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Ecdysterone Regulatory Elements Function as Both Transcriptional Activators and Repressors

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A synthetic, 23-bp ecdysterone regulatory element (EcRE), derived from the upstream region of the *Drosophila melanogaster hsp27* gene, was inserted adjacent to the herpes simplex virus thymidine kinase promoter fused to a bacterial gene for chloramphenicol acetyltransferase (CAT). Hybrid constructs were transfected into *Drosophila* S3 cells and assayed for ecdysterone-inducible CAT expression. In the absence of ecdysterone a tandem pair of EcREs repressed the high constitutive level of CAT activity found after transfection with the parent reporter plasmid alone. After hormone addition very high levels of CAT activity were observed. Insertion of the EcRE pair 3′ of the CAT gene also led to high levels of ecdysterone-induced CAT expression, but the repression of high constitutive levels of CAT activity failed to occur. The EcRE-CAT construct was cotransfected with plasmids containing tandem 10-mers or 40-mers of the EcRE but lacking a reporter gene. These additional EcREs led to a reduced level of ecdysterone-induced CAT activity and to an elevation of basal CAT activity in the absence of hormone. The data suggest that the receptor binds to the EcRE in the absence of hormone, blocking basal transcription from a constitutive promoter. In the presence of ecdysterone, receptor-hormone binding to the EcRE leads to greatly enhanced transcription.

The small heat shock protein (*s-hsp*) genes of *Drosophila melanogaster* (7, 8, 56) are expressed in response to high-temperature shock (42, 52), to chemical and physical stress agents (2), and at several specific stages in development (14, 15, 18, 28, 48, 63). In both larval imaginal disks (4, 21) and continuous cell lines (20, 31–33), *s-hsp* gene expression can also be induced by treatment with physiological doses of the moulting hormone, ecdysterone. Functional tests involving the use of P-element transformation, or transient transfection, have shown that the *cis*-acting DNA regulatory elements involved in heat-induced expression and in ecdysterone-induced expression are physically separate and nonoverlapping (1, 3, 6, 17–19, 22, 26, 29, 30, 36, 37, 40, 41). In the case of heat shock, a so-called heat shock element (HSE), conforming to a short consensus sequence, has been identified and characterized. The HSE is known to serve as a binding site for a *trans*-acting heat shock transcription factor (HSF). In *D. melanogaster*, at least, the heat shock response is mediated by the interaction of activated HSF with redundant HSEs located 5′ of each *hsp* gene (12, 38, 39, 46, 50, 53, 57, 60, 61). Although the precise mechanism of HSF activation and function is not completely understood, the HSEs appear to interact cooperatively or synergistically in activating *hsp* gene transcription (34, 47, 49).

Earlier analysis of *s-hsp* gene induction following ecdysterone treatment, in cultured cells or in imaginal disk tissue, has established several important features of the response. First, ecdysterone induction is based on an increased level of new transcription. This is true for expression of both endogenous *s-hsp* genes (55) and a reporter gene put under the regulatory control of an *s-hsp* gene promoter (32, 33). There is no evidence to suggest that ecdysterone alters the stability of endogenous *s-hsp* transcripts (55), or mRNAs transcribed from reporter genes, such as the chloramphenicol acetyl transferase gene (*CAT*) or thymidine kinase gene (*tk*) (32, 33). In addition, the response is quite specific. Biologically active ecdysteroids produce high levels of *s-hsp* gene expression, whereas none of the mammalian steroids or biologically inactive ecdysteroids tested functioned as inducers (21). In cell lines deficient in ecdysterone receptor activity (hormone binding) *s-hsp* gene expression did not occur following ecdysterone treatment, but did occur following heat shock (33). Attempts to identify and characterize *cis*-acting regulatory elements involved in ecdysterone-mediated *s-hsp* gene activation have only recently been successful (41, 51). However, no definitive ecdysterone regulatory element (EcRE) consensus sequence has been established, in the sense that vertebrate sequences for glucocorticoid (13, 43, 45, 58), estrogen (13), progesterone (13, 44), and thyroid hormone (9, 10, 13, 23, 25) response elements have been defined.

The first direct demonstration of a functional EcRE was reported by Riddihough and Pelham for the *hsp27* gene (41). They identified a 23-bp sequence, located between positions −549 and −527 (where +1 is the initiation site for transcription), that is sufficient to confer ecdysterone-regulated expression on a contiguous reporter gene, which in their case encoded the bacterial enzyme chloramphenicol acetyltransferase (CAT). This EcRE sequence contains a region of hyphenated dyad symmetry which, they suggest, may serve as a recognition and/or binding site for some nuclear transcription factor, perhaps the ecdysterone receptor (24, 62).

While attempting to confirm these findings, we obtained results which both extend the characterization of EcRE structure and activity and identify a new and unexpected feature of the EcRE. In the absence of the hormone ecdysterone, EcREs located 5′ of the *CAT* reporter gene behave as repressors of constitutive transcription. We also show that both the repressor and the hormone-dependent activator functions probably involve the same *trans*-acting factor, which we hypothesize is the ecdysterone receptor.

