Identification of ecdysone response elements by analysis of the *Drosophila Eip28/29* gene

Lucy Cherbas,¹ Keesook Lee,¹ and Peter Cherbas

Program in Molecular, Cellular, and Developmental Biology, Institute for Molecular and Cellular Biology, and the Department of Biology, Indiana University, Bloomington, Indiana 47405 USA

We have identified ecdysone-response elements (EcREs) by studying regulation of the steroid-responsive *Drosophila Eip28/29* gene. First, functional assays of deletion mutants identified large sequence regions required for the response; then a blotting method using the specifically labeled steroid receptor as probe identified receptor-binding regions. Three short receptor-binding regions near *Eip28/29* have been identified: *Prox* and *Dist* [521 and 2295 nucleotides, respectively, downstream of the poly(A) site] are probably required for the *Eip28/29* response in cell lines; *Upstream* (–440) is unnecessary for that response. We have also demonstrated that an EcRE-containing region from *hsp27* contains a receptor-binding site. Each of these four receptor-binding regions functions as an EcRE when placed upstream of an ecdysone nonresponsive promoter and each contains an imperfect palindrome, suggesting the consensus 5'-RG(GT)TCANTGA(CA)CY-3'. Furthermore, a synthetic 15-bp fragment containing an imperfect palindrome similar to the consensus is a fully functional EcRE. The presence of any of the EcREs leads, in the absence of hormone, to depressed gene expression. When hormone is added, it relieves this repression and causes additional activation. The similarity of the EcRE sequence to response elements for estrogen, thyroid hormone, and retinoic acid receptors suggests that the steroid receptors and their signal transduction mechanisms have been strongly and broadly conserved.

[Key Words: Ecdysone response elements; ecdysone receptor; steroid hormones; *Drosophila melanogaster*; *Eip28/29*; Kc cells]

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Ecdysone is the steroid molting hormone of arthropods; it regulates the molting cycle and provokes the remarkable developmental events associated with metamorphosis. Ever since the pioneering work of Clever (1964) and Ashburner (see, e.g., Ashburner et al. 1974) on hormone-induced puffing in salivary gland chromosomes suggested that ecdysone might act by regulating transcription, it has been attractive to suppose that all of the steroid hormones are functionally related and that they control transcription by similar mechanisms. If so, studies of ecdysone action in *Drosophila* should contribute significantly to our understanding of the mechanistic bases of the developmental specificity of steroid effects.

Strong evidence has accumulated demonstrating that the vertebrate steroids do share a common mechanism of action, a mechanism that depends on a conserved family of ligand-dependent transcription factors, the steroid receptors. Each receptor binds its cognate ligand tightly and specifically and, in the presence of steroid, these zinc-finger proteins also bind to specific hormone response elements (HREs) in the vicinity of responsive genes. The HREs function as hormone-dependent tran-

scriptional enhancers. The HREs for the vertebrate steroid hormones constitute a family of short, more-or-less palindromic, and closely related DNA sequences, which are strongly conserved among vertebrates (for review, see Evans 1988; Beato 1989; Carson-Jurica et al. 1990).

In the case of ecdysone, considerable indirect evidence is consistent with a similar mechanism of action, but direct evidence for transcriptional regulation by ecdysone receptors has been elusive. Thus, active ecdysones are known to bind with appropriate affinities to a nuclear protein (or proteins) present at low abundance in responsive cells and thought to be receptor(s) (Maroy et al. 1978; Yund et al. 1978; Sage et al. 1982; Cherbas et al. 1988), and a number of genes with the response characteristics of immediate hormone targets ("early effects") are known (for review, see Cherbas et al. 1986a; Ashburner 1990; Segraves and Richards 1990). Using one of these genes (*hsp27*), Riddihough and Pelham (1987) described an upstream sequence sufficient to confer hormonal inducibility in transient assays. They proposed, but did not establish, that a protein binding that sequence is the ecdysone receptor.

Our goal has been to identify ecdysone-response elements (EcREs); to do so we have begun by identifying sequences necessary for the hormonal regulation of the gene *Eip28/29*, which is ecdysone-inducible in some

¹The two first authors are listed alphabetically.

Drosophila cell lines (Savakis et al. 1980, 1984) and in some larval tissues (Andres 1990). In cell lines this induction is known to be transcriptional and rapid (Savakis et al. 1980, 1984; Bieber 1986); in these respects, its regulation can be considered analogous to that of the "early puffs" in the salivary gland. The region just upstream of *Eip28/29* is transcriptionally complex; the genes *z600*, *gdl*, and *Eip28/29* are represented by overlapping transcription units on a single DNA strand, but the three genes are expressed at very different times and places in development (Schulz et al. 1989; Schulz and Butler 1989; Schulz and Miksch 1989). Of particular interest in the present context is the fact that *gdl*, whose 3'-noncoding region overlaps the 5' end of *Eip28/29*, is expressed at a low level in Kc cells but is entirely unaffected by ecdysone (Schulz et al. 1989). Hence, any satisfactory explanation for the ecdysone responsiveness of *Eip28/29* must also account for the fact that its overlapping upstream neighbor is hormone refractory.

In the present communication we describe the sequences of several functional EcREs. These elements have been identified by their roles in the *cis* regulation of *Eip28/29*, by their ability to bind ecdysone receptors, and by their ability to confer hormonal inducibility on suitable reporters. Taken together, these sequences (along with the sequence described by Riddihough and Pelham 1987) provide a consistent picture of the nature of an EcRE. Both the fact that the ecdysone receptor binds to EcREs and the sequence content of the EcREs themselves provide strong support for the idea that ecdysone and its receptor are elements of a highly conserved signal response system very similar to that of the vertebrate steroid hormones.

Results

We began by using deletion mutagenesis and transient expression in *Drosophila* cells to map sequences involved in the ecdysone responsiveness of *Eip28/29*. Both the Kc167/M3 and EH34A3/M3 cell lines show excellent *Eip28/29* induction, and both yield good gene expression following transfection; we have used both lines, with indistinguishable results, in the experiments that follow (see Materials and methods).

All of the cell lines we have tried share the serious handicap that simple exposure to the conditions of transfection reduces the magnitude of the ecdysone response as judged by *Eip28/29* induction. Thus, after any transfection (see, e.g., Fig. 1) or mock transfection (no exogenous DNA; data not shown) the endogenous *Eip28/29* gene is induced only 3- to 4-fold (Fig. 1, lanes 3–6) rather than 10- to 15-fold as it is under normal conditions (lanes 1–2). Although this constriction of the dynamic range of the response may eventually prove limiting, it has not, in the present instance, prevented us from identifying regions with strong effects on the hormone response.

For our initial experiments (Fig. 2), plasmids were constructed in which *Eip28/29*-flanking sequences of varying extents were combined with either of two re-

Figure 1. Induction of tagged *Eip28/29* genes by ecdysone. EH34A3/M3 cells were transfected with plasmids bearing tagged *Eip28/29* genes, and RNA was isolated 24 hr later for analysis by Northern blots; during the final 4 hr of incubation, cells for some lanes were treated with hormone (10^{-6} M 20-hydroxyecdysone). (For the structures of the plasmids, see Fig. 2.) (A) The blot was probed with a single-stranded *Eip28/29*-specific DNA probe to reveal transcripts from both the exogenous (solid arrowhead) and the endogenous (open arrowhead) genes. (Lanes 1 and 2) Untransformed cells; (lanes 3 and 4) construct 12; (lanes 5 and 6) construct 1. (Lanes 1, 3, and 5) No hormone treatment; (lanes 2, 4, and 6) hormone-treated. (B) Cells were cotransformed with an equimolar mixture of the indicated plasmid and *Eip188-Adh* as internal control (see Materials and methods). Duplicate blots were probed with a single-stranded RNA probe for λ (top) and a single-stranded RNA probe for *Adh* (bottom). (Lanes 1 and 2) Construct 1; (lanes 3 and 4) construct 4; (lanes 5 and 6) construct 10; (lanes 7 and 8) construct 12. (Lanes 1, 3, 5, and 7) no hormone treatment; (lanes 2, 4, 6, and 8) hormone treated.

porters: (1) a modified *Eip28/29* transcription unit tagged, in the 3'-untranslated region, by insertion of a 499-bp fragment of bacteriophage λ and detectable by Northern analysis, or (2) the chloramphenicol acetyltransferase (*cat*) gene.

