C for the duration of the depletion procedure, and were diluted with EcR buffer to match the final concentration of the depleted aliquots.

The methods of Cherbas et al. (1988) were used for [^{125}I]iodoponasterone synthesis and were modified for hormone-binding assays. Assay tubes contained 140 μl of extract, 14 μl of [^{125}I]iodoponasterone in EcR 40 buffer, and either 14 μl of EcR 40 buffer or 14 μl of unlabeled 20-hydroxyecdysone in EcR 40 buffer as a competitor. [^{125}I]iodoponasterone was at 2177 Ci/mM and was used at a final concentration of 5×10^{-10} M in the assay; 20-hydroxyecdysone was at a final concentration of 2×10^{-5} M in the assay. After incubation for 1 hr at 25°C, each reaction was spotted on a dry Whatman GF/C filter (2.4 cm), and after 30 s the filter was washed by using a vacuum to draw 10 ml of EcR buffer through the filter over a period of 1 min. Filters were placed in 800 μl of 4% SDS, and radioactivity measured in a γ counter (LKB model 1275). The hormone-binding activities shown are saturable binding activities, calculated as the total binding activity measured in assays with no added competitor, minus the unsaturable binding activity measured in the assays with excess unlabeled ecdysone added. In assays of the most active extracts, the unsaturable activity, representing binding to the large number of low affinity binding sites in the extract, contributed less than 10% of the total counts.

Immunoprecipitation DNA-Binding Assay

Cesium-purified pUC18 or pUC18 containing seven *hsp27* EcRE oligonucleotides cloned in the SalI site was digested with ApaLI and HindIII and end labeled with [α-^{32}P]dATP and the large fragment of DNA polymerase I. Preliminary experiments were run to optimize ionic strength, type, and amount of competitor DNA, type and amount of antibody, and extract concentration in this assay. In the final assay, 0.2 fmol of digested, labeled plasmid DNA was mixed with 2 μg of poly(dI-dC)·poly(dI-dC) (Sigma) in 10 μl of TE (10 mM Tris–HCl [pH 8.0], 1 mM EDTA), and 90 μl of the Mt/EcR/Hy extract, diluted to 0.9 mg/ml in EcR buffer and adjusted to 180 mM KCl, was added. After binding for 15 min at 25°C, 2 μl of affinity-purified anti-EcR polyclonal antibody, diluted 1.5-fold in EcR 180 buffer, was added, and this incubation was continued at 25°C for 40 min. Then, 50 μl of anti-rabbit Ig–coated magnetic beads (Dupont Magnasort-R) exchanged into 180 mM KCl EcR buffer was added and the incubation continued for 15 min. The beads were washed twice in 400 μl of EcR 180 buffer, and DNA was eluted from the beads by soaking twice in 200 μl of 1% SDS in TE at 65°C. The eluted DNA was ethanol precipitated and run on an agarose gel, which was dried and autoradiographed. For comparison, one-half of the input DNA (0.1 fmol) was run on the gel. As a control, the antibody was left out of the binding assay.

The binding preference of EcR protein was determined by carrying out binding reactions using a mixture of α-^{32}P end-labeled DNA fragments containing the seven EcRE cassettes and ^{3}H end-labeled pUC18 DNA fragments. ^{32}P and ^{3}H counts in the input DNA and precipitated DNA were measured by scintillation counting. Whereas 64% of the EcRE-containing fragment was antibody precipitated, only 0.12% of similar length pUC18 fragments were precipitated. If the assumption is made that binding to each of the seven EcREs is independent, EcR protein is calculated to be bound to one 30 bp EcRE ~850-fold more frequently than to an average 30 bp of pUC18 DNA.

Gel Electrophoresis DNA-Binding Assay

Binding reactions were carried out in a total volume of 20 μl containing 22.5 mM HEPES (pH 7.0), 1 mM Tris (pH 7.5), 9% (v/v) glycerol, 90 mM KCl, 1 mM EDTA, 0.9 mM DTT, 0.5 mM PMSF, 2 μg of poly(dI-dC)·poly(dI-dC), 0.5–1.0 ng of ^{32}P-labeled probe, and 4 μg of cell extract. After incubation for 10 min at room temperature, 10 μl of each sample was electrophoresed on a 4% polyacrylamide native gel prepared as described by Buratowski et al. (1989). Assay mixtures that included competitor oligonucleotides contained 1 μl of 10 ng/μl double-stranded oligonucleotide in 10 mM Tris (pH 7.4), 1 mM EDTA. Assay mixtures that included antibodies contained 1 μl of the affinity-purified anti-EcR polyclonal antibody, or as a control, 1 μl of an identically prepared polyclonal antibody against the Drosophila steroid receptor homolog DHR3 (Koelle et al., 1991); these reaction mixtures were incubated at 37°C instead of room temperature.