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MATERIALS AND METHODS

Cell culture and transfection. *Drosophila* S3 cells were cultured at 25°C in Schneider medium (GIBCO) containing 10% fetal bovine serum. For transfection, 20 μg of CsCl-purified plasmid DNA was added as a $CaCl_2$ coprecipitate (11, 59) to each 25-cm^2 flask containing confluent cells in 4 ml of medium. In studies involving hormone, ecdysterone (Calbiochem) was added to 1 μm (final concentration) 24 h after DNA addition and the flasks were cultured for an additional 24 h at 25°C before the cells were harvested.

CAT assay. Following treatment, the cells were harvested by centrifugation. After a wash in *Drosophila* Ringer solution, a cell pellet was recovered and resuspended in 250 μl of 0.25 M Tris (pH 8.0). Cell lysates, prepared by brief sonication, were clarified by a 5-min spin at 5°C in a microcentrifuge. Aliquots of the clear supernatant were then assayed for CAT activity (16), and the reaction products were visualized by ascending thin-layer chromatography and autoradiography. CAT activity was quantitated by excising spots containing [^{14}C]chloramphenicol and its acetylated products and conducting scintillation counting.

Luciferase assay. Following treatment, the cells were harvested by centrifugation. After a wash in cold buffer A (100 mM potassium phosphate buffer [pH 7.8], 1 mM dithiothreitol), the cell pellet was recovered and resuspended in 150 μl of buffer A. Cell lysates were prepared as detailed for the CAT assay. Typically 50 μl of cell lysate was assayed with 350 μl of luciferase buffer (100 mM potassium phosphate buffer [pH 7.8], 15 mM $MgSO_4$, 10 mM ATP) followed by an injection of 100 μl of a 1 mM solution of D-luciferin in a 1251 LKB Wallac Luminometer for luciferase activity (given in millivolt seconds). Specific luciferase activity was determined relative to the protein concentration (35) by using the Bio-Rad protein assay for each sample.

Plasmids. For the purpose of constructing plasmids containing multiple copies of the EcRE, plasmid pUC18Ec, a gift from Guy Riddihough (41), was digested with *Sma*I, and a *Bgl*II linker was inserted. A 47-bp *Bam*HI-*Bgl*II fragment containing the EcRE was purified from low-melting-point agarose and ligated in the presence of *Bam*HI and *Bgl*II at room temperature for 1 h. Polymers with a head-to-tail arrangement of the *Bam*HI-*Bgl*II fragment were separated on and purified from low-melting-point agarose (FMC Corp.).

The plasmid designated ptkATO (Fig. 1) contains the *tk* TATA box and start site of transcription on a 150-kp *Sal*I-*Bgl*II fragment from ptkSO (33) directionally cloned into the multicloning site (35) of pBLCAT3 (51). Plasmid pT81 luc has been described previously (35).

RESULTS

EcREs as Activators and Repressors. A synthetic 23bp oligonucleotide conforming to the *hsp27* EcRE sequence defined by Riddihough and Pelham (41) was inserted into the multicloning site of the reporter plasmid ptkATO (Fig. 1), 30 bp upstream of a functional TATA box. Constructs were transfected into ecdysterone-responsive S3 cells, and the transfected cells were cultured in medium with or without 1 μM ecdysterone for 24 h. Cell extracts were prepared and assayed radiometrically for CAT activity. Transfection with a plasmid containing one EcRE, inserted in either orientation, led to the appearance of high CAT activity levels following hormone treatment (Fig. 2). In both cases, however, a high basal level of CAT activity was also found in