Table 1 indicates that basal expression (i.e., hormone-independent expression) does not require an extended 5'-flanking region; constructs containing only 188 bp of 5'-flanking DNA yield essentially full basal expression. Relative basal expression levels were determined for most of the *Eip* transcription unit-based constructs shown in Figure 2 by cotransfecting with an *Adh*-expressing plasmid, normalizing, and comparing the results with construct 1 (see Materials and methods). For construct 1 itself, our impression, on the basis of results from numerous permanently transformed clonal cell lines, is that expression per gene copy is comparable to that for the endogenous gene (data not shown). Save for a very minor decrease in expression when the 5'-flanking region extends upstream of -657 —a decrease that may be related to the presence (and function; data not shown) of the *gdl* promoter—exceptional levels of basal expression were exhibited by only two constructs, 3 and 12. Construct 3 lacks introns, and we suspect that its diminished expression reflects instability of the primary transcript. Construct 12 is more interesting. This construct, which has the shortest 3'-flanking region tested, exhibits reproducibly elevated basal expression (e.g., Fig. 1A, lane 3; Fig. 1B, lane 7), a phenomenon to which we shall return later.

Figure 2. Deletion analysis of ecdysone inducibility at *Eip28/29*. (Left) The overlapping transcription units for *Eip28/29* (Cherbas et al. 1986b), *gdl*, and *z600* (Schulz et al. 1989; Schulz and Butler 1989) are shown at the top; the axis (in kb) represents the entire sequenced region, with the origin taken as +1 of the *Eip28/29* transcription unit. [For clarity, we have included only two (short form *gdl^M* and long form *gdl^L*) of the four *gdl* transcription units.] Rectangles represent exons; solid rectangles represent coding sequence. The flanking sequences (of *Eip28/29*) are represented by thin open bars. Within the constructs, the site of the 499-bp bacteriophage λ insertion in the 3'-noncoding region is indicated. Open rectangular boxes labeled *cat* represent a 1.6-kb fragment containing a bacterial *cat* gene; for simplicity it is drawn as if it were the same length as the *Eip28/29* transcription unit. In each case the *cat* fragment is inserted between bases +11 and +2205 of *Eip28/29* (where the transcribed region is +1 to +2147). (For details of the constructions, see Materials and methods.) Individual construct numbers are given at left; the constructs have been grouped to facilitate comparison. Regions A–D, described in the text, are indicated at bottom. (Right) The histogram shows the induction ratio for each construct (λ -tagged constructs tested in EH34A3 cells; *cat* constructs tested in Kc167 cells). Error bars indicate standard errors; the number of independent experiments is shown in brackets. The dashed base line has been drawn at an induction ratio of 1 to emphasize the decreased expression in the null constructs 15 and 16.

Because the *cat* data are not directly comparable, we cannot eliminate the possibility that sequences internal to the *Eip* transcription unit contribute to basal expression. We know from experiments using *cat* reporters that sequences just 3' of the transcription unit, in region B, are important for full basal expression in Kc167 and EH34A3 cells (data not shown).

Numerous elements, distributed widely within both flanking sequences, contribute to a full ecdysone response

Figure 1A (lanes 5 and 6) demonstrates that the tagged *Eip28/29* gene in construct 1 is fully inducible in the

Table 1. Relative basal expression

Constructs	Relative basal expression
4, 10, 11	0.99 ± 0.08 (9)
5, 6, 7	1.26 ± 0.08 (8)
3	0.43 ± 0.15 (3)
12	2.87 ± 0.32 (3)

Basal expression was measured by Northern analysis, and the measurements were corrected by reference to the cotransfected control plasmid *Eip188-Adh*. Construct 1 was included in each experiment, and all values were normalized by setting the expression of construct 1 to 1.00 [for construct structures, see Fig. 2]. Data are presented as relative expression $\pm$ SE (independent experiments). Inter se differences among the four groups were highly significant ($P < 5\%$ for the first two groups; $P < 1\%$ for the others by t-tests).

sense that its induction ratio (3.2) is comparable to that for the endogenous gene.

Comparison of the first group of constructs in Figure 2 shows that 3'-flanking sequences are important to the full ecdysone response. Constructs 1 and 4 do not differ significantly; hence, we doubt that significant elements reside to the right of +5882 on the map. However, constructs 10 and 11 (deleted for region D) show a modestly reduced hormone response. Further 3' truncation as in construct 12 (deleted for both regions C and D) leads to a marked additional reduction of the response.

These results show the essentially full induction of constructs 1 and 4 (Fig. 1A, lanes 5 and 6; Fig. 1B, lanes 1–4), the modestly diminished induction of construct 10 (Fig. 1B, lanes 5 and 6), and the substantially reduced induction of construct 12 (Fig. 1A, lanes 3 and 4; Fig. 1B, lanes 7 and 8). The weak induction of construct 12 appears to be due, at least in part, to the significant elevation in its basal expression noted earlier.

The second group of constructs was derived from mini-genes and lacks introns. Comparison of the ecdysone responses of appropriate pairs (constructs 3 with 1 and 14 with 12) suggests that the introns do not contribute significantly to the response.

A third group of constructs lack the 5' region A. This set (constructs 5–7) can be compared fruitfully with construct 4, which has the same 3' extension. The comparison shows that deletion of region A reduces the ecdysone effect modestly.

Finally, to eliminate effects of all the *Eip28/29* transcription unit sequences, we have constructed a series of

cat fusions (constructs 8, 9, 15, and 16). Constructs (15 and 16) that contain 188 or 657 bp of 5'-flanking sequence are not induced by ecdysone. Ecdysone treatment leads to a reduction ($\sim 20\%$) in their expression. Previously, we had observed just such a reduction with a variety of reporter plasmids assumed to be wholly nonresponsive to ecdysone, for example, pSV2.*cat* (Gorman et al. 1982; L. Cherbas and N. Rosenthal, unpubl.). We suppose that this is due to some unknown effect of ecdysone on the uptake, retention, or expression of exogenous DNA, and we take an induction ratio of 0.8 to represent a truly "null" response. In contrast, *cat* constructs (8 and 9) that contain the 3' regions *B*, *C*, and *D* coupled with the same 5' sequences give ecdysone responses comparable to those of constructs 5–7, which contain essentially identical flanking regions.

Our results indicate that some element (or elements) within each of the four regions can contribute, at least in certain circumstances, to expression of a full ecdysone response. This is more clearly shown in Table 2, where the data have been aggregated according to the flanking regions included. Plainly, we observe four distinct levels of inducibility, and these reflect the complement of important flanking regions included. The residual inducibility of group III must be due to elements located within either region *A* or *B*. We are confident that *B* contains a weak EcRE because *cat* constructs containing region *B* coupled with only 188 bp of upstream sequence have inducibilities comparable to those of constructs 12 and 14 and because constructs containing extensive upstream sequences, but lacking downstream sequences, are not inducible (data not shown).

