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Selective gene expression induced by ecdysterone in cultured fat bodies of *Drosophila*

(steroid hormone/*Drosophila* development/gene regulation)

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ABSTRACT **Expression of the *LSP-2* and *P1* genes was induced in cultured fat bodies of *Drosophila* third-instar larvae by supplementing the culture medium with ecdysterone. The fat bodies were isolated from ecdysterone-deficient larvae of the temperature-sensitive mutant *ecd*^{1}, which were shifted from the permissive to the restrictive temperature either at the beginning of the third instar for the detection of *LSP-2* induction or several hours later for the detection of *P1* induction. During normal larval development, the *LSP-2* gene is expressed before the *P1* gene, and this order is also observed in the cultured fat bodies. Induction was demonstrated by increased amounts of *LSP-2* and *P1* transcripts in the ecdysterone-supplemented fat bodies. The amount of *P1* transcript was determined by two methods: one involved measuring the hybridization of a labeled *P1* DNA probe to total fat body RNA; the other involved labeling the newly synthesized RNA in nuclei isolated from cultured fat bodies and measuring the hybridization of the labeled RNA to *P1* DNA. Only the second method was used for the *LSP-2* transcript because the earlier expression of the *LSP-2* gene results in a measurable accumulation of the transcript in the fat bodies before ecdysterone supplementation. The maximal level of *P1* induction was reached within 2 hr after supplementation, and the induction was not affected by a concentration of cycloheximide that strongly inhibited total protein synthesis, suggesting that ecdysterone acts directly on the *P1* gene rather than indirectly by inducing formation of proteins required for the subsequent induction of *P1*. Ecdysterone appears to function normally in the cultured fat body system because the *LSP-2* and *P1* genes are induced in culture in the same order as *in vivo* and a third gene, *G12*, that is not induced in fat bodies *in vivo* also is not induced in culture.**

During the third-instar stage of *Drosophila* development, several genes become strongly expressed in the fat bodies, as indicated by the sharp increase in the amounts of the gene transcripts and encoded polypeptides (1–3). For two of the genes, *LSP-2* and *P1*, this increase is blocked in the temperature-sensitive mutant *ecd*^{1} after the mutant larvae are shifted from a permissive to a restrictive temperature, which causes a deficiency of the steroid hormone ecdysterone (4). The block can be released by supplementing the larvae with an exogenous source of ecdysterone (1, 3). These responses *in vivo* to changes in the level of ecdysterone suggest that expression of the *LSP-2* and *P1* genes is hormonally regulated by ecdysterone.

In this report, the effect of ecdysterone on *LSP-2* and *P1* expression is examined in dissected fat bodies maintained in culture. The results demonstrate that supplementation of the culture medium with ecdysterone also induces selective expression of the two genes in ecdysterone-deficient *ecd*^{1} fat bodies, consistent with the effects observed *in vivo*.

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MATERIALS AND METHODS

***Drosophila* Strains.** The temperature-sensitive mutant *ecd*^{1} (4) was maintained as a homozygous stock at a permissive temperature of 20°C. For the temperature shifts, the larvae were staged at the second molt by microscopic examination, kept at 20°C for the additional period indicated in the experiment, and then incubated at 29°C for about 40 hr. The standard wild-type strain was an inbred population of Oregon-R.

Culturing of Fat Body Tissue. Fat bodies were dissected by hand and transferred immediately to the basal culture medium (5) at room temperature; in some dissections the entire tissue remained intact and in others a few large fragments separated from the main body of tissue. The dissections were completed within 20–60 min, depending on the number, and then the medium was supplemented as indicated and the timing of the incubation period was started. The tissue samples were incubated at 25°C, and the tissue and the medium were processed together for determination of *LSP-2* and *P1* transcripts.