The probe was prepared from the plasmid pUC/EcRE (one *hsp27* EcRE oligo cloned into the SalI site of pUC18). The EcRE-containing probe fragment was excised with EcoRI and SphI and gel purified. The fragment was end labeled with [α-^{32}P]dATP and the large fragment of DNA polymerase I (Boehringer Mannheim). The specific competitor oligonucleotide was the *hsp27* EcRE oligonucleotide described above. The nonspecific competitor oligonucleotide was prepared by annealing the two single-stranded 40-mers 5′-CTAG$(AAT)_{12}$-3′ and 5′-CTAG$(ATT)_{12}$-3′.

Hormonal Induction and Assay of β-Gal in Tissue Culture Cells

Twenty-four hours after transfection, each dish of cells was split in half: one-half was treated with 2×10^{-6} M 20-hydroxyecdysone, and one-half received no hormone treatment. 20-Hydroxyecdysone (Sigma) was kept as a 10 mg/ml stock in ethanol. To examine induction of the transfected β-gal reporter construct, cells were cultured for 24 hr after addition of the hormone. Under the assay conditions used, the activity of the endogenous β-gal is insignificant compared with the β-gal expressed from the reporter plasmid, and therefore does not interfere with interpretation of experiments with this reporter plasmid. To make extracts, 2 ml of cells was washed once in PBS (137 mM NaCl, 27 mM KCl, 65 mM Na_2HPO_4, 15 mM KH_2PO_4 [pH 6.8]), and was resuspended in 100 μl of freeze/thaw buffer (0.25 M sucrose, 10 mM Tris [pH 7.4], 10 mM EDTA) and repeatedly frozen in liquid nitrogen and thawed in a 37°C water bath, for a total of three freeze/thaw cycles. Cell debris was removed by a 10 min centrifugation in a microcentrifuge at 4°C. The concentration of protein in the supernatant (cell extract) was determined by the method of Bradford (1976), with bovine serum albumin as a standard, and was typically 1.5–2.5 mg/ml. Extracts were assayed immediately or frozen and assayed up to 2 weeks later with no loss in activity. To 10 μl of extract, or an appropriate dilution in freeze/thaw buffer containing 0.1 mg/ml bovine serum albumin, 500 μl of assay buffer (0.1 M sodium phosphate [pH 8.0], 5 mM $MgCl_2$, 0.6 mM 4-methylumbelliferyl-β-D-galactoside) was added. After incubation at 37°C for 30 min, the reactions were stopped by adding 500 μl of 300 mM glycine, 15 mM EDTA (pH 11.2). The fluorescent reaction product was quantified on a Perkin-Elmer LS-5B luminescence spectrometer, with λ_{ex} = 365 nm and λ_{em} = 450 nm. β-Gal activities are given as fluorescence units per μg of protein assayed.

Preparation of Anti-EcR Antibodies

The region between the conserved DNA- and hormone-binding domains was chosen as an immunogen for EcR polyclonal antibodies. The BsmI–HindII cDNA fragment, containing coding sequences for amino acids 335–447 of EcR (Figure 1), was cloned into both pUR (Ruther and Muller, 1983) and pATH (Rimm and Pollard, 1989) vectors to make E. coli β-gal and TrpE fusion proteins, respectively. The TrpE fusion protein was used as an immunogen and the β-gal fusion protein was used on immunoblots to test sera for immunoreactivity to the EcR portion of the fusion proteins. The TrpE fusion protein was highly insoluble, allowing further purification as follows: E. coli from a 2 liter culture of induced cells (Rimm and Pollard, 1989) were centrifuged, and the cell pellet was subjected to several freeze/thaw cycles. The cells were resuspended in 18 ml of 50 mM Tris–HCl (pH 7.5), 0.5 mM EDTA, and 1.8 ml of 10 mg/ml lysozyme was added. After 15 min on ice, the cells were lysed by passing three times through a French pressure cell at 10,000 psi. The insoluble fraction was collected by centrifugation at 27,000 × g for 15 min, and washed by resuspension, using a Dounce homogenizer, in ice-cold 50 mM Tris–HCl, 0.5 mM EDTA, 0.3 M NaCl, followed by centrifugation as above. The washing step was repeated, and the final pellet was dissolved in 10 ml of 4 M urea, 2% (w/v) SDS, 50 mM Tris–HCl (pH 7.5), 1 mM EDTA, 5% (v/v) 2-mercaptoethanol. Material remaining insoluble was centrifuged out and discarded.