Evidently, sequences contributing to the ecdysone response, while absent from the transcription unit per se, are widely distributed in the regions flanking *Eip28/29*, with at least one significant element in the upstream region between -2007 and -658 and three dispersed elements within the 3735 bp downstream of the polyadenylation site. Each of these elements is a candidate to be an EcRE. Given the extent of the DNA to be tested, we elected to proceed by searching for sites that bind the ecdysone receptor.

A blotting assay identifies three receptor-binding sites near Eip28/29

We devised an approach to facilitate the screening of extended regions of DNA for receptor-binding sites. Briefly, separated restriction fragments were blotted, as native double-stranded molecules, to a cationic membrane. After membrane blocking, receptor-binding sites were probed by incubating the membrane with a crude extract in which the ecdysone receptor had been specifically labeled with the hormone analog 26-[125 I]iodoponasterone A (for details, see Materials and methods; Cherbas et al. 1988).

The sensitivity of such a screen ought to be dependent on restriction fragment length. Typically, steroid receptors display only modest DNA-binding specificity, affinities for specific sites being only $\sim 10^3$ those for

Table 2. Flanking sequence content defines four induction ratio levels

Group	Constructs	Regions included	Induction ratio
I	1, 3, 4	A, B, C, D	3.03 ± 0.18 (23)
II	10, 11	A, B, C	2.40 ± 0.14 (13)
	5–9	B, C, D	2.43 ± 0.18 (8)
III	12, 14	A, B	1.38 ± 0.08 (25)
IV	15, 16	none	0.82 ± 0.04 (30)

Data are presented as induction ratio $\pm$ SE (independent experiments). Constructs and regions are defined in Fig. 2. Inter se differences among the four groups were highly significant ($P < 2\%$ for every comparison by t-tests).

random sites (see, e.g., Pfahl 1982). If the ecdysone receptor behaves similarly, and if a signal/background of 2 is selected as a practical threshold, fragments should not exceed 1 kb.

Figure 3 shows some typical results. A low-resolution experiment in which the starting DNA (-657 to $+7100$) was cut into mostly large restriction fragments is shown in Figure 3A. Most fragments show nonspecific binding giving an autoradiographic signal roughly proportional to fragment length (a conclusion confirmed by densitometry; data not shown). However, in this instance, it is clear that the two smallest fragments (*Pst*I-d, 648 bp and *Bgl*III-d, 432 bp) yield disproportionately strong signals indicative of high-affinity sites. As expected, neither site could have been discerned by inspection of the corresponding large fragments (*Bgl*III-a and *Pst*I-a, both >5 kb). In such low-resolution experiments, only restriction fragments less than ~ 1 kb in length are fully informative, although impressions based on somewhat larger fragments have generally survived further testing.

When the restriction fragments are smaller, as in Figure 3B, the identification of high-affinity sites becomes unambiguous. An experiment involving the 432-bp fragment *Bgl*III-d from Figure 3A is shown in Figure 3B. A series of four bifurcations of the fragment delineate a 27-bp region that is evidently critical for binding. This site is designated *Prox* since it is the more proximal of two downstream binding sites.

All of the binding that we observe, both nonspecific and specific, is due to ecdysone receptor: (1) All of the binding can be blocked by competition with unlabeled steroid (muristerone A, 10^{-4} M); and (2) previously, it has been demonstrated for similar extracts labeled under comparable conditions that all competition-sensitive binding is due to a protein (or proteins) with the steroid specificity of ecdysone receptor (Cherbas et al. 1988). Thus, unbound (or nonspecifically bound) ligand does not contribute to the signals that we observe; similarly, prior removal of unbound label (by gel filtration) does not affect the results (data not shown).

Using this blotting assay we have looked for receptor-binding sites throughout most of a 9-kb region around *Eip28/29*, covering virtually all of the sequence from -1929 to $+7100$ with overlapping restriction fragments of <1 kb, as summarized in Figure 4. As shown in Figure

Figure 3. Mapping ecdysone receptor-binding sites by blotting. (A) Coarse mapping of binding sites in the *Eip28/29* region. A plasmid whose insert is similar to construct 5 but extends downstream to +7100 (see Fig. 2) was digested with *Pst*I or *Bgl*II, and the resulting fragments were separated by electrophoresis (1% agarose in TAE, 4 μ g DNA/lane) in duplicate panels. After electrophoresis, one panel was stained with ethidium bromide, and the entire gel was electroblotted. The stained portion of the blot was photographed (left); the unstained portion was incubated with a 125 I-labeled receptor-ligand mixture (see Materials and methods) and autoradiographed (right). In the map of the insert, fragments are labeled simply a–d in decreasing order of size. There are no sites for

either enzyme in 2.7 kb of pUC8 vector sequence. The positions of the *Eip28/29* transcription unit, the λ insert (hatched), and fragments that bound the labeled receptor disproportionately (*Upstream* and *Prox*) are indicated below the map. (B) Fine mapping of the binding site *Prox*. The starting fragment was an isolated 432-bp *Bgl*II fragment (fragment d in A). To control for blotting efficiency and for the effects of washing, all operations were carried out in parallel on two DNA samples: unlabeled DNA and "tracer DNA" that had been labeled with 32 P by nick translation. Aliquots of each DNA (0.2 μ g) were digested with the enzymes indicated and electrophoresed (8% polyacrylamide in TBE); the entire gel was electroblotted. The unlabeled panel was incubated with 125 I-labeled receptor–ligand complex, and washed; the tracer panel was autoradiographed, incubated, and washed similarly, except that cell extract and 125 I-labeled steroid were omitted from the incubation. Both panels were visualized by autoradiography. (Left) A 3-hr exposure (with intensifying screen) of the tracer panel after incubation and washing showing the two bands that result from cleavage with each restriction enzyme. [An autoradiogram of the same panel prior to incubation and washing appeared identical (not shown).] (Right) A 48-hr exposure (with intensifying screen) of the 125 I-labeled portion; it demonstrates that only one of each pair of restriction fragments contains a receptor-binding site. The overlap of the binding-positive regions (27 bp) defines *Prox*.

Figure 4. A survey of the *Eip28/29* region for ecdysone receptor-binding sites. Each thin horizontal line shows the extent of a restriction fragment that has been tested, by experiments similar to those in Fig. 3, for ecdysone receptor binding. Fragments above the dashed line exhibited no high-affinity binding; those below it exhibited clear evidence of one or more high-affinity sites. Alignments of these fragments identified the three binding sites *Upstream*, *Prox*, and *Dist*, whose positions and extents are indicated below. For orientation, the transcription units for *Eip28/29* and *gdlF* and the positions of regions A–D are given as in Fig. 2.

4, three sites within this large region tested positive: *Upstream* (–457 to –415); *Prox* (+2651 to +2681, within region C); and *Dist* (+4433 to +4469, within region D). The sequences of these sites are shown in Figure 5.

For comparison, we applied the same assay to the upstream region of *hsp27* in which a 23-bp region (–551 to –529; Riddihough and Pelham 1987) confers ecdysone responsiveness. Our survey covered the 5'-flanking region of *hsp27* from –757 to –124 and revealed high-affinity binding due to a site (or sites) within the region –562 to –514, a region that includes the element as described previously (data not shown).