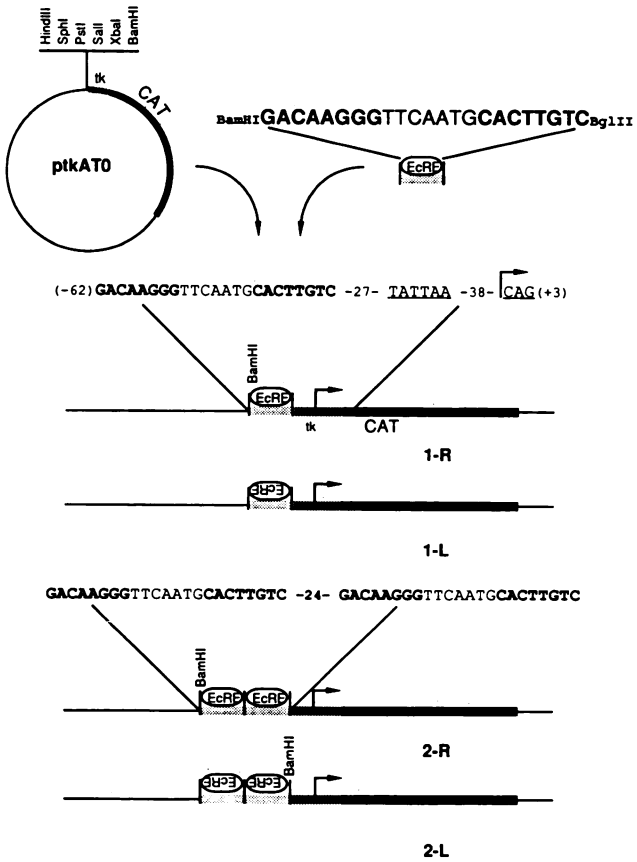

FIG. 1. Construction of EcRE-*CAT* expression plasmids. The plasmid ptkATO was linearized by digestion with *Bam*HI, which cuts once in the multicloning site. A 47-bp synthetic oligonucleotide containing the 23-bp EcRE and flanked by *Bam*HI and *Bgl*II overhangs was then ligated into the sticky ends, creating a functional *Bam*HI site and a nondigestible *Bam*HI-*Bgl*II junction. Tandem EcREs, created by ligation of EcRE monomers in the presence of *Bam*HI and *Bgl*II and size selected from low-melting-point agarose gels, were inserted in some cases. EcRE orientation was determined from individual minipreparations by using appropriate restriction sites. The letter R refers to insertion in the correct, i.e., in vivo, orientation, and L designates the inverted orientation.

control cells that were transfected but cultured in the absence of ecdysterone. As a consequence, the level of CAT activity induced by ecdysterone in the experiment was only twofold higher than the level in control cells.

To determine whether the high basal level of CAT activity found in untreated cells was due to leaky transcription from the inserted EcRE, we examined the level of CAT activity in S3 cells transfected with the original reporter vector, ptkATO, alone. We found (Fig. 2) that cells transfected with only ptkATO also showed a high basal level of CAT activity but that this activity was unaffected by hormone treatment (Fig. 2) or the absence of serum in the medium. We surmise that the ptkATO plasmid contains a constitutive promoter capable of initiating *CAT* gene transcription in *Drosophila* cells.

Because the ptkATO vector displays a high level of basal expression, it is difficult to assess the actual level of hormone induction in constructs containing a single EcRE. To circumvent this problem we tested a different vector, pT81 luc, which contains the luciferase (*luc*) gene as a reporter, for

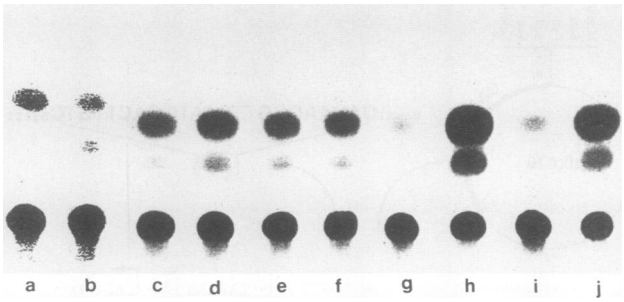

FIG. 2. CAT assays. Levels of CAT activity were assayed by using extracts of *Drosophila* S3 cells that had been treated for 1 day without (lanes a, c, e, g, and i) or with (lanes b, d, f, h, and j) 1 μM ecdysterone. Plasmids used for transfection include ptkATO (lanes a and b); EcRE-1R-CAT (lanes c and d, where the 1R designates that the insert contains one EcRE in the correct [R] orientation); EcRE-1L-CAT (lanes e and f where L indicates inverted orientation); EcRE-2R-CAT (lanes g and h); and EcRE-2L-CAT (lanes i and j).

basal expression in *Drosophila* cells. When transfected into line S3 cells the pT81 luc plasmid alone was essentially inactive and negligible levels of luciferase activity accumulated in cells (Fig. 3). One or two EcRE sequences were then inserted into the multicloning site of pT81 luc, and the analysis was repeated. With the plasmid containing a single EcRE, hormone treatment for 1 day led to a 5- to 10-fold increase in luciferase specific activity (Fig. 3). In the presence of a pair of head-to-tail EcREs, ecdysterone treatment led to a 20- to 30-fold increase in luciferase specific activity.

Constructs containing a pair of head-to-tail EcREs, inserted into ptkATO in either orientation, were next used for transfection. Ecdysterone treatment of cells transfected with the ptkATO-derived vector led to a 40-fold increase in CAT activity (Fig. 2). This enhanced effect on induction was due to a combination of low or negligible basal levels of CAT activity in non-hormone-treated cells and a substantially enhanced level of CAT induction by ecdysterone. After repeating the analyses with constructs with one or two EcREs several times, we could not identify a consistent difference based on EcRE orientation (Table 1).

EcRE specificity. To further characterize the transcriptional response of EcREs, we performed several additional experiments. We found, first, that ecdysteroids known to show high levels of biological activity (β-ecdysterone) in vivo were potent inducers of CAT activity in transfected cells, whereas steroids known to have little or no biological activity (α-ecdysone) were weak inducers of CAT activity (after treatment with 1 μm ecdysteroid for 24 h, the CAT activity obtained with α-ecdysone was 7.8% of the activity obtained with β-ecdysterone; the activities with inokosterone and the control were 60.4 and 3.9%, respectively). We then examined the dose-response profile of CAT induction by using the 2R construct for transfection. The results show a physiological dose-response profile with half-maximum activity occurring between 10^{-7} and 10^{-6} M (Fig. 4), a profile characteristic of other bona fide ecdysterone-mediated gene responses (5), indeed including the induction of endogenous *s-hsp* gene expression (21).