For immunization, fusion protein was electrophoresed by SDS–PAGE: proteins were visualized by soaking the gel for 15 min in ice-cold 0.25 M KCl, and the fusion protein band was cut out. Approximately 100 μg of fusion protein in 0.25 ml of gel slice was liquified by passing through successively smaller hypodermic needles, or by sonication, and mixed with 0.25 ml of a sterile saline solution and 0.5 ml of Freund's

complete adjuvant. Two New Zealand White rabbits were injected at multiple intramuscular sites, and after 1 month, boosted at 2 week intervals, omitting the Freund's adjuvant.

Polyclonal antiserum was affinity purified according to the method of Redding et al. (1991), by passing the antibodies through two columns. A "nonspecific" affinity column, containing resin coupled to insoluble protein from E. coli expressing unmodified TrpE protein, was used to absorb out antibodies directed against TrpE and other insoluble E. coli proteins. Then a specific column, containing resin coupled to the EcR–TrpE fusion protein, was used to purify antibodies directed against the *EcR* portion of the TrpE fusion.

Anti-EcR monoclonal antibodies were raised against a different EcR–TrpE fusion protein, containing amino acids 67–878 of EcR (W. S. T. and D. S. H., unpublished data). The epitopes for the monoclonal antibodies DDA2.7 and IID9.6, used in Figures 8 and 9, are encoded by the third exon from the 5′ terminus (Figures 1 and 3) and, like the epitopes for the polyclonal antibody described above, are common to the EcR protein of Figure 1 and the two other EcR proteins noted in the Discussion.

Northern Analysis

The developmental Northern blot in Figure 8A is the same filter used by Thummel et al. (1990), serially rehybridized with *EcR* and *RP49* probes, allowing time for decay of the radioactive signal between probings. For *EcR*, a single-stranded DNA probe (Segraves and Hogness, 1990) containing nucleotides 729–2561 of the cDNA sequence was used (Figure 1). Slightly less than half of the 1833 nucleotides in this hybridization probe are complementary to the nucleotide sequence in the other two *EcR* mRNAs (see Discussion). For *RP49* (O'Connell and Rosbash, 1984), an EcoRI–XhoI genomic fragment containing the entire gene was used as a double-stranded probe.

Western Analysis

Samples for the developmental Western blot were prepared by collecting Canton S eggs for 3 hr and harvesting the developing animals at 3 hr intervals during embryogenesis, and at 12 hr intervals thereafter. Gel samples were made by grinding 1 g of tissue per 4 ml of cracking buffer (0.125 M Tris [pH 6.8], 5% β-mercaptoethanol, 2% sodium dodecyl sulfate, 4 M urea) using a motorized tissue homogenizer (Polytron). Preliminary gels, stained with Coomassie blue, were used to equalize the amount of protein loaded from the various samples. Gel samples of tissue culture cells were prepared by washing the cells in PBS, and solubilizing 3.3×10^7 cells per ml of cracking buffer. The immunoblots in Figures 6A and 8B were probed with the anti-EcR monoclonals AD4.4 and DDA2.7, respectively, followed by a horseradish peroxidase–conjugated secondary antibody (Bio-Rad). Bands were visualized using enhanced chemiluminescence reagents (Amersham), according to the specifications of the manufacturer.

Immunohistochemistry

Embryos were dechorionated, fixed, and devitellinized as described by Irvine et al. (1991), and stained by a horseradish peroxidase detection method (MacDonald and Struhl, 1986). The fixed embryos were rinsed in PBS and then washed twice for 30 min each in PBS containing 1% bovine serum albumin and 0.5% NP-40 (PBN). Primary antibodies were diluted in PBN 1:2 to 1:5 for anti-EcR monoclonal antibody culture supernatants, or 1:100 for the affinity-purified anti-EcR rabbit polyclonal antibody. The remainder of the staining procedure was as described by Irvine et al. (1991), except that PBN was substituted for BSN, and $NiSO_4$ and $CoCl_2$ were omitted from the developing reaction. Dissected tissues and copper-treated cultured cells were mounted on poly-L-lysine–coated slides, fixed in 4% paraformaldehyde, and stained as described above. Samples were stained using a number of monoclonal and polyclonal anti-EcR antibodies. The results shown in Figure 9 are representative of those obtained with multiple independent antibodies. These antibodies generally have similar reactivity in immunoblots to that shown for DDA2.7 in Figure 8B.

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References

Arriza, J. L., Weinberger, C., Cerelli, G., Glaser, T. M., Handelin, B. L., Housman, D. E., and Evans, R. M. (1987). Cloning of human mineralocorticoid receptor complementary DNA: structural and functional kinship with the glucocorticoid receptor. Science *237*, 268–275.

Ashburner, M., and Richards, G. (1976). The role of ecdysone in the control of gene activity in the polytene chromosomes of Drosophila. In Insect Development, P. A. Lawrence, ed. (New York: Wiley & Sons), pp. 203–225.