Isolation of Fat Body Nuclei. For each preparation of nuclei, cultured fat bodies from 30 larvae were suspended in 0.5 ml of ice-cold medium [15 mM *N*-(2-hydroxyethyl)piperazinepropanesulfonic acid (Epps), pH 7.5/15 mM NaCl/60 mM KCl/15 mM 2-mercaptoethanol/0.5 mM spermidine/0.15 mM spermine/0.34 M sucrose/2 mM EDTA/0.5 mM EGTA/0.5 mM phenylmethylsulfonyl fluoride ($PhMeSO_2F$) (modified formula from ref. 6)] and the cells were broken with about 20 strokes in a glass/Teflon homogenizer. All subsequent steps were done at about 4°C. The homogenate was centrifuged at 1,700 × *g* for 10 min and the pellet was suspended in the same medium except that EDTA and EGTA were decreased to 0.1 mM. The suspension was centrifuged again and the pellet was suspended in 0.025 ml of a medium containing 50 mM Epps (pH 7.5), 5 mM $MgCl_2$, 0.07 mM EDTA, 0.07 mM EGTA, 75 mM KCl, 11 mM NaCl, 11 mM 2-mercaptoethanol, 0.36 mM spermidine, 0.11 mM spermine, 0.36 mM $PhMeSO_2F$, 0.24 M sucrose, 25 mM $(NH_4)_2SO_4$, 0.25 mM ATP, 0.25 mM CTP, 0.25 mM UTP, and 0.025 mM GTP. The nuclear suspensions were used immediately for the experiments reported in Table 2.

Preparation of Cloned DNA. For the hybridizations involving ^{32}P-labeled *P1* and *LSP-2* DNA probes, as reported in Figs. 1, 2, and 3 and Table 1, the *P1* DNA contained 2.1 kilobase pairs (kbp) of the *P1* coding region, which was purified from a *Bgl* II digest of the λ/*Drosophila* clone 117 (3) subcloned into pBR322. The *LSP-2* DNA consisted of two fragments, one 4.4 kbp and the other 4.8 kbp, purified from an *Eco*RI/*Sal* I digest of the λ/*Drosophila* clone 104 (3). The *G12* DNA was isolated intact from a λ/*Drosophila* clone containing the *G12* gene.

Abbreviations: Epps, *N*-(2-hydroxyethyl)piperazinepropanesulfonic acid; $PhMeSO_2F$, phenylmethylsulfonyl fluoride; kbp, kilobase pair(s).

For the hybridizations of ^{32}P-labeled RNA to immobilized cloned DNA, as reported in Table 2, the DNA was isolated intact from λ/*Drosophila* clones 117 for the *P1* gene and 104 for the *LSP-2* gene (3).

RESULTS

The cultured fat body system was prepared from homozygous *ecd*^{1} larvae that were grown at 20°C until either 5 or 10 hr after the second molt and were shifted to 29°C for about 40 hr; fat bodies were dissected from the shifted larvae and incubated in culture medium either with or without a supplement of ecdysterone. The assay for *P1* or *LSP-2* transcript was done by extracting RNA from the cultured fat bodies, spotting the RNA on nitrocellulose paper, and hybridizing with ^{32}P-labeled DNA from the cloned genes. After hybridization, the paper was autoradiographed and the film was scanned in the regions of the RNA spots with a laser beam densitometer. The first set of such experiments provided a test of the effects of ecdysterone concentration in the culture medium and of incubation time on *P1* expression, as measured by the amounts of *P1* transcript that accumulated in the cultured *ecd*^{1} fat bodies. At ecdysterone concentrations >0.1 μM, the amount of *P1* transcript increased, reaching a plateau between 10 and 100 μM (Fig. 1). The time required to achieve the maximal increase with 100 μM ecdysterone was 1–2 hr (Fig. 2).