Finally, the sequence specificity of EcREs was examined in a preliminary way by inserting random DNA fragments about the length of an EcRE into the MCS of ptkATO. In this experiment two different 50-bp DNA restriction fragments derived from the *hsp22* coding region were inserted

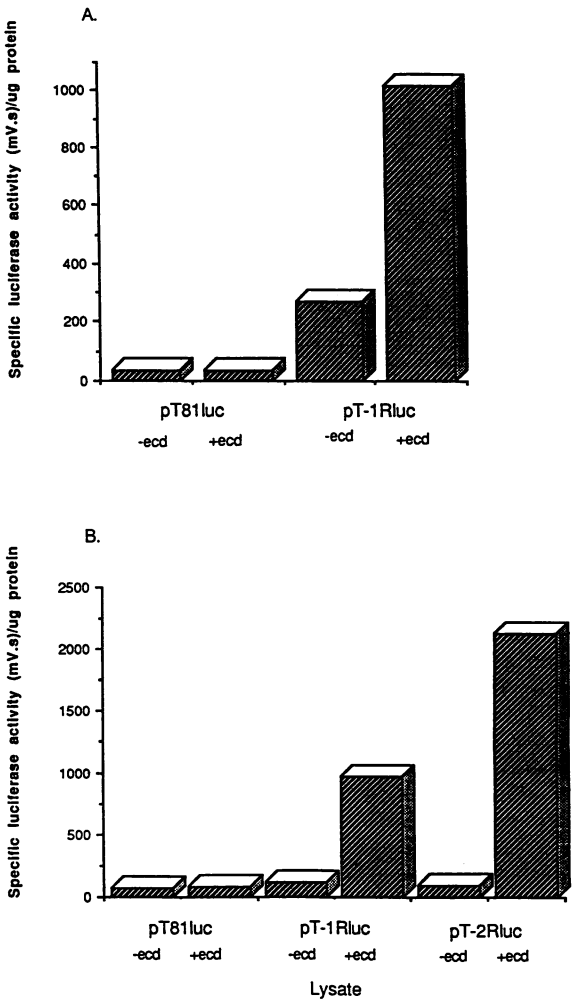

FIG. 3. Specific luciferase activity for pT81 luc, pT-1R luc, and pT-2R luc. pT81 luc vector contains only the thymidine kinase promoter from simian virus 40 (−81 to +53), cloned upstream of the luciferase gene. The constructs pT-1R luc and pT-2R luc were made by cloning the *Bam*HI-*Bgl*II monomer or dimer fragment into the pT81 luc vector at the *Bam*HI-*Bgl*II region of the multicloning site and assayed in *Drosophila* S3 cells which were cultured in the presence or absence of 1 μM ecdysterone for 24h. All luciferase assays were done in duplicate. (A) Basal and ecdysterone-induced activity of pT-1R luc with respect to the activity of the control vector, pT81 luc. (B) Basal and induced activities for pT-1R luc and pT-2R luc relative to pT81 luc, in a second independent assay. The luciferase vectors were kindly provided by the American Type Culture Collection.

into the site of ptkATO. These constructs were transfected into S3 cells, and CAT enzyme activity was determined following hormone addition. The results (Fig. 5) show that neither inserted fragment was effective in either reducing the high basal level of CAT activity seen with the parent plasmid alone or activating *CAT* gene expression in the presence of hormone. Thus, both repressor and activator function show DNA sequence specificity.

EcREs inserted 3′ of the *CAT* gene. To determine the importance of EcRE location, in terms of activator and repressor function, we made a construction in which a head-to-tail pair of EcREs was inserted into a multicloning site located 3′ of the *CAT* gene, 73 bp downstream of the

TABLE 1. Induction and repression of the *CAT* reporter gene is enhanced by multiple EcREs

Construct	Activity ratios[a]	
	Induced	Basal
1R	1.63	0.90
1L	2.28	0.24
2R	13.05	0.16
2L	12.50	0.08
(2R) 3′	2.74	1.10
(2L) 3′	3.75	1.09
ptkATO	0.87	1.00

[a] The results shown are the average of at least three independent transfection experiments. Induction is calculated as the ratio of CAT activity in the presence of 1 μM ecdysterone to CAT activity in the absence of hormone. Basal activity is calculated as the ratio of CAT activity in the absence of hormone to CAT activity of ptkATO.

poly(A) addition site, in the ptkATO vector (Fig. 6A; Table 1). Plasmids containing insertions in both orientations were recovered and assayed, following transfection, for CAT activity by using cells cultured in the presence or absence of 1 μM ecdysterone. The results of the analysis are summarized in Fig. 6B. We found that in the absence of ecdysterone, transfected cells contain a high basal level of CAT activity, characteristic of the parent plasmid ptkATO, but that in cells incubated with ecdysterone for 24 h, levels of CAT activity were greatly elevated and were comparable to