Ashburner, M., Chihara, C., Meltzer, P., and Richards, G. (1974). Temporal control of puffing activity in polytene chromosomes. Cold Spring Harbor Symp. Quant. Biol. *38*, 655–662.

Baker, A. R., McDonnell, D. P., Hughes, M., Crisp, T. M., Mangelsdorf, D. J., Haussler, M. R., Pike, J. W., Shine, J., and O'Malley, B. W. (1988). Cloning and expression of full-length cDNA encoding human vitamin D receptor. Proc. Natl. Acad. Sci. USA *85*, 3294–3298.

Barton, M. C., and Shapiro, D. J. (1988). Transient administration of estradiol-17 beta establishes an autoregulatory loop permanently inducing estrogen receptor mRNA. Proc. Natl. Acad. Sci. USA *85*, 7119–7123.

Belyaeva, E. S., Protopopov, M. O., Baricheva, E. M., Semeshin, V. F., Izquierdo, M. L., and Zhimulev, I. F. (1987). Cytogenetic analysis of region 2B3-4-2B11 of the X-chromosome of *Drosophila melanogaster*. VI. Molecular and cytological mapping of the ecs locus and the 2B puff. Chromosoma *95*, 295–310.

Benbrook, D., and Pfahl, M. (1987). A novel thyroid hormone receptor encoded by a cDNA clone from a human testis library. Science *238*, 788–791.

Best-Belpomme, M., Courgeon, A. M., and Rambach, A. (1978). β-Galactosidase is induced by hormone in *Drosophila melanogaster* cell cultures. Proc. Natl. Acad. Sci. USA *75*, 6102–6106.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. *72*, 248–254.

Bunch, T. A., Grinblat, Y., and Goldstein, L. S. (1988). Characterization and use of the *Drosophila* metallothionein promoter in cultured *Drosophila melanogaster* cells. Nucl. Acids Res. *16*, 1043–1061.

Buratowski, S., Hahn, S., Guarente, L., and Sharp, P. A. (1989). Five intermediate complexes in transcription initiation by RNA polymerase II. Cell *56*, 549–561.

Burtis, K. C. (1985). Isolation and characterization of an ecdysone inducible gene from *Drosophila melanogaster*. PhD thesis, Stanford University, Stanford, California.

Burtis, K. C., Thummel, C. S., Jones, C. W., Karim, F. D., and Hogness, D. S. (1990). The Drosophila 74EF early puff contains *E74*, a complex ecdysone-inducible gene that encodes two *ets*-related proteins. Cell *61*, 85–99.

Cavener, D. R. (1987). Comparison of the consensus sequence flanking translational start sites in *Drosophila* and vertebrates. Nucl. Acids Res. *15*, 1353–1361.

Chao, A. T., and Guild, G. M. (1986). Molecular analysis of the ecdysterone-inducible 2B5 "early" puff in *Drosophila melanogaster*. EMBO J. *5*, 143–150.

Cherbas, L., Yonge, C. D., Cherbas, P., and Williams, C. M. (1980).

The morphological response of Kc-H cells to ecdysteroids: hormonal specificity. Roux's Arch. Dev. Biol. *189*, 1–15.

Cherbas, L., Lee, K., and Cherbas, P. (1991). Identification of ecdysone response elements by analysis of the *Drosophila Eip28/29* gene. Genes Dev. *5*, 120–131.

Cherbas, P., Cherbas, L., Lee, S. S., and Nakanishi, K. (1988). 26-[^{125}I]-iodoponasterone A is a potent ecdysone and a sensitive radioligand for ecdysone receptors. Proc. Natl. Acad. Sci. USA *85*, 2096–2100.

Danielsen, M., Hinck, L., and Ringold, G. M. (1989). Two amino acids within the knuckle of the first zinc finger specify DNA response element activation by the glucocorticoid receptor. Cell *57*, 1131–1138.

de The, H., Marchio, A., Tiollais, P., and Dejean, A. (1989). Differential expression and ligand regulation of the retinoic acid receptor alpha and beta genes. EMBO J. *8*, 429–433.

DiBello, P. R., Withers, D. A., Bayer, C. A., Fristrom, J. W., and Guild, G. M. (1991). The *Drosophila Broad-Complex* encodes a family of related, zinc finger-containing proteins. Genetics, in press.

Dinan, L. (1985). Ecdysteroid receptors in a tumorous blood cell line of *Drosophila melanogaster*. Arch. Insect Biochem. Physiol. *2*, 295–317.

Dollé, P., Ruberte, E., Kastner, P., Petkovich, M., Stoner, C. M., Gudas, L. J., and Chambon, P. (1989). Differential expression of genes encoding alpha, beta and gamma retinoic acid receptors and CRABP in the developing limbs of the mouse. Nature *342*, 702–705.