Sequences of the four binding fragments (including the 23-bp *hsp27* element as described by Riddihough and Pelham 1987) are presented in Figure 5A, where they have been aligned to emphasize an obvious common element: Each fragment contains at least one copy of the sequence 5'-TGA(CA)C-3', usually followed by two pyrimidines, as the right arm of an imperfect palindrome with a single nucleotide at the center of symmetry. When a fragment containing *Upstream* was cut with *Mse*I (recognition site T/TAA), to disrupt the left half of the palindromic region, binding was eliminated (data not shown), supporting the idea that sequences near the conserved box are significant for binding. Further evidence in support of that conclusion is discussed below. *Dist* contains two copies of the conserved palindrome,

Figure 5. Sequences and functional tests of fragments containing EcREs. (A) Sequences are shown for the three ecdysone receptor-binding sites *Upstream*, *Prox*, and *Dist*, defined by the experiments depicted in Figs. 3 and 4. Both strands are shown, to indicate the structures of their termini. *Dist** is an alternate alignment of *Dist*, drawing attention to the presence of a more proximal TGACC, adjacent sequence (not part of the smallest binding-positive fragments) has been given in lowercase letters to indicate that this element is also partially symmetric. The *hsp27* region also contains a receptor-binding site (see text). Its sequence corresponds (left to right) to bases –529 to –551 of *hsp27* (Riddihough and Pelham 1986). The vertical arrow (top) indicates the center of symmetry of each partial dyad; dots are shown above bases that contribute to this symmetry. The box encloses a hexameric half-palindrome, for which the consensus sequence is indicated below. (B) Each of the sequences shown in A was inserted into X-188-cc–cat (see text) and tested for activity as an EcRE. (Left) The sequence of the inserted fragment (solid underline), of the intervening polylinker, and of a portion of the *Eip28/29* promoter fragment (dashed underline); base –188, the 5' end of the *Eip28/29* fragment, is indicated by an arrow (top). Each copy of the consensus half-palindrome is boxed (as is the functional cognate in *Upstream*). *Prox* was inserted into two different sites with results shown as Prox-A and Prox-B. *Dist* was inserted both as a shorter fragment (*Dist*) and as the 5-bp longer fragment *Dist**. The constructs 11 N *hsp* and 15 N *hsp* contain synthetic oligonucleotides designed to differ (lowercase letters) from the *hsp27* 23-mer at their ends. Thus, their central bases (uppercase) are identical to those of the *hsp27* sequence and either contain (15 N *hsp*) or do not contain (11 N *hsp*) a full consensus palindrome. Tabular data (right) summarize expression from each construct. The data are derived from CAT activities following transient expression in Kc167/M3 cells. (C) No hormone treatment; (E) 24-hr treatment with 10^{-6} M 20-hydroxyecdysone. Induction means induction ratio (E/C). The unmodified vector was included in each experiment, and expression levels were normalized to its expression in untreated cells. Mean values, standard deviations, and the number of experiments (in parentheses) are shown.

as indicated by the two alignments drawn in Figure 5A. Appropriate digestions can release either *Dist**, which contains two “complete” palindromes or *Dist* (5 bp shorter), in which the left arm of the more proximal dyad is truncated.

To establish that the fragments shown in Figure 5A contain all of the sequence necessary to give high-affinity binding, we subcloned them into pUC18 or pUCL1 (see Materials and methods) and tested the resulting plasmids for receptor binding. The tested fragments were precisely those shown in Figure 5A (with any 5' extensions filled in prior to cloning). In each case,

insertion of a binding element converted the polylinker from binding-negative to binding-positive (data not shown). Both *Dist* and *Dist** give positive results, indicating that the more downstream consensus is functional for binding; the functionality of the more upstream palindrome remains to be tested (but see below).

Receptor binding regions contain functional EcREs

To establish that these binding regions contain EcREs, we inserted each into a similar location ~240 bp upstream of the transcriptional start. Our test vector was

very similar to construct 16 in which the only *Eip28/29*-derived sequence consists of a short promoter region (−188 to +11) driving the *cat* reporter. In the test vector X-188-cc-*cat*, a long polylinker has been added directly upstream of the *Eip* segment. As shown in Figure 5B, the vector is ecdysone nonresponsive, giving the usual 20–30% reduction in *cat* expression following hormone treatment.

That the *hsp27*-derived element functions well, giving inductions of ~65-fold, is confirmation in a new test environment of the observations of Riddihough and Pelham (1987). In addition, each of the receptor-binding sites derived from *Eip28/29* functions as an EcRE, though their potencies vary: Element *Dist** is most effective (inductions of ~20-fold), *Prox* and *Dist* appear to be intermediate in effect, and *Upstream* gives marginal, although reproducible, induction. In the case of *Prox*, two insertion sites differing by 13 bp were tried (*Prox-A*, *Prox-B* in Fig. 5B), and a small but probably genuine position effect was observed. Although the activity of *Dist* has been measured only once, two separate experiments measured the relative effects of *Dist** and *Dist*; *Dist** is evidently 2.4- to 3-fold more active, suggesting that the more proximal palindrome present in *Dist** contributes to its potency as an EcRE.

To identify the critical nucleotides with greater certainty, the plasmids 11 N *hsp* and 15 N *hsp* were constructed by synthesizing oligonucleotides whose sequences match the *hsp27* 23-mer internally but diverge at the ends. As shown in Figure 5B, 15 N *hsp* was fully active as an EcRE; 11 N *hsp* was nonfunctional in these assays, and it also failed to bind ecdysone receptor (data not shown). These observations confirm our conclusion that the critical sequence for both receptor-binding and EcRE activity is the palindromic region indicated in Figure 5A.

The data in Figure 5B demonstrate a more remarkable aspect of the hormone response: Ecdysone inducibility arises as a result of both derepression and activation. In every construct containing an EcRE, basal expression is depressed about fourfold relative to the vector. Hormone both alleviates this repression and activates expression to a level substantially above that of the unmodified vector. It is interesting that *Upstream*, which is a poor EcRE, appears to be equivalent to *Dist** as an inhibitory element. Both aspects of EcRE activity, depression of basal expression and induction by ecdysone, have also been observed in constructs using a heterologous promoter (K. Lee and P. Cherbas, in prep.).

Discussion

Our principal goal in this work has been to recover EcREs and so begin the process of understanding their functions. We have succeeded in identifying three new examples of short regions bearing functional EcREs, and the properties of these regions along with those of the *hsp27* region, studied originally by Riddihough and Pelham (1986), are immediately suggestive.

Sequence comparisons (Fig. 5A) and functional tests

(Fig. 5B) are consistent with the idea that functional EcREs are composed of half-sites of the hexamer 5'-TGA(AC)CY-3', with the most effective elements organized as imperfect palindromes having a single undefined base at the center of symmetry. As described previously, a single restriction cut within this palindrome can obliterate high-affinity receptor binding. Similarly, it is significant that *Dist* contains two imperfect but palindromic copies of this consensus and that both appear to contribute to its function. The 23-bp region from *hsp27* is complex, containing two partially overlapping palindromes. Riddihough and Pelham (1987) focused their attention on a perfect interrupted palindrome composed of the terminal hexamers. Those hexamers bear little similarity to the vertebrate steroid HREs. The test vectors 15 N *hsp* and 11 N *hsp* demonstrate clearly that the functional feature of the *hsp27* 23-mer is the central imperfect palindrome, which is also the conserved feature of EcREs. Our data are consistent with either a hexameric or a heptameric half-site, since we have not yet tested the relevant 13-bp cognates. This palindrome is strikingly similar to one group of vertebrate HREs (see below). Similarly, we believe that our conclusions concerning EcRE structure are more compelling than previous inferences (Pongs 1988; Beato 1989), which implicated other portions of the *hsp27* 23-mer, solely on the basis of sequence comparisons among promoters thought to be ecdysone responsive.