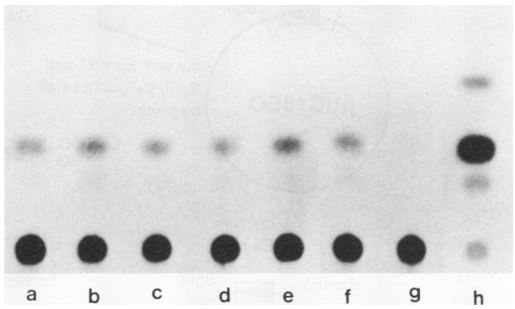

FIG. 5. Random DNA fragments from the *hsp22* coding region inserted into ptkATO are not ecdysterone inducible, nor do they repress basal CAT activity. Following transfection with ptkATO (lane a) or plasmids containing 50-bp fragments in the *Bam*HI site of the multicloning site (lanes b to e), cells were cultured for 1 day either with 1 μM ecdysterone (lanes c and e) or without hormone (lanes b and d). CAT activity was then determined from cell extracts, as discussed in the legend to Fig. 2.

those found by using the tandem EcREs inserted 5′ of the *CAT* genes (Fig. 2). From these results we conclude that EcRE activator function, i.e., ecdysterone-inducible CAT activity, occurs even when EcREs are located 3′ of the reporter gene, but that the EcRE repressor activity is absent.

Transfection-competition studies. The synthetic *hsp27* EcRE, then, appears to possess two activities. It acts as a transcriptional repressor of high basal *CAT* transcription in

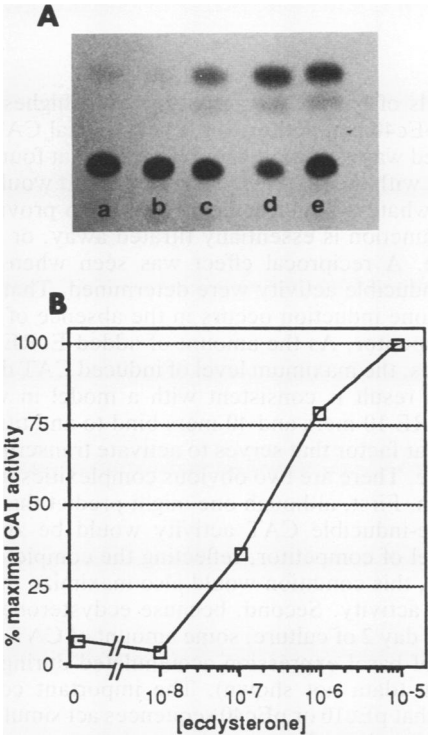

FIG. 4. Dose-response of EcRE-2R-CAT. Cells were transfected with EcRE-2R-CAT DNA for 1 day and then treated with ecdysterone at several final concentrations. After 24 h the level of CAT activity was assayed by using extracts of transfected cells. Maximum induction, at 10^{-5} M, was used as the standard for comparison. (A) CAT assays. Lanes: a, no hormone; b, 10^{-8} M hormone; c, 10^{-7} M; d, 10^{-6} M; e, 10^{-5} M. (B) Dose-response curve.

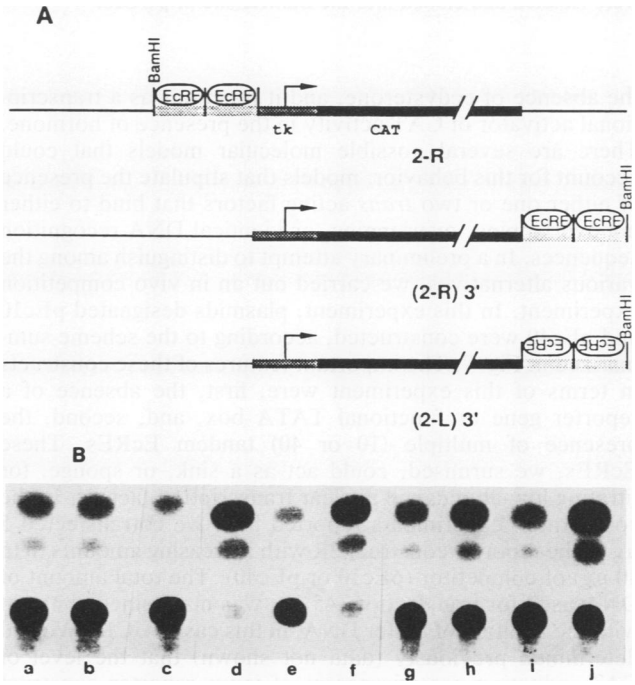

FIG. 6. EcRE insertion 3′ and 5′ of the *CAT* gene. (A) A tandem pair of EcREs was inserted either 5′ or 3′ of the *CAT* gene. (B) Following transfection into responsive S3 cells with DNA from ptkATO (lanes a and b), the 5′ insertions EcRE-2R-CAT (lanes c and d) and EcRE-2L-CAT (lanes e and f), or the 3′ insertions EcRE-2R 3′-CAT (lanes g and h) and EcRE-2L 3′-CAT (lanes i and j), cells were treated without (lanes a, c, e, g, and i) or with (lanes b, d, f, h, and j) 1 μM ecdysterone for 1 day. CAT assays were carried out with cell lysates 1 day after hormone addition.

