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, 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 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 fat bodies, consistent with the effects observed in vivo.

MATERIALS AND METHODS

Drosophila Strains. The temperature-sensitive mutant ecd (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/0.15 mM spermine/0.15 mM spermidine/0.34 M sucrose/2 mM EDTA/0.5 mM EGTA/0.5 mM phenylmethyisulfonyl fluoride (PhMeSO2F) (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 MgCl2, 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 PhMeSO2F, 0.24 M sucrose, 25 mM (NH4)2SO4, 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 32P-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 A/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 EcoRI/Sal I digest of the A/Drosophila clone 104 (3). The G12 DNA was isolated intact from a A/Drosophila clone containing the G12 gene.

Abbreviations: Epps, N-(2-hydroxyethyl)piperazinepropanesulfonic acid; PhMeSO2F, phenylmethyisulfonyl fluoride; kbp, kilobase pair(s).
For the hybridizations of 32P-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 ecd3 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 32P-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 amount of P1 transcript that accumulated in the cultured ecd3 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 ecd3 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 ecd3 fat bodies was analyzed by electrophoresis of the extracted RNA in agarose gel, transfer of the RNA to nitrocellulose paper, hybridization with 32P-labeled DNA, and autoradiography (Fig. 3 Left). The autoradiographed film shows a single P1 hybridization band in the induced ecd3 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 ecd3 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 ecd3 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 ecd3 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.

The test for ecdysterone-induced accumulation of a gene transcript in cultured fat bodies depends on timing the temperature shift of the ecd3 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 ecd1 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-32P]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 (10), was included in the incubation medium (data not shown).

In the experiments using nuclei isolated from fat bodies of ecd1 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 ecd1 fat bodies were not yet competent to express the P1 gene (1). When the shift of the ecd1 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 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

<table>
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<th>Culture medium</th>
<th>Cycloheximide</th>
<th>Relative amount of protein synthesis</th>
<th>Relative amount of P1 transcript</th>
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The ecd1 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 [35S]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.
of genes that are regulated by ecdysone 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^i 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^i 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^i 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-instar larval 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^i 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 by 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^i 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 uninjured fat bodies. However, when 32P-labeled P1 and LSP-2 transcripts were incubated with an extract from uninjured ecd^i 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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