Taken together with the new observation that the ecdysone receptor is a specific DNA-binding protein that interacts with EcREs (see below), the EcRE consensus suggests strongly that ecdysone receptors are members of the steroid receptor superfamily and that ecdysone acts by mechanisms homologous to those for the other steroids. The vertebrate receptors can be classified according to the sequences of their HREs (Evans 1988; Beato 1989). One group (which includes the glucocorticoid, progestin, androgen, and mineralocorticoid receptors) recognizes the half-site 5'-TGTTCT-3'. A second group (which includes the estrogen, thyroid hormone, retinoic acid, and vitamin D receptors) recognizes the half-site 5'-TGACCT-3'. This classification is certainly an oversimplification because differences in spacing and small sequence differences are apparently reflected in significant binding specificity (Glass et al. 1988, 1989; Sucov et al. 1990). Nonetheless, the classification correlates well with sequence relationships among the receptors, particularly with relationships among their DNA-binding domains (Danielson et al. 1989; Mader et al. 1989; Umesono and Evans 1989). Thus, the EcRE consensus suggests a close relationship between the ecdysone receptor and the estrogen, thyroid, retinoic acid, and vitamin D receptors. Studies of a cloned ecdysone receptor have confirmed this inference (M. Koelle, W. Talbot, W. Segraves, M. Bender, P. Cherbas, and D. Hogness, in prep.).

Although crude receptor extracts labeled by specific steroid binding have been used previously to localize HREs (see, e.g., Pfahl 1982; Glass et al. 1987), the extension of this procedure by blotting has proved to be a par-

ticularly simple and effective mapping tool. Still, our use of it has thus far been entirely pragmatic and the assay is relatively undefined. Most importantly, our experiments suggest but do not prove that ecdysone receptors bind directly to EcREs. Assuming such binding, the ecdysone receptor is comparable to the other steroid receptors in the magnitude of its DNA specificity as well, because in our assays the presence of a single EcRE approximately doubled the signal due to a 1- to 2-kb fragment of nonspecific DNA (cf. Pfahl 1982).

Although our binding survey gave no false-positive results—in the sense that all of the sequences identified by blotting proved to be functional EcREs—we know little about its sensitivity and about the frequency of false negative results. On the one hand, *Upstream* gave a signal indistinguishable from *hsp27* despite large differences in their biological effects; on the other, we know that additional EcREs must exist, for example, within region *B*, and that these were not detected. We have occasionally observed intermediate levels of binding by some small DNA fragments (L. Cherbas, unpubl.), but none of these sites has been mapped. The sequenced region around *Eip28/29* contains many copies of the half-site sequence 5'-TGA(AC)CY-3', only a few of which tested positive for binding. One possibility is that the blotting assay selectively detects palindromic sequences, but that in vivo "isolated" half-sites can interact over longer distances. Another is that cooperative interactions involving other proteins stabilize some weak EcRE–receptor interactions in vivo but not in our assays.

In any event, until the purified ecdysone receptors become available, the blotting procedure appears to be a practical way to map EcREs. We suspect that with appropriate modifications, it may prove more generally useful in situations where it is possible to use a ligand to label specifically one component of a crude extract.

Although we have identified EcREs in the context of a more general analysis of the hormonal regulation of *Eip28/29*, our studies of that gene have not yet progressed to the point where we can unambiguously identify the three known EcREs as the necessary elements. Still, it is clear that the ecdysone sensitivity of *Eip28/29* depends largely on the presence of sequences downstream of the transcription unit, a result that appears reasonable in light of the insensitivity of the *gdl^F* promoter to the hormone. Similarly, it is satisfying that two of the EcREs recovered fall within regions required for a full ecdysone response (*Prox* within region *C* and *Dist* within region *D*). We have noted previously that the third downstream region (*B*) must contain a weak EcRE that has not yet been identified. For reasons explained earlier we doubt that the stimulatory element(s) in region *A* is an EcRE; nor does this region exert any obvious effect on basal transcription. Thus, its stimulatory effect on the *Eip28/29* ecdysone response is intriguing, particularly because region *A* falls largely within an independent and nonresponsive transcription unit (*gdl^F*) and includes its promoter.

The role of *Upstream* is puzzling, as our experiments

have revealed no effect of that element on the *Eip28/29* hormone response. *Upstream* does not fall within any of the known critical regions (*A–D*). In constructs containing regions *B–D*, inclusion of *Upstream* does not increase the response (cf. constructs 5–9), and constructs containing only *Upstream* give no detectable response (cf. constructs 15 and 16). Yet when the *Upstream* sequence is moved from its normal location (–453) to –188, it yields a small but easily detectable response. Were *Upstream* a substantially weaker binding site than *Prox* or *Dist*, its inactivity in situ might plausibly be ascribed to distance. Although we cannot eliminate that possibility, we suspect that affinity differences of the magnitude necessary would have been detectable in the binding assays. Interesting alternative explanations are possible: A sequence between –453 and –188 may "block" the effects of receptor bound at *Upstream*, or a DNA-binding protein whose specificity overlaps that of receptor may compete for binding at *Upstream* in vivo. Our interest in this problem is considerably enhanced by the observations (Andres 1990) that (1) *Eip28/29* is expressed and regulated by ecdysone in numerous tissues of fly larvae; and (2) downstream sequences are required for its regulation in only a subset of those tissues, while sequences upstream of –188 are required in another subset (including epidermis and fat body). Thus, we consider it plausible that *Upstream* is a functional EcRE under some circumstances and is involved in the regulation of *Eip28/29* in some tissues. If so, the specificity might well hinge on the tissue-specific expression of proteins regulating the function of *Upstream*.

Finally, we turn to the observation that in the absence of hormone, the presence of an EcRE represses expression. The repression itself and the derepression and superactivation induced by hormone are clear in the data of Figure 5B, and all of the effects become still more pronounced when EcREs are introduced as multimers (K. Lee and P. Cherbas, in prep.). Whether the downstream EcREs have the same effects in situ is an open question. Certainly, this would provide an economical explanation for the elevated basal expression of construct 12 (Table 1; Fig. 1A,B), which lacks both of the strong EcREs *Prox* and *Dist*. The alternative explanation, that basal expression in that construct is elevated due to the removal of a distinct inhibitory element downstream of region *B*, will require testing by more detailed mutagenesis.

Inhibitory effects of HREs in the absence of hormone are not unknown, the most notable example being the thyroid HRE, which is comparable to an EcRE in its effects (Damm et al. 1989). Although it is possible that the "unliganded" ecdysone receptor binds to an EcRE and represses, we favor the alternative explanation that inhibition results from competition between DNA-binding proteins with overlapping but distinguishable specificities. According to this explanation, the ecdysone–receptor complex displaces an inhibitory protein bound previously at the EcRE. The fact that *Upstream* gives comparable inhibitions to far stronger EcREs (Fig. 5B) provides some (albeit inconclusive) support for this hy-

pothesis. There is precedent in the literature for competitive interactions between steroid receptors and other proteins for binding to HREs (Brüggemeier et al. 1990; Crawford and Chapman 1990). It is intriguing to note that the steroid receptor-like proteins encoded by the *Drosophila* genes *E75* (Segraves and Hogness 1990), *CF1* (Shea et al. 1990), *tll* (Pignoni et al. 1990), *svp* (Mlodzik et al. 1990), *kni* (Nauber et al. 1988), *knrl* (Oro et al. 1988), and *egon* (Rothe et al. 1989) all have sequences suggesting binding to TGACCT-related half-sites (for a summary, see Pignoni et al. 1990). The *CF1* product has been shown experimentally to bind to such a sequence (Shea et al. 1990). Thus, it is not only true that each of these proteins is a candidate for the role of the putative inhibitory factor but that a network of competitive regulatory interactions with important developmental consequences may exist.

Materials and methods

Nomenclature

We use ecdysone as a generic name for compounds with the appropriate biological activity, in analogy with the terms estrogen or progesterone (Cherbas et al. 1984). This reflects common usage. 20-Hydroxyecdysone is the major naturally occurring ecdysone.