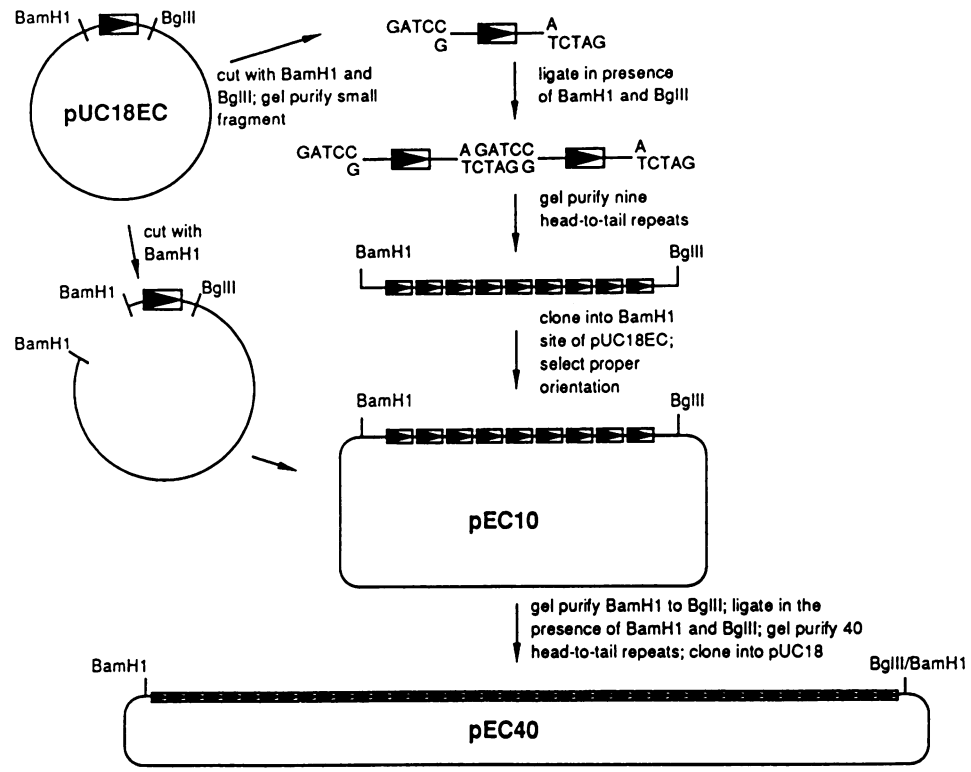

FIG. 7. Construction of plasmids pEc10 and pEc40. Concatomers of EcRE were created by excising the *Bam*HI-*Bgl*II EcRE fragment from pUC18EC and ligating in the presence of *Bam*HI and *Bgl*II. Head-to-tail monomers were inserted into the unique *Bam*HI site of pUC18Ec to produce pEC10. To produce pEC40, the *Bam*HI-*Bgl*II EcRE 10-mer fragment generated from pEC10 was ligated and 40 repeats were isolated and cloned into the *Bam*HI site of pUC18.

the absence of ecdysterone, and it functions as a transcriptional activator of CAT activity in the presence of hormone. There are several possible molecular models that could account for this behavior: models that stipulate the presence of either one or two *trans*-acting factors that bind to either nonoverlapping, overlapping, or identical DNA recognition sequences. In a preliminary attempt to distinguish among the various alternatives, we carried out an in vivo competition experiment. In this experiment, plasmids designated pEc10 and pEc40 were constructed, according to the scheme summarized in Fig. 7. The important features of these constructs in terms of this experiment were, first, the absence of a reporter gene or functional TATA box, and, second, the presence of multiple (10 or 40) tandem EcREs. These EcREs, we surmised, could act as a sink, or sponge, for titrating low-abundance nuclear transcription factors. In the competition experiments reported here we cotransfected 5 μg of the reporter construct 2R with increasing amounts (0 to 40 μg) of competitor (pEc10 or pEc40). The total amount of DNA used for transfection, 45 μg, was maintained constant with the addition of buffer DNA, in this case pUC18. We had determined previously (data not shown) that the level of CAT induction per microgram of input reporter construct DNA increased linearly between 1 and 50 μg. Following the 24-h transfection, cells were cultured in the presence or absence of 1 μM ecdysterone for an addition 24-h period, and, finally, cell lysates were prepared and assayed for CAT activity. We found that the competitor DNA containing multiple, tandem EcREs had two important effects (Fig. 8). First, in the absence of ecdysterone, cells transfected with increasing amounts of competitor showed progressively higher levels of basal CAT activity. At the highest concentration of pEc40 competitor, the level of basal CAT activity accumulated was essentially equivalent to that found in cells transfected with the ptkATO plasmid alone. It would appear, then, that whatever nuclear factor serves to provide EcRE repressor function is essentially titrated away, or somehow unavailable. A reciprocal effect was seen when levels of hormone-inducible activity were determined. That is, maximum hormone induction occurs in the absence of pEc10 or pEc40 competitor. As the amount of added EcRE competitor increases, the maximum level of induced CAT decreases. Again, this result is consistent with a model in which the tandem EcRE 10-mers and 40-mers bind to and titrate away some nuclear factor that serves to activate transcription with ecdysterone. There are two obvious complexities in terms of quantitation. First, although one might predict that the level of hormone-inducible CAT activity would be zero at the highest level of competitor, reflecting the complete titration of receptor, this condition would also maximize the level of basal CAT activity. Second, because ecdysterone is added only during day 2 of culture, some amount of CAT activity is the result of basal expression accumulated during day 1 of transfection (data not shown). The important conclusion, though, is that pEc10 or pEc40 sequences act simultaneously to elevate basal CAT expression and to lower hormone-induced levels of CAT expression.