Evans, R. M. (1988). The steroid and thyroid hormone receptor superfamily. Science *240*, 889–895.

Feigl, G., Gram, M., and Pongs, O. (1989). A member of the steroid hormone receptor gene family is expressed in the 20-OH-ecdysone inducible puff 75B in *Drosophila melanogaster*. Nucl. Acids Res. *17*, 7167–7178.

Forrest, D., Sjöberg, M., and Vennström, B. (1990). Contrasting developmental and tissue-specific expression of alpha and beta thyroid hormone receptor genes. EMBO J. *9*, 1519–1528.

Freedman, L. P., Luisi, B. F., Korszun, Z. R., Basavappa, R., Sigler, P. B., and Yamamoto, K. R. (1988). The function and structure of the metal coordination sites within the glucocorticoid receptor DNA binding domain. Nature *334*, 543–546.

Galceran, J., Llanos, J., Sampedro, J., Pongs, O., and Izquierdo, M. (1990). Transcription at the ecdysone-inducible locus 2B5 in *Drosophila*. Nucl. Acids Res. *18*, 539–545.

Gatermann, K. B., Rosenberg, G. H., and Kaufer, N. F. (1988). Double-stranded sequencing, using mini-prep plasmids, in eleven hours. Biotechniques *6*, 951–952.

Gietz, R. D., and Hodgetts, R. B. (1985). An analysis of dopa decarboxylase expression during embryogenesis in *Drosophila melanogaster*. Dev. Biol. *107*, 142–155.

Giguere, V., Ong, E. S., Segui, P., and Evans, R. M. (1987). Identification of a receptor for the morphogen retinoic acid. Nature *330*, 624–629.

Giguere, V., Yang, N., Segui, P., and Evans, R. M. (1988). Identification of a new class of steroid hormone receptors. Nature *331*, 91–94.

Glass, C. K., Lipkin, S. M., Devary, O. V., and Rosenfeld, M. G. (1989). Positive and negative regulation of gene transcription by a retinoic acid–thyroid hormone receptor heterodimer. Cell *59*, 697–708.

Green, S., and Chambon, P. (1987). Oestradiol induction of a glucocorticoid-responsive gene by a chimaeric receptor. Nature *325*, 75–78.

Green, S., and Chambon, P. (1988). Nuclear receptors enhance our understanding of transcription regulation. Trends Genet. *4*, 309–314.

Green, S., Walter, P., Kumar, V., Krust, A., Bornert, J. M., Argos, P., and Chambon, P. (1986). Human oestrogen receptor cDNA: sequence, expression and homology to v-erb-A. Nature *320*, 134–139.

Greene, G. L., Gilna, P., Waterfield, M., Baker, A., Hort, Y., and Shine, J. (1986). Sequence and expression of human estrogen receptor complementary DNA. Science *231*, 1150–1154.

Guiochon-Mantel, A., Loosfelt, H., Lescop, P., Sar, S., Atger, M., Perrot-Applanat, M., and Milgrom, E. (1989). Mechanisms of nuclear localization of the progesterone receptor: evidence for interaction between monomers. Cell *57*, 1147–1154.

Handler, A. M. (1982). Ecdysteroid titres during pupal and adult development in *Drosophila melanogaster*. Dev. Biol. *93*, 73–82.

Handler, A. M., and Maroy, P. (1989). Ecdysteroid receptors in *Drosophila melanogaster* adult females. Mol. Cell Endocrinol. *63*, 103–109.

Härd, T., Kellenbach, E., Boelens, R., Maler, B. A., Dahlman, K., Freedman, L. P., Carlstedt-Duke, J., Yamamoto, K. R., Gustafsson, J., and Kaptein, R. (1990). Solution structure of the glucocorticoid receptor DNA-binding domain. Science *249*, 157–160.

Haynes, S. R., Rebbert, M. L., Mozer, B. A., Forquignon, F., and Dawid, I. B. (1987). pen repeat sequences are GGN clusters and encode a glycine-rich domain in a *Drosophila* cDNA homologous to the rat helix destabilizing protein. Proc. Natl. Acad. Sci. USA *84*, 1819–1823.

Heberlein, U., England, B., and Tjian, R. (1985). Characterization of Drosophila transcription factors that activate the tandem promoters of the alcohol dehydrogenase gene. Cell *41*, 965–977.

Henikoff, S. (1984). Unidirectional digestion with exonuclease III creates targeted breakpoints for DNA sequencing. Gene *28*, 351–359.

Henrich, V. C., Sliter, T. J., Lubahn, D. B., MacIntyre, A., and Gilbert, L. I. (1990). A steroid/thyroid hormone receptor superfamily member in *Drosophila melanogaster* that shares extensive sequence similarity with a mammalian homologue. Nucl. Acids Res. *18*, 4143–4148.