Cell lines

We selected suitable host cell lines after a preliminary survey of *Drosophila* lines available to us, checking both induction of *Eip28/29* and expression of transfected DNA. The lines S2/M3 [(Schneider 1972) adapted by S. Lindquist to growth in M3 medium (Shields et al. 1975), supplemented with 10% fetal calf serum], G2 (a gift from W. Gehring), GM3 (Mosna and Dolfini 1972), DM1 (Schneider 1972; a gift of C. Benyajati), and E-Adh^{N1} (a line derived from *Adh*-null flies by H.-P. Bernhard; a gift of W. Gehring) exhibited poor induction; the lines S3 (Schneider 1972; a gift of C. Wyss) and Kc-H (clone A3A11; Cherbas et al. 1986b) were very poorly transfectable. Both Kc167/M3 and EH34A3/M3 cells gave satisfactory results.

Kc167/M3 cells (Cherbas et al. 1988) were grown in M3 medium (Shields and Sang 1977; as modified by Lindquist et al. 1982) supplemented with 5% heat-inactivated fetal calf serum. EH34A3/M3 cells were derived from the *shibire* clone EH34A3 (Woods and Poodry 1983) by adaptation to growth in M3 medium supplemented with 12.5% heat-inactivated fetal calf serum.

Steroids

20-Hydroxyecdysone was purchased from Simes spa (Milan), stored, and administered as described previously (Cherbas et al. 1980). Iodoponasterone labeled with ¹²⁵I was prepared according to Cherbas et al. (1988).

Plasmids

Unless otherwise specified, constructions were in pUC8 (Vieira and Messing 1982) with the 5' end of the insert oriented toward the *HindIII* site of the polylinker. All *Eip28/29* fragments were derived from pCg101-1 (Cherbas et al. 1986b) or from the Charon 4 clone Cs500 (Eickbush 1987). Many constructs con-

tained tagged *Eip28/29* genes in which a 499-bp *SalI* fragment from bacteriophage λ was inserted at a *SnaBI* site at position +2070 (using an *XhoI* linker). The 5' ends of the constructs shown in Figure 2 are an *XhoI* site at -4600 (construct 11), a *HindIII* site at -2007 (constructs 1, 3, 4, 10, 12, and 14), a *PstI* site at -657 (constructs 5, 8, and 15), and BAL-31-generated, *HindIII*-linked sites at -188 (constructs 7, 9, and 16) and at about -400 (construct 6, whose 5' end has not been sequenced). 3' Ends of the constructs are a *HindIII* site at +7100 (constructs 1 and 3), a *HpaI* site at +5882 (constructs 4-9), an *XhoI* site at +3756 (constructs 10 and 11), or a *BglII* site at +2484 (constructs 12 and 14). [Coordinates given represent the terminal *Eip28/29* base contained in the construct. We note that an earlier statement (Cherbas et al. 1986b) that the region contained no sites for *HpaI* was in error.]

In constructs 3 and 14 the introns were deleted by substituting a *SacI*-*PstI* fragment from the EIP28 cDNA clone pKc45 (Cherbas et al. 1986b) for the corresponding genomic region; this fragment spans all 4 exons of *Eip28/29*. *Eip28/29*-*cat* fusions contain the first 11 bases of the *Eip28/29* transcription unit, joined by a short polylinker to the *HindIII* (filled-in)-*BamHI* fragment of pSV2-*cat* (Gorman et al. 1982), which contains the structural gene for CAT and an SV40 polyadenylation region. The 3' *Eip28/29* fragment used in constructs 8 and 9 was a *SpeI*-*HpaI* fragment consisting of bases +2204 to +5882, filled in to convert the *SpeI* site to a blunt end, and inserted into the *SmaI* polylinker site downstream of the *cat*-SV40 fragment. *Eip188-Adh* consists of bases -188 to +11 of *Eip28/29*, fused by means of a polylinker to a *Drosophila Adh* fragment extending from base -23 of the larval transcription unit to ~1 kb 3' of the polyadenylation site (derived from pSAC; Goldberg 1980).

X-188-cc-*cat*, our vector for testing EcREs, was constructed as follows: pUC18 (Yannisch-Perron et al. 1985) was modified by insertion of an additional synthetic polylinker into the *HindIII* site. This oligonucleotide, 5'-AGCTATCGATAGGCCTGAT-ATCTCGAGATCTCTAGATACGTCGCGTCTCGACTCGCGA-3', was inserted with its 3' end (of the strand shown) toward the pUC18 polylinker. The resulting plasmid, pUCL1, retains all of the sites of pUC18 and has additional sites for *Clal*, *StuI*, *EcoRV*, *XhoI*, *BglII*, *XbaI*, *SnaBI*, *MluI*, *SalI*, and *NruI*. The insert of 188-cc-*cat* (construct 16) was subcloned as a *HindIII*-*BamHI* fragment between the *HindIII* and *BamHI* sites of pUCL1. Fragments to be tested for EcRE activity (Fig. 5B) were either isolated from digests or synthesized de novo and inserted into one of the polylinker sites upstream of the insert.

The *hsp27* promoter region was derived from the clone J1PR1 (a gift from D. Osterbur, originally from E. Craig). J1PR1 is a subclone of J1 (Craig and McCarthy 1980). The sequence of the region is given by Riddihough and Pelham (1986).

The published sequence for *Eip28/29* (Cherbas et al. 1986b) extends only to +2197. Since elements important for the ecdysone response are not included in this region, we have extended the sequence to +6436 (submitted to GenBank).

Transient expression assays

Cells at 3×10^6 /ml (10 ml/100-mm plate) were transfected by the addition of 1 ml/plate (20 μ g DNA/plate) of a DNA-calcium phosphate coprecipitate (Wigler et al. 1979); no carrier DNA was used. In all experiments except our initial screen of cell lines for transfectability, we prepared the precipitate at pH 7.05 rather than pH 7.10; the lower pH leads to a much stronger ecdysone response for many constructs (K. Lee, unpubl.). Four plates were transfected with each precipitate.

In experiments where expression was assayed by Northern

analysis, each construct was introduced as an equimolar mixture with *Eip188-Adh*, with total introduced DNA remaining 20 µg/plate. Cells were washed 12 hr after the addition of the precipitate, 20-hydroxyecdysone was added to two of the four plates (final concentration of 10^{-6} M) 8 hr later, and cells were harvested after an additional 4 hr. The contents of duplicate plates were pooled before preparing RNA.

In experiments where expression was assayed by CAT activity, the cells were washed 16 hr after the addition of the precipitate and ecdysone was added to two plates immediately. Each plate was harvested individually 24 hr later. CAT was assayed as described by Gorman et al. (1982) and modified by Rosenthal (1987), corrected for protein concentration (Bio-Rad protein assay), and CAT specific activities from duplicate plates were averaged. No internal control for transfection was included in these experiments.

Northern analysis

RNA was isolated by the method of Chomczynski and Sacchi (1987), separated on 1.5% formaldehyde-agarose gels (Lehrach et al. 1977), using 10 µg of RNA per lane, and blotted onto nitrocellulose. Except where otherwise indicated, duplicate Northern blots were probed with ³²P-labeled RNA probes for the 499-bp *Sall* fragment of bacteriophage λ and for *Drosophila Adh* to correct for loading errors and RNA degradation, using probes prepared from BlueScribe-based plasmids. Hybridized blots were exposed to Kodak XAR-5 film, without an intensifying screen, and the resulting autoradiograms were quantified by scanning densitometry. Controls for linearity were included in each set of scans.

Kc cell extracts

Kc167 cells were expanded in spinner flasks and harvested by centrifugation. They were washed once in phosphate-buffered saline (0.13 M NaCl, 0.007 M Na₂HPO₄, 0.003 M NaH₂PO₄ at pH 6.7). In some cases, the cell pellets were frozen in liquid nitrogen and stored at -80°C for several weeks before use; in other cases, they were used fresh. Whole-cell extracts were prepared according to Manley et al. (1983), fast-frozen in small aliquots, and stored at -80°C. Aliquots were thawed once and used immediately. Such extracts have a binding capacity of 50–100 fmoles of steroid per milligram of protein and are stable at -80°C for several months at least.