Having established conditions for inducing accumulation of *P1* transcript in *ecd*^{1} cultured fat bodies, the effect of adding cycloheximide, an inhibitor of protein synthesis but not of RNA synthesis, to the culture medium was tested. Addition of 1 mM cycloheximide had no effect on the accumulation of *P1* transcript, although protein synthesis was inhibited by about 94% (Table 1). Because ecdysterone continued to induce the maximal *P1* response in fat bodies under conditions that drastically decreased protein synthesis, it appears that ecdysterone is not acting indirectly by inducing formation of a protein required for the *P1* response.

The size of the *P1* transcript induced by ecdysterone in cultured *ecd*^{1} fat bodies was analyzed by electrophoresis of the extracted RNA in agarose gel, transfer of the RNA to nitrocellulose paper, hybridization with ^{32}P-labeled DNA, and autoradiography (Fig. 3 *Left*). The autoradiographed film shows a single *P1* hybridization band in the induced *ecd*^{1} sample at the same position as the hybridization band in the control sample, which contained fat body RNA from late-third-instar standard larvae, indicating that the size of the *P1* transcript induced in the cultured *ecd*^{1} fat bodies is normal.

The *LSP-2* and *P1* genes are expressed sequentially during the third instar, *LSP-2* preceding *P1* by several hours (3). For the tests of *P1* expression reported in Figs. 1, 2, and 3, the *ecd*^{1} larvae were shifted from the permissive to the restrictive temperature 10 hr after the second molt, before *P1* expression and after *LSP-2* expression begins. Therefore, in contrast to the *P1* transcript, the *LSP-2* transcript should be present not only in the cultured fat bodies treated with ecdysterone but also in the untreated fat bodies; the results of testing for *LSP-2* transcripts in the 10-hr shift experiment confirm this expectation (Fig. 3 *Middle*).

If ecdysterone-induced gene expression in cultured fat bodies is a specific rather than a general effect, it should be limited to genes that are normally induced *in vivo* in third-instar fat bodies. As a control for the specificity of induction, the RNA from cultured *ecd*^{1} fat bodies was hybridized with a DNA probe containing the cloned gene *G12*, which is strongly expressed *in vivo* in early embryos but not in late-third-instar fat bodies (unpublished data). The *G12* transcripts in the 10-hr shift experiments were hardly detectable even when *P1* expression was strongly induced by ecdysterone (Fig. 3 *Right*), consistent with the specificity observed *in vivo*.