Hollenberg, S. M., Weinberger, C., Ong, E. S., Cerelli, G., Oro, A., Lebo, R., Thompson, E. B., Rosenfeld, M. G., and Evans, R. M. (1986). Primary structure and expression of a functional human glucocorticoid receptor cDNA. Nature *318*, 635–641.

Irvine, K. D., Helfand, S. L., and Hogness, D. S. (1991). The large upstream control region of the *Drosophila* homeotic gene *Ultrabithorax*. Development *111*, 407–424.

Issemann, I., and Green, S. (1990). Activation of a member of the steroid hormone receptor superfamily by peroxisome proliferators. Nature *347*, 645–650.

Janknecht, R., Taube, W., Ludecke, H. J., and Pongs, O. (1989). Characterization of a putative transcription factor gene expressed in the 20-OH-ecdysone inducible puff 74EF in *Drosophila melanogaster*. Nucl. Acids Res. *17*, 4455–4464.

Karim, F. D., and Thummel, C. S. (1991). Ecdysone coordinates the timing and amounts of *E74A* and *E74B* transcription in *Drosophila*. Genes Dev. *5*, 1067–1079.

Kaufman, P. D., Doll, R. F., and Rio, D. C. (1989). Drosophila P element transposase recognizes internal P element DNA sequences. Cell *59*, 359–371.

King, W. J., and Greene, G. L. (1984). Monoclonal antibodies localize oestrogen receptor in the nuclei of target cells. Nature *307*, 745–747.

Kiss, I., Beaton, A. H., Tardiff, J., Fristrom, D., and Fristrom, J. W. (1988). Interactions and developmental effects of mutations in the Broad-Complex of *Drosophila melanogaster*. Genetics *118*, 247–259.

Koelle, M. R., Segraves, W. A., and Hogness, D. S. (1991). DHR3: a new *Drosophila* steroid receptor homolog. Proc. Natl. Acad. Sci. USA, in press.

Kozak, M. (1984). Compilation and analysis of sequences upstream from the translational start site in eukaryotic mRNAs. Nucl. Acids Res. *12*, 857–872.

Kraminsky, G. P., Clark, W. C., Estell, M. A., Gietz, R. D., Sage, B. S., O'Connor, J. D., and Hodgetts, R. B. (1980). Proc. Natl. Acad. Sci. USA *77*, 4175–4179.

Krasnow, M. A., Saffman, E. E., Kornfeld, K., and Hogness, D. S. (1989). Transcriptional activation and repression by *Ultrabithorax* proteins in cultured Drosophila cells. Cell *57*, 1031–1043.

Krust, A., Green, S., Argos, P., Kumar, V., Walter, P., Bornert, J. M., and Chambon, P. (1986). The chicken oestrogen receptor sequence: homology with *v-erbA* and the human oestrogen and glucocorticoid receptors. EMBO J. *5*, 891–897.

Kumar, V., and Chambon, P. (1988). The estrogen receptor binds

tightly to its responsive element as a ligand-induced homodimer. Cell *55*, 145–156.

Lavorgna, G., Ueda, H., Clos, J., and Wu, C. (1991). FTZ-F1, a steroid hormone receptor-like protein implicated in the activation of *fushi tarazu*. Science *252*, 848–851.

Lubahn, D. B., Joseph, D. R., Sar, M., Tan, J., Higgs, H. N., Larson, R. E., French, F. S., and Wilson, E. M. (1988). The human androgen receptor: complementary deoxyribonucleic acid cloning, sequence analysis, and gene expression in prostate. Mol. Endocrinol. *2*, 1265–1275.

MacDonald, P. M., and Struhl, G. (1986). A molecular gradient in early *Drosophila* embryos and its role in specifying the body pattern. Nature *324*, 537–545.

Mader, S., Kumar, V., de Verneuil, H., and Chambon, P. (1989). Three amino acids of the oestrogen receptor are essential to its ability to distinguish an oestrogen from a glucocorticoid-responsive element. Nature *338*, 271–274.

Mangelsdorf, D. J., Pike, J. W., and Haussler, M. R. (1987). Avian and mammalian receptors for 1,25-dihydroxyvitamin D3: in vitro translation to characterize size and hormone-dependent regulation. Proc. Natl. Acad. Sci. USA *84*, 354–358.

Mangelsdorf, D. J., Ong, E. S., Dyck, J. A., and Evans, R. M. (1990). Nuclear receptor that identifies a novel retinoic acid response pathway. Nature *345*, 224–229.