Blot assays of ecdysone receptor DNA binding

DNA was cut with restriction enzymes and fractionated by electrophoresis on agarose gels in TAE or on acrylamide gels in TBE (Maniatis et al. 1982). Each lane contained ~1 µg DNA/kb of starting material. No ethidium bromide was included in the gels. Following electrophoresis, gels were electroblotted (Bio-Rad TransBlot Cell) onto a GeneScreen Plus filter (New England Nuclear), using the same buffer system that was used for the initial electrophoresis. For agarose gels, the transfer was at 15 V (0.2 A) for 1–2 hr, followed by 45 V (0.6 A) for 1–2 hr. For acrylamide gels, we used 10 V (0.04 A), followed by 20 V (0.13 A). Blotting was monitored by including a duplicate gel in which the DNA fragments were either labeled with ³²P or stained with ethidium bromide; fragments as small as 80 bp are efficiently blotted by this procedure and are retained on the blot during the ensuing steps. The blot was wrapped in plastic wrap and stored at -20°C until it was probed with the ecdysone receptor. Immediately before treatment with the receptor extract, the blot was shaken for ~1 hr at 4°C in milk buffer (10% Car-

nation nonfat dry milk, 40 mM KCl, 40 mM HEPES at pH 7.0, 0.1 mM dithiothreitol).

The ecdysone receptor in a Kc cell extract was labeled with [¹²⁵I]iodoponasterone (Cherbas et al. 1988) by incubating the undiluted extract with the ligand at ~20 µC/ml, at room temperature for 1 hr. An aliquot was tested to confirm binding (glass fiber filter technique, as described in Cherbas et al. 1988); typically ~15–20% of the ligand was bound to receptor. The remainder of the ligand-extract mixture was diluted with 20 volumes of milk buffer and incubated with the blocked DNA blot for 3 hr at 4°C with gentle shaking. The volume of the incubation solution was just sufficient to cover the filter, typically 5–10 ml. After incubation with the labeled receptor, the filter was washed at 4°C (four changes, each about five times the volume used for incubation, over a period of 2 hr) in milk buffer, and exposed to Kodak XAR film overnight with an intensifying screen.

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References

- Andres, A.J. 1990. An analysis of the temporal and spatial patterns of expression of the ecdysone-inducible genes *Eip28/29* and *Eip40* during development of *Drosophila melanogaster*. Ph.D. thesis, Indiana University, Bloomington.
- Ashburner, M. 1990. Puffs, genes, and hormones revisited. *Cell* **61**: 1–3.
- Ashburner, M., C. Chihara, P. Meltzer, and G. Richards. 1974. Temporal control of puffing activity in polytene chromosomes. *Cold Spring Harbor Symp. Quant. Biol.* **38**: 655–662.
- Beato, M.J. 1989. Gene regulation by steroid hormones. *Cell* **56**: 335–344.
- Bieber, A.J. 1986. Ecdysteroid-inducible polypeptides in *Drosophila* Kc cells: Kinetics of mRNA induction and aspects of protein structure. Ph.D. thesis, Harvard University, Cambridge.
- Brüggemeier, U., L. Rogge, E.-L. Winnacker, and M. Beato. 1990. Nuclear factor I acts as a transcription factor on the MMTV promoter but competes with steroid hormone receptors for DNA binding. *EMBO J.* **9**: 2233–2239.
- Carson-Jurica, M.A., W.T. Schrader, and B.W. O'Malley. 1990. Steroid receptor family: Structure and functions. *Endocr. Rev.* **11**: 201–220.
- Cherbas, L., C.D. Yonge, P. Cherbas, and C.M. Williams. 1980. The morphological response of Kc-H cells to ecdysteroids: Hormonal specificity. *Wilhelm Roux's Arch. Dev. Biol.* **189**: 1–15.
- Cherbas, L., J.W. Fristrom, and J.D. O'Connor. 1984. The action of ecdysone in imaginal discs and Kc cells of *Drosophila melanogaster*. In *Biosynthesis, metabolism and mode of action of invertebrate hormones* (ed. J. Hoffmann and M. Porchet), pp. 305–322. Springer-Verlag, Berlin.
- Cherbas, L., H. Beneš, M. Bourouis, K. Burtis, A. Chao, P. Cherbas, M. Crosby, M. Garfinkel, G. Guild, D. Hogness, J. Jami, C.W. Jones, M. Koehler, J.-A. Lepesant, C. Martin, F.