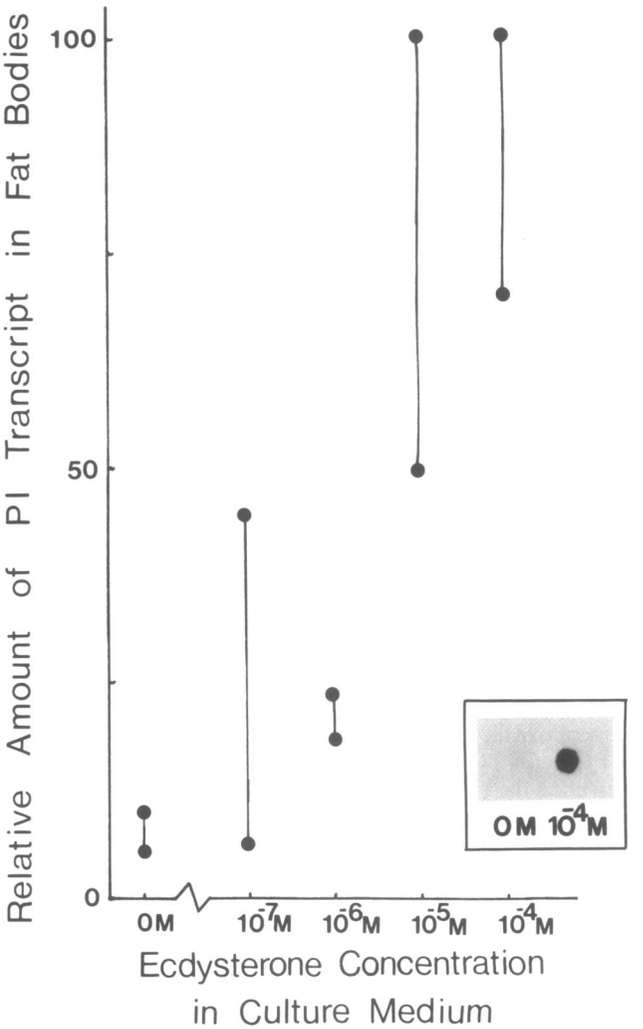

FIG. 1. Effect of ecdysterone concentration on the accumulation of *P1* transcript in cultured fat bodies. The homozygous *ecd*^{1} larvae were synchronized at the second molt and kept at 20°C for 10 hr; afterward, the temperature was shifted to 29°C and the larvae were kept for an additional 40 hr. Fat bodies were dissected from about 10 larvae for each sample and cultured at 25°C in 0.1 ml of a basal medium (5) with the indicated concentration of ecdysterone. After 3-hr incubation, total RNA was extracted from the fat bodies (1) and immobilized on nitrocellulose paper by the following procedure. The RNA was denatured by heating to 60°C for 7 min in 2.2 M formaldehyde/7.5 mM sodium phosphate, pH 7.0, and then the solution was chilled on ice. Aliquots of the solution were spotted onto nitrocellulose paper (Schleicher & Schuell BA85) that had been wetted with 3 M NaCl/0.3 M sodium citrate, and then the paper was heated to 80°C for 5 hr in a vacuum oven. The immobilized RNA was hybridized to a ^{32}P-labeled cloned *P1* DNA probe (10^8 cpm per μg of DNA) prepared by nick-translation (7). Conditions for hybridization were as described (8) except that the hybridization solution did not contain dextran sulfate. After hybridization, the filter was washed and autoradiographed, and the intensity of the film darkening in the region of each RNA spot was measured with a laser beam densitometer; the highest intensity was assigned a value of 100. The duplicate points for each ecdysterone concentration are the results from two experiments. (*Inset*) Autoradiograph of the spots for two of the samples analyzed, from fat bodies cultured in a medium without ecdysterone and in a medium with 100 μM ecdysterone.

The test for ecdysterone-induced accumulation of a gene transcript in cultured fat bodies depends on timing the temperature shift of the *ecd*^{1} larvae so that the larvae become deficient in ecdysterone before a significant amount of the tran-

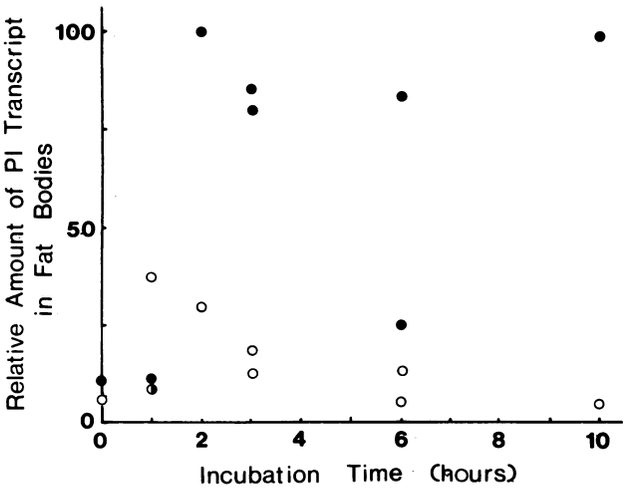

FIG. 2. Effect of incubation time on the accumulation of *P1* transcript in cultured fat bodies. The homozygous *ecd*^{1} larvae were grown as in Fig. 1, and fat bodies were dissected and cultured at 25°C for the indicated times, either in a medium without ecdysterone (○) or with 100 μM ecdysterone (●). The amount of *P1* transcript was measured as in Fig. 1. The results from two experiments are shown.