DISCUSSION

A 23-bp DNA sequence located upstream of the *Drosophila hsp27* gene, at positions −549 to −527, is sufficient to

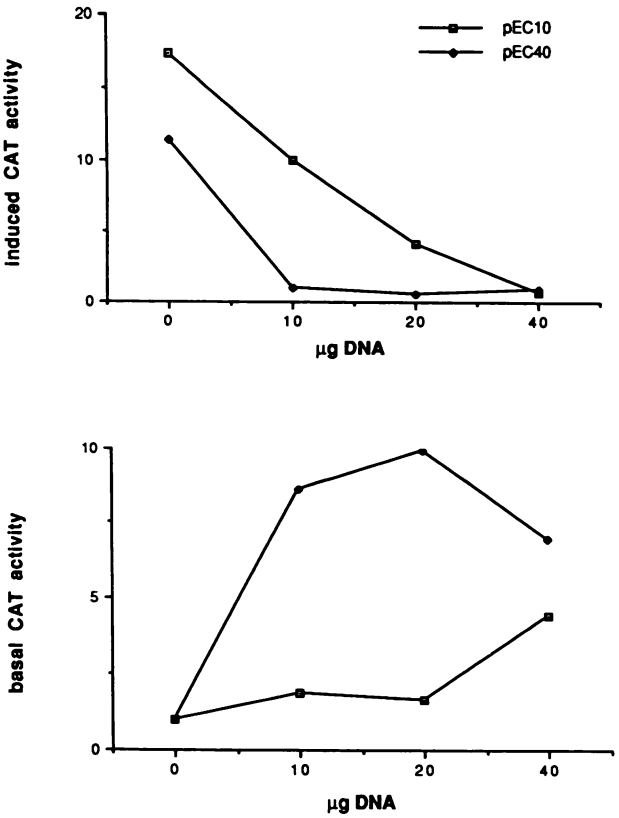

FIG. 8. Results from in vivo competition assays. Cells were transfected in duplicate with 5 μg of reporter plasmid, 2R, and increasing amounts of competitor DNA, pEC10 or pEC40. After 24 h, cells were treated with hormone or left untreated; 24 h later they were harvested and CAT activity was measured. "Induced CAT activity" is calculated to be the ratio of CAT activity measured for hormone-treated and untreated cells. "Basal CAT activity" is calculated to be the ratio of CAT activity in cells incubated with and without competitor DNA; this ratio is taken to be 1 at 0 μg of competitor DNA.

Gene	EcRE sequence	Positions
hsp 27	TGCACTT TGAACCC	-538 --> -532 -540 --> -546
hsp 22	TGACCTT	-286 --> -280
hsp 26	TGATCCC TGACTTT	-166 --> -160 -170 --> -176
Gene 1	TGACCCT	-289 --> -294
EIP28/29	TGACCTC TGACCCC TGAACTT	Proximal-A Distal Distal
Consensus	TGANCPyPy	

FIG. 9. DNA sequences homologous or related to the *Drosophila hsp27* EcRE. Sequences for the *s-hsp* genes are located at the positions indicated, where +1 is the start site of transcription.

confer ecdysterone-inducible expression on a contiguous reporter gene, as shown in a transient-expression assay with cultured *Drosophila* cells (11, 31). The results presented here have confirmed those observations and extend them in several important ways. First, we have shown that insertion of a tandem pair of elements near the reporter gene confers substantially higher levels of ecdysterone-induced CAT or luciferase activity than does the insertion of a single EcRE. Similar results, indicating cooperative interactions between adjacent hormone response elements, have been made in studies of glucocorticoid- (45, 51), estrogen- (51), and progesterone-regulated (44) gene expression. Second, the data show no consistent effect of EcRE orientation in terms of either repressing basal CAT expression or activating CAT expression following hormone addition. Third, we found that the induction of CAT activity conforms to a physiological dose-response curve, with half-maximal induction occurring at about 10^{-7} M. Similar dose-response patterns have been seen for endogenous *hsp* gene expression (20, 21) and for acetylcholinesterase activity induced by ecdysterone (5). Fourth, the response appears highest when biologically active ecdysteroids (such as ecdysterone) are present and low or absent when less active steroids (α-ecdysone or inokosterone) are used.

In the case of HSEs, which show cooperative interactions in the form of activating transcription, tandem or adjacent copies are normally found near each of the *hsp* genes. Although EcREs show additive or, perhaps, cooperative interactions in experiments with synthetic constructs, the hormone-responsive *s-hsp* genes do not appear to be flanked by multiple, tandem elements. One explanation is that cryptic EcREs exist which differ from the EcRE consensus sequence (Fig. 9). Another explanation is that the functional requirement for heat shock proteins in cells following heat shock or chemical stress is great, while much lower levels of heat shock protein are needed during normal development. Although assembling tandem EcREs would certainly lead to higher levels of hormone responsiveness, the additional accumulation of heat shock proteins might be gratuitous, or even deleterious. The function of the *s-hsp* gene products remains unknown.