- Maschat, P. Mathers, E. Meyerowitz, R. Moss, R. Pictet, J. Rebers, G. Richards, J. Roux, R. Schulz, W. Segraves, C. Thummel, and K. Vijayraghavan. 1986a. Structural and functional analysis of some moulting hormone-responsive genes from *Drosophila*. *Insect Biochem.* **16**: 241–248.
- Cherbas, L., R.A. Schulz, M.M.D. Koehler, C. Savakis, and P. Cherbas. 1986b. Structure of the *Eip28/29* gene, an ecdysone-inducible gene from *Drosophila*. *J. Mol. Biol.* **189**: 617–631.
- Cherbas, P., L. Cherbas, S.S. Lee, and K. Nakanishi. 1988. 26-[¹²⁵I]-Iodoponasterone is a potent ecdysone and a sensitive radioligand for ecdysone receptors. *Proc. Natl. Acad. Sci.* **85**: 2095–2100.
- Chomczynski, P. and N. Sacchi. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* **162**: 156–159.
- Clever, U. 1964. Actinomycin and puromycin: Effects on sequential gene activation by ecdysone. *Science* **146**: 794–795.
- Craig, E.A. and B.J. McCarthy. 1980. Four *Drosophila* heat shock genes at 67B: Characterization of recombinant plasmids. *Nucleic Acids Res.* **8**: 4441–4457.
- Crawford, L. and K. Chapman. 1990. Identification of a high molecular weight steroid response element binding protein. *Mol. Endocrinol.* **4**: 685–692.
- Damm, K., C.C. Thompson, and R.M. Evans. 1989. Protein encoded by *v-erbA* functions as a thyroid-hormone receptor antagonist. *Nature* **339**: 593–597.
- Danielson, M., L. Hinck, and G.M. Ringold. 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.
- Eickbush, D. 1987. Molecular characterization of the 71CE region of *Drosophila melanogaster*. Ph.D. thesis, Harvard University, Cambridge.
- Evans, R.M. 1988. The steroid and thyroid hormone receptor superfamily. *Science* **240**: 889–895.
- Glass, C.K., R. Franco, C. Weinberger, V.R. Albert, R.M. Evans, and M.G. Rosenfeld. 1987. A *c-erb-A* binding site in rat growth hormone gene mediates *trans*-activation by thyroid hormone. *Nature* **329**: 738–741.
- Glass, C.K., M.J. Holloway, O.V. Devary, and M.G. Rosenfeld. 1988. The thyroid hormone receptor binds with opposite transcriptional effects to a common sequence motif in thyroid hormone and estrogen response elements. *Cell* **54**: 313–323.
- Glass, C.K., S.M. Lipkin, O.V. Devary, and M.G. Rosenfeld. 1989. Positive and negative regulation of gene transcription by a retinoic acid-thyroid hormone receptor heterodimer. *Cell* **59**: 697–708.
- Goldberg, D.A. 1980. Isolation and partial characterization of the *Drosophila* alcohol dehydrogenase gene. *Proc. Natl. Acad. Sci.* **77**: 5794–5798.
- Gorman, C.M., L.P. Moffatt, and B.H. Howard. 1982. Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. *Mol. Cell. Biol.* **2**: 1044–1051.
- Lehrach, H., D. Diamond, J.M. Wozney, and H. Boedtker. 1977. RNA molecular weight determinations by gel electrophoresis under denaturing conditions, a critical reexamination. *Biochemistry* **16**: 4743–4751.
- Lindquist, S.L., S. Sonada, T. Cox, and K. Slusser. 1982. Instant medium for *Drosophila* tissue culture cells. *Dros. Inf. Serv.* **58**: 163.
- Mader, S., V. Kumar, H. de Verneuil, and P. Chambon. 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.
- Maniatis, T., E.F. Fritsch, and J. Sambrook. 1982. *Molecular cloning: A laboratory manual*. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
- Manley, J.L., A. Fire, M. Samuel, and P.A. Sharp. 1983. *In vitro* transcription: Whole-cell extract. *Methods Enzymol.* **101**: 568–582.
- Maroy, P., R. Dennis, C. Beckers, B.A. Sage, and J.D. O'Connor. 1978. Demonstration of an ecdysteroid receptor in a cultured cell line of *Drosophila melanogaster*. *Proc. Natl. Acad. Sci.* **75**: 6035–6038.
- Mlodzik, M., Y. Hiromi, U. Weber, C.S. Goodman, and G.M. Rubin. 1990. The *Drosophila seven-up* gene, a member of the steroid receptor gene superfamily, controls photoreceptor cell fates. *Cell* **60**: 211–224.
- Mosna, G. and S. Dolfini. 1972. New continuous cell lines of *Drosophila melanogaster*. Morphological characteristics and karyotypes. *Dros. Inf. Serv.* **48**: 144–145.
- Nauber, U., M.J. Pankratz, A. Kienlin, E. Seifert, U. Klemm, and H. Jäckle. 1988. Abdominal segmentation of the *Drosophila* embryo requires a hormone receptor-like protein encoded by the gap gene *knirps*. *Nature* **336**: 489–492.
- Oro, A.E., E.S. Ong, J.S. Margolis, J.W. Posakony, M. McKeown, and R.M. Evans. 1988. The *Drosophila* gene *knirps-related* is a member of the steroid-receptor gene superfamily. *Nature* **336**: 493–496.
- Pfahl, M. 1982. Specific binding of the glucocorticoid-receptor complex to the mouse mammary tumor proviral promoter region. *Cell* **31**: 475–482.
- Pignoni, F., R.M. Baldarelli, E. Steingrímsson, R.J. Diaz, A. Papatoutian, J.R. Merriam, and J.A. Lengyel. 1990. The *Drosophila* gene *tailless* is expressed at the embryonic termini and is a member of the steroid receptor superfamily. *Cell* **62**: 151–163.
- Pongs, O. 1988. Ecdysteroid-regulated gene expression in *Drosophila melanogaster*. *Eur. J. Biochem.* **175**: 199–204.
- Riddihough, G. and H.R.B. Pelham. 1986. Activation of the *Drosophila* hsp27 promoter by heat shock and by ecdysone involves independent and remote regulatory sequences. *EMBO J.* **5**: 1653–1658.
- . 1987. An ecdysone response element in the *Drosophila* hsp27 promoter. *EMBO J.* **6**: 3729–3934.
- Rosenthal, N. 1987. Identification of regulatory elements of cloned genes with functional assays. *Methods Enzymol.* **152**: 704–720.
- Rothe, M., U. Nauber, and H. Jäckle. 1989. Three hormone receptor-like *Drosophila* genes encode an identical DNA-binding finger. *EMBO J.* **8**: 3087–3094.
- Sage, B.A., M.A. Tanis, and J.D. O'Connor. 1982. Characterization of ecdysteroid receptors in cytosol and naive nuclear preparations of *Drosophila* K_c cells. *J. Biol. Chem.* **257**: 6373–6379.
- Savakis, C., G. Demetri, and P. Cherbas. 1980. Ecdysteroid-inducible polypeptides in a *Drosophila* cell line. *Cell* **22**: 665–674.
- Savakis, C., M.M.D. Koehler, and P. Cherbas. 1984. cDNA clones for the ecdysone-inducible polypeptide (EIP) mRNAs of *Drosophila* K_c cells. *EMBO J.* **3**: 235–243.
- Schneider, I. 1972. Cell lines derived from late embryonic stages of *Drosophila melanogaster*. *J. Embryol. Exp. Morphol.* **27**: 353–365.
- Schulz, R.A. and B.A. Butler. 1989. Overlapping genes of *Drosophila melanogaster*: Organization of the *z600-gonadal-Eip28/29* gene cluster. *Genes Dev.* **3**: 232–242.
- Schulz, R.A. and J.L. Miksch. 1989. Dorsal expression of the *Drosophila* *z600* gene during early embryogenesis. *Dev.*

- Biol.* **136**: 211–221.
- Schulz, R.A., W. Shlomchik, L. Cherbas, and P. Cherbas. 1989. The *Drosophila* *Eip28/29* gene and its upstream neighbors: Structural overlap and diverse expression. *Dev. Biol.* **131**: 515–523.
- Segraves, W.A. and D.S. Hogness. 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.
- Segraves, W.A. and G. Richards. 1990. Regulatory and developmental aspects of ecdysone-regulated gene expression. *Invert. Reprod. Dev.* **18**: 67–76.
- Shea, M.J., D.L. King, M.J. Conboy, B.D. Mariani, and F.C. Kafatos. 1990. Proteins that bind to *Drosophila* chorion *cis*-regulatory elements; A new C₂H₂ zinc finger protein and a C₂C₂ steroid receptor-like component. *Genes Dev.* **4**: 1128–1140.
- Shields, G. and J.H. Sang. 1977. Improved medium for culture of *Drosophila* embryonic cells. *Dros. Inf. Serv.* **52**: 161.
- Shields, G., A. Dübendorfer, and J. Sang. 1975. Differentiation *in vitro* of larval cell types from early embryonic cells of *Drosophila melanogaster*. *J. Embryol. Exp. Morphol.* **33**: 159–175.
- Sucov, H.M., K.K. Murakami, and R.M. Evans. 1990. Characterization of an autoregulated response element in the mouse retinoic acid receptor type β gene. *Proc. Natl. Acad. Sci.* **87**: 5392–5396.
- Vieira, J. and J. Messing. 1982. The pUC plasmids, an M13mp7-derived system for insertion mutagenesis and sequencing with synthetic universal primers. *Gene* **19**: 259–268.
- Umesono, K. and R.M. Evans. 1989. Determinants of target gene specificity for steroid/thyroid hormone receptors. *Cell* **57**: 1139–1146.
- Wigler, M., A. Pellicer, S. Silverstein, R. Axel, G. Urlaub, and L. Chasin. 1979. DNA mediated transfer of the adenine-phosphoribosyltransferase locus into mammalian cells. *Proc. Natl. Acad. Sci.* **76**: 1373–1376.
- Woods, D.F. and C.A. Poodry. 1983. Cell surface proteins of *Drosophila*. I. Changes induced by 20-hydroxyecdysone. *Dev. Biol.* **96**: 23–31.
- Yanisch-Perron, C., J. Vieira, and J. Messing. 1985. Improved M13 phage cloning vectors and host strains: Nucleotide sequences of the M13mp19 and pUC19 vectors. *Gene* **33**: 103–119.
- Yund, M.A., D.S. King, and J.W. Fristrom. 1978. Ecdysteroid receptors in imaginal discs of *Drosophila melanogaster*. *Proc. Natl. Acad. Sci.* **75**: 6039–6043.

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L Cherbas, K Lee and P Cherbas

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