script accumulates. When the shift occurred 10 hr after the second molt, accumulation of *P1* transcript was blocked but the amount of *LSP-2* transcript already was high (Fig. 3). When the shift occurred 5 hr after the second molt, the amount of *LSP-2* transcript still was too high to permit detection of an inducing effect of ecdysterone (data not shown). As an alternative procedure for testing *LSP-2* induction, nuclei were isolated from cultured fat bodies and incubated with [α-^{32}P]GTP in order to label newly synthesized RNA, and the labeled *LSP-2* transcripts were assayed by hybridization to *LSP-2* DNA immobilized on nitrocellulose. This procedure eliminated the background from *LSP-2* transcripts that accumulate in the cytoplasm before ecdysterone is added to the culture medium. In a control experiment, using nuclei isolated from fat bodies of normal larvae at the climbing stage when the levels of *P1* and *LSP-2* transcripts are high (3), there was strong incorporation of the label into both transcripts; the incorporation was blocked when α-amanitin (9 μg/ml), which inhibits RNA polymerase II activity

Table 1. Effect of cycloheximide on ecdysterone-induced accumulation of *P1* transcript in cultured fat bodies

Culture medium		Relative amount of protein synthesis	Relative amount of *P1* transcript accumulation
Ecdysterone	Cycloheximide		
0	0	1.0	1.0
100 μM	0	1.5	5.0
100 μM	1 mM	0.1	5.5

The *ecd*^{1} larvae were grown as in Fig. 1, and fat bodies were dissected and cultured at 25°C for 3 hr in a basal medium (5), supplemented as indicated in the table. The amount of protein synthesis was measured by adding [^{35}S]methionine to the medium and, after incubation, precipitating the labeled protein with 5% trichloroacetic acid and assaying the radioactivity in a scintillation counter. The amount of *P1* transcript was measured as described in Fig. 1. The mean values for two experiments are shown.

(10), was included in the incubation medium (data not shown). In the experiments using nuclei isolated from fat bodies of *ecd*^{1} larvae shifted 5 hr after the second molt, the amount of newly synthesized *LSP-2* transcript in the isolated nuclei increased about 10-fold when the fat bodies were cultured in the medium with ecdysterone, compared to the medium without ecdysterone (Table 2). No synthesis of *P1* transcript was detected in these nuclei, probably because the *ecd*^{1} fat bodies were not yet competent to express the *P1* gene (1). When the shift of the *ecd*^{1} larvae was delayed until 10 hr after the second molt, there was a strong effect of ecdysterone on the amount of newly synthesized *P1* transcript in isolated nuclei (Table 2); the effect of ecdysterone on the amount of *LSP-2* transcript was weaker than in the previous experiment because the level in the uninduced fat bodies already was high.

DISCUSSION

Earlier studies with the temperature-sensitive mutant *ecd*^{1} showed that ecdysterone has an essential role in *Drosophila* development at virtually all stages (4). Although ecdysterone induces such diverse physiological effects as pupariation, chromosome puffing, and imaginal disc differentiation, some of the effects, and probably all, result primarily from the induced expression of various genes.