Maniatis, T., Hardison, R. C., Lacy, E., Lauer, J., O'Connell, C., Quon, D., Sim, G. K., and Efstratiadis, A. (1978). The isolation of structural genes from libraries of eucaryotic DNA. Cell *15*, 687–701.

Maroy, P., Dennis, R., Beckers, C., Sage, B., and O'Connor, J. D. (1978). Demonstration of an ecdysteroid receptor in a cultured cell line of *Drosophila melanogaster*. Proc. Natl. Acad. Sci. USA *75*, 6035–6038.

Milbrandt, J. (1988). Nerve growth factor induces a gene homologous to the glucocorticoid receptor gene. Neuron *1*, 183–188.

Misrahi, M., Atger, M., d'Auriol, L., Loosfelt, H., Meriel, C., Fridlansky, F., Guiochon, M. A., Galibert, F., and Milgrom, E. (1987). Complete amino acid sequence of the human progesterone receptor deduced from cloned cDNA. Biochem. Biophys. Res. Commun. *143*, 740–748.

Miyajima, N., Horiuchi, R., Shibuya, Y., Fukushige, S., Matsubara, K., Toyoshima, K., and Yamamoto, T. (1989). Two *erbA* homologs encoding proteins with different T_3 binding capacities are transcribed from opposite DNA strands of the same genetic locus. Cell *57*, 31–39.

Mlodzik, M., Hiromi, Y., Weber, U., Goodman, C. S., and Rubin, G. M. (1990). The Drosophila *seven-up* gene, a member of the steroid receptor gene superfamily, controls photoreceptor cell fates. Cell *60*, 211–224.

Mount, S. M. (1982). A catalogue of splice junction sequences. Nucl. Acids Res. *10*, 459–472.

Nauber, U., Pankratz, M. J., Kienlin, A., Seifert, E., Klemm, U., and Jackle, H. (1988). Abdominal segmentation of the *Drosophila* embryo requires a hormone receptor-like protein encoded by the gap gene *knirps*. Nature *336*, 489–492.

O'Connell, P. O., and Rosbash, M. (1984). Sequence, structure, and codon preference of the *Drosophila* ribosomal protein 49 gene. Nucl. Acids Res. *12*, 5495–5513.

Oro, A. E., Ong, E. S., Margolis, J. S., Posakony, J. W., McKeown, M., and Evans, R. M. (1988). The *Drosophila* gene *knirps-related* is a member of the steroid-receptor gene superfamily. Nature *336*, 493–496.

Oro, A. E., McKeown, M., and Evans, R. M. (1990). Relationship between the product of the *Drosophila ultraspiracle* locus and the vertebrate retinoid X receptor. Nature *347*, 298–301.

Osterbur, D. L., and Yund, M. A. (1982). Ecdysteroid binding activity in embryos of *Drosophila melanogaster*. J. Cell Biochem. *20*, 277–282.

Perrot-Applanat, M., Logeat, F., Groyer, P. M., and Milgrom, E. (1985). Immunocytochemical study of mammalian progesterone receptor using monoclonal antibodies. Endocrinology *116*, 1473–1484.

Petkovich, M., Brand, N. J., Krust, A., and Chambon, P. (1987). A human retinoic acid receptor which belongs to the family of nuclear receptors. Nature *330*, 444–450.

Picard, D., and Yamamoto, K. R. (1987). Two signals mediate hormone-dependent nuclear localization of the glucocorticoid receptor. EMBO J. *6*, 3333–3340.

Pignoni, F., Baldarelli, R. M., Steingrimsson, E., Diaz, R. J., Patapoutian, A., Merriam, J. R., and Lengyel, J. A. (1990). The Drosophila gene *tailless* is expressed at the embryonic termini and is a member of the steroid receptor superfamily. Cell *62*, 151–163.

Pratt, W. B., Jolly, D. J., Pratt, D. V., Hollenberg, S. M., Giguere, V., Cadepond, F. M., Schweizer, G. G., Catelli, M. G., Evans, R. M., and Baulieu, E. E. (1988). A region in the steroid binding domain determines formation of the non-DNA-binding, 9 S glucocorticoid receptor complex. J. Biol. Chem. *263*, 267–273.

Redding, K., Hokomb, C., and Fuller, R. S. (1991). Immunolocalization of Kex2 protease identifies a putative late Golgi compartment in yeast *Saccharomyces cerevisiae*. J. Cell Biol. *113*, 527–538.

Richards, G. P. (1981a). Insect hormones in development. Biol. Rev. *56*, 501–549.

Richards, G. P. (1981b). The radioimmune assay of ecdysteroid titres in *Drosophila melanogaster*. Mol. Cell. Endocrinol. *21*, 181–197.

Riddihough, G., and Pelham, H. R. B. (1987). An ecdysone response element in the *Drosophila hsp27* promoter. EMBO J. *6*, 3729–3734.