The most unexpected result obtained is that in the absence of ecdysterone the EcRE can function to repress the high constitutive level of *CAT* gene expression found after transfection with the parent plasmid, ptkATO, alone. As is the case for its activator function, a pair of EcRE sequences is more effective at repression than are single EcRE inserts. The importance of orientation is not clear, but the effect, if any, is small. In marked contrast to its activator function, EcRE location is critical for repressor function. Insertion of a tandem EcRE pair 3′ of the *CAT* gene has essentially no affect on repressing basal *CAT* gene expression, while insertion 5′ of the *CAT* gene nearly abolishes basal CAT expression. Both activator and repressor activities are DNA sequence specific: two random DNA fragments of approximately similar length to an EcRE pair were ineffective in terms of either repression or hormone-induced increase of *CAT* gene expression.

Although it is generally true that steroid hormone receptors bind to their target DNA recognition sequences in a ligand-dependent manner, the data presented here and previous observations on thyroid hormone receptor activity challenge this view (10, 13, 23, 25). The ability of the thyroid hormone receptor to recognize and bind to the cognate target sequence is known to occur in the absence of hormone (10, 13, 23, 25). In fact, as in the case reported here for ecdysterone, tandem thyroid response elements potentiate both positive and negative transcriptional effects. It has been proposed that the free glucocorticoid receptor also shows DNA sequence-dependent glucocorticoid response element binding (44, 58), but this has been disputed, with the evidence suggesting that an hsp90 capping protein has been

eluted and lost during receptor purification. This capping protein is believed to alter the conformation or actually cover the DNA-binding site in unliganded receptors. It is likely, then, that the distinction is between free receptor not binding hormone response elements (in the case of glucocorticoid, estrogen, and progesterone) and the facilitated binding of unliganded receptor (thyroid and ecdysterone). In the latter case the critical and perhaps sole function of hormone is to trigger a conformational change in the DNA-bound receptor that somehow converts its repressor activity into enhancer function. It will become important to purify ecdysterone receptor in order to assess this aspect of the model by using gel shift and DNA protection and to carry out detailed in vitro mutagenesis studies of the EcRE to learn whether individual base pair changes can affect repressor or activator activity independently. The simplest model one can propose is that the hormone-receptor complex, bound to the EcREs, potentiates transcription by physically interacting with RNA polymerase and/or with other nonspecific transcription factors. This may occur even when the EcREs and the TATA-promoter region are located at different ends of the *CAT* gene. Repressor activity, however, is found only when EcREs are adjacent to the TATA-promoter region. This suggests that repressor function involves a steric block of RNA polymerase extension from an upstream start site or a local masking or distortion of the TATA-promoter region by the bound but unliganded receptor. To distinguish between these and other models (27), it will be necessary to determine the 5′ initiation site of CAT mRNA in the experiment. Our prediction is that repressor activity will be lost when EcRE insertion occurs far upstream of the *CAT* gene. The only evidence we have that both activator and repressor activities involve receptor-EcRE interactions comes from the titration of transfection competition experiments, but these results could also be explained by a two-factor (activator and repressor) model in which both factors are at equally low abundance. The availability of purified receptor and ecdysterone receptor specific antibodies will resolve this question.

Several years ago, Wyss (59) isolated a series of ecdysterone-insensitive cell lines, selected for by their ability to continue cell proliferation in the presence of ecdysterone, a condition which normally produces rapid mitotic arrest (59). Although all the hormone-insensitive cell line variants tested were deficient in ecdysterone receptor-binding activity (with [^{3}H]ponasterone A as the ligand) (24, 62), they fell into two distinct classes based on their phenotypes when fused to a normal, hormone-responsive cell. Several variants showed a recessive phenotype. That is, variant-normal cell hybrids were hormone responsive. The rest displayed a dominant phenotype in that hybrid cells were nonresponsive and failed to show receptor activity. We suspect that the recessive variants lack receptor activity because the receptor protein is either absent or unable to bind to recognition site DNA. The dominant variants, in contrast, may contain a defective receptor that binds to recognition site DNA but is unable to bind to or become activated by hormone. Our suspicion, then, is that when hormone-insensitive cell lines are transfected with the 2R construct containing a pair of EcREs fused to the *CAT* gene, the recessive class will show a high basal level of CAT activity that is noninducible, whereas the dominant class may contain a defective receptor that is able to bind and suppress transcription but cannot become activated.

The similarities between the vertebrate thyroid hormone and the insect ecdysterone gene response systems are intriguing. Both utilize hormone to transform a bound hormone receptor from a transcriptional suppressor to a transcriptional activator. In fact, the DNA recognition sequences corresponding to the consensus EcRE and thyroid response element share remarkable homology (Fig. 9). Also, it has not escaped our notice that ecdysterone (in insects) and thyroxin (in amphibians) are both developmental hormones required for the transformation of larval to adults forms.

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