The *P1* and *LSP-2* genes analyzed in this report are examples

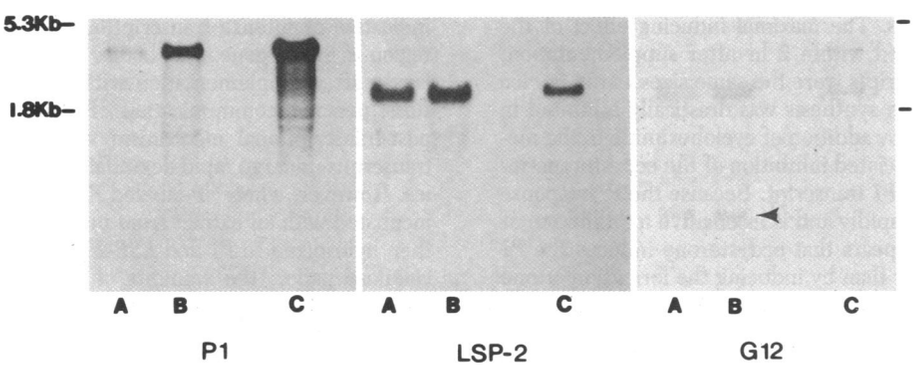

FIG. 3. Assay of transcripts in cultured fat bodies after gel electrophoresis. The *ecd*^{1} larvae were grown as in Fig. 1, and fat bodies were dissected and cultured at 25°C for 3 hr. Total RNA was extracted from fat bodies and electrophoresed in 1.5% agarose gel containing 2.2 M formaldehyde, and the RNA was transferred to nitrocellulose paper by blotting (9). The immobilized RNA was hybridized to the indicated ^{32}P-labeled DNA probe as in Fig. 1, and the nitrocellulose paper was washed and autoradiographed. Lanes: A and B, 2.6 μg of RNA extracted from fat bodies cultured either in a medium without ecdysterone (lane A) or with 100 μM ecdysterone (lane B); C, control containing 0.4 μg of RNA extracted from fat bodies of wild-type climbing larvae, in which the level of *P1* transcript is maximal (4). Mouse ribosomal RNA was used for the molecular size standardization. The same RNA blot was used for three successive hybridizations with the indicated DNA probes, first with *P1* DNA, then with *LSP-2* DNA, and finally with *G12* DNA; the paper was regenerated after the first and second hybridizations by immersion in water at 90°C and subsequent incubation until the water cooled to room temperature. The *G12* hybridization result shows three bands, of which the lowest at the 0.6-kilobase (kb) position, indicated by an arrow, is the *G12* band; the others are the residual *P1* and *LSP-2* bands which are visible because of the long exposure time.

Table 2. Synthesis of *LSP-2* and *P1* transcripts in nuclei isolated from cultured *ecd*[1] fat bodies

Ecdysterone in culture medium	DNA used for hybridization to nuclear RNA	Relative amounts of nuclear RNA hybridized			Inducing effect of ecdysterone (means)
		Exp. 1	Exp. 2	Exp. 3	
		Temperature shift at 5 hr			
0	*LSP-2*	1.0	1.0		9.9
100 μM	*LSP-2*	11.7	8.1		
0	*P1*	<0.1	<0.1		
100 μM	*P1*	<0.1	<0.1		
		Temperature shift at 10 hr			
0	*LSP-2*	1.0	1.0	1.0	1.7
100 μM	*LSP-2*	2.7	1.5	0.9	
0	*P1*	0.3	0.2	0.2	5.4
100 μM	*P1*	2.3	1.0	0.5	

The *ecd*[1] larvae were grown at 20°C until either 5 or 10 hr after the second molt and then were shifted to 29°C for about 40 hr. Fat bodies were dissected and cultured at 25°C for 2 hr in a basal medium either without or with added ecdysterone, and a nuclear fraction was prepared. Nuclei from 30 dissected fat bodies in 25 μl of the suspension buffer were labeled with 50–100 μCi of [α-^{32}P]GTP (757 Ci/mmol; 1 Ci = 3.7 × 10^{10} Bq), and the suspension was kept at 25°C for 30 min. The RNA was extracted as described (11) except that the treatment with DNase was omitted. The RNA was hybridized to an excess of cloned *P1* or *LSP-2* DNA immobilized on nitrocellulose paper (8). The amount of hybridized transcript was measured as in Fig. 1. Two independent experiments were done for the 5-hr shift and three for the 10-hr shift. For both shifts, the amount of *LSP-2* transcript detected in the samples without ecdysterone was assigned a value of 1.0 for comparison with the ecdysterone-supplemented samples; the actual amount, however, was about 20-fold higher in the 10-hr shift than in the 5-hr shift because of the early induction of the *LSP-2* gene.

of genes that are regulated by ecdysterone in a tissue-specific and stage-specific manner: intense expression of the two genes occurs in the larval fat bodies during the third-instar stage in response to an increased concentration of ecdysterone (1, 3). The procedure used to detect ecdysterone-induced expression of the two genes *in vivo* involved establishing conditions of ecdysterone deficiency in the temperature-sensitive mutant *ecd*[1] by shifting the larvae from permissive to restrictive temperature shortly before gene expression would normally begin and then relieving the deficiency by providing the larvae with an exogenous source of ecdysterone. Intense expression of *P1* and *LSP-2* was blocked in the shifted larvae and restored when the ecdysterone titer increased (1, 3).