Rimm, D. L., and Pollard, T. D. (1989). New plasmid vectors for high level synthesis of eukaryotic fusion proteins in *Escherichia coli*. Gene *75*, 323–327.

Rio, D. C., Laski, F. A., and Rubin, G. M. (1986). Identification and immunochemical analysis of biologically active Drosophila P element transposase. Cell *44*, 21–32.

Rothe, M., Nauber, U., and Jackle, H. (1989). Three hormone receptor-like *Drosophila* genes encode an identical DNA-binding finger. EMBO J. *8*, 3087–3094.

Ruther, U., and Muller, H. B. (1983). Easy identification of cDNA clones. EMBO J. *2*, 1791–1794.

Sage, B. A., Tanis, M. A., and O'Connor, J. D. (1982). Characterization of ecdysteroid receptors in cytosol and naive nuclear preparations of *Drosophila* Kc cells. J. Biol. Chem. *257*, 6373–6379.

Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989). Molecular Cloning: A Laboratory Manual, Second Edition (Cold Spring Harbor, New York: Cold Spring Harbor Laboratory).

Sanger, F., Nicklen, S., and Coulson, A. R. (1977). DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA *74*, 5463–5467.

Schneider, I., and Blumenthal, A. B. (1978). *Drosophila* cell and tissue culture. In Genetics and Biology of Drosophila, Volume 2A, M. Ashburner and T. R. F. Wright, eds. (London: Academic Press), pp. 265–315.

Schüle, R., Umesono, K., Mangelsdorf, D. J., Bolado, J., Pike, J. W., and Evans, R. M. (1990). Jun–Fos and receptors for vitamins A and D recognize a common response element in the human osteocalcin gene. Cell *61*, 497–504.

Scott, M. P., and Carroll, S. B. (1987). The segmentation and homeotic gene network in early Drosophila development. Cell *51*, 689–698.

Segraves, W. A. (1988). Molecular and genetic analysis of the *E75* ecdysone-responsive gene of *Drosophila melanogaster*. PhD thesis, Stanford University, Stanford, California.

Segraves, W. A., and Hogness, D. (1990). The *E75* ecdysone-inducible gene responsible for the 75B early puff in *Drosophila* encodes two new members of the steroid receptor superfamily. Genes Dev. *4*, 204–219.

Shea, M. J., King, D. L., Conboy, M. J., Mariani, B. D., and Kafatos, F. C. (1990). Proteins that bind to *Drosophila* chorion cis-regulatory elements: a new C_2H_2 zinc finger protein and a C_2C_2 steroid receptor-like component. Genes Dev. *4*, 1128–1140.

Stevens, B., and O'Connor, J. D. (1982). The acquisition of resistance to ecdysteroids in cultured *Drosophila* cells. Dev. Biol. *94*, 176–182.

Thummel, C. S., Burtis, K. C., and Hogness, D. S. (1990). Spatial and temporal patterns of *E74* transcription during Drosophila development. Cell *61*, 101–111.

Umesono, K., and Evans, R. M. (1989). Determinants of target gene specificity for steroid/thyroid hormone receptors. Cell *57*, 1139–1146.

Umesono, K., Giguere, V., Glass, C. K., Rosenfeld, M. G., and Evans, R. M. (1988). Retinoic acid and thyroid hormone induce gene expression through a common responsive element. Nature *336*, 262–265.

Urness, L. D., and Thummel, C. S. (1990). Molecular interactions within the ecdysone regulatory hierarchy: DNA binding properties of the Drosophila ecdysone-inducible *E74A* protein. Cell *63*, 47–61.

Wang, L. H., Tsai, S. Y., Cook, R. G., Beattie, W. G., Tsai, M. J., and O'Malley, B. W. (1989). COUP transcription factor is a member of the steroid receptor superfamily. Nature *340*, 163–166.

Weinberger, C., Thompson, C. C., Ong, E. S., Lebo, R., Gruol, D. J., and Evans, R. M. (1986). The *c-erb-A* gene encodes a thyroid hormone receptor. Nature *324*, 641–646.

Yund, M. A. (1979). Specific binding of 20-hydroxyecdysone to nuclei of imaginal discs of *Drosophila melanogaster*. Mol. Cell. Endocrinol. *14*, 19–35.

Yund, M. A., King, D. S., and Fristrom, J. W. (1978). Ecdysteroid receptors in imaginal discs of *Drosophila melanogaster*. Proc. Natl. Acad. Sci. USA *75*, 6039–6043.

Zelent, A., Krust, A., Petkovich, M., Kastner, P., and Chambon, P. (1989). Cloning of murine alpha and beta retinoic acid receptors and a novel receptor gamma predominantly expressed in skin. Nature *339*, 714–717.

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