These effects of ecdysterone on *P1* and *LSP-2* expression *in vivo* have now also been obtained in cultured fat bodies from shifted *ecd*[1] larvae: supplementation of the culture medium with ecdysterone induced the accumulation of *P1* and *LSP-2* transcripts in the fat bodies. The maximal inducing effect of the ecdysterone was reached within 2 hr after supplementation, and the resulting transcripts were the same sizes as the *in vivo* products. When protein synthesis was drastically inhibited in the cultured fat bodies by addition of cycloheximide to the medium, there was no associated inhibition of the ecdysterone-induced accumulation of *P1* transcript. Because the *P1* response to ecdysterone occurs rapidly and is insensitive to inhibition of protein synthesis, it appears that ecdysterone induces the *P1* response directly rather than by inducing the formation of one or more proteins required for the response.

The fat bodies respond similarly to ecdysterone in culture and *in vivo*. When the *ecd*[1] larvae were shifted 5 hr after the second molt, ecdysterone induced the *LSP-2* response but not the *P1* response in both systems. When the shift was delayed until 10 hr after the second molt, the *P1* response was induced in both systems. Furthermore, another gene that is not induced by ecdysterone *in vivo* also did not respond to induction in culture, indicating that ecdysterone acts selectively in the cultured fat bodies. The concentration of ecdysterone required to achieve a maximal *P1* response in the cultured fat bodies is about 100 times greater than the estimated physiological concentration at the end of the third-instar stage (12, 13). For another culture system, which used second-intermolt salivary glands to detect ecdysterone-induced chromosome puffs (12), the concentration of ecdysterone required for induction of puff formation was also in excess of the physiological concentration at the intermolt stage (12, 13). The reason for the apparently excessive ecdysterone requirements in the cultured systems is not understood. The use of ecdysterone-deficient *ecd*[1] larvae to prepare the fat bodies could affect the capacity of that system to respond to ecdysterone. Nevertheless, ecdysterone appears to elicit a normal response in the cultured fat bodies, inducing expression of the same genes, and in the same order, in culture as *in vivo*.

Ecdysterone could induce gene expression either by a transcriptional mechanism involving stimulation of gene transcription or a post-transcriptional mechanism involving inhibition of transcript degradation. In support of a transcriptional mechanism, it was observed that a large chromosomal puff, which is indicative of intense transcriptional activity, appeared in the region of the *P1* gene in fat bodies of ecdysterone-deficient *ecd*[1] larvae after supplementation with exogenous ecdysterone (P. Ross, personal communication). Furthermore, the alternative post-transcriptional mechanism would require that the two transcripts undergo rapid degradation in the uninduced fat bodies. However, when ^{32}P-labeled *P1* and *LSP-2* transcripts were incubated with an extract from uninduced *ecd*[1] fat bodies and then hybridized to *P1* and *LSP-2* DNA immobilized on nitrocellulose paper, the amounts of hybridizable *P1* and *LSP-2* transcripts remained constant during a 20-min or longer incubation period (unpublished data), suggesting that these transcripts are not subject to rapid degradation in the fat bodies, at least not to an extent that would affect the hybridization assay. Thus, the available evidence supports a transcriptional rather than a post-transcriptional mechanism for ecdysterone-induced expression of the *P1* and *LSP-2* genes. This conclusion is consistent with the finding that ecdysterone binds to salivary gland chromosomes at specific sites where puffs are formed (14).

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