Characterization of an EcR/USP heterodimer target site that mediates ecdysone responsiveness of the Drosophila Lsp-2 gene

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Abstract The Larval serum protein-2 gene (Lsp-2) of Drosophila melanogaster is uniquely expressed in the fat body tissue from the beginning of the third instar to the end of adult life. Accumulation of the larval Lsp-2 transcript is enhanced by 20-hydroxyecdysone. To study the molecular basis for ecdysone regulated Lsp-2 activity, deletion mutants of the Lsp-2 5'-flanking region were constructed by fusion to either the Escherichia coli chloramphenicol acetyltransferase (CAT) gene or to an hsp70-lacZ hybrid gene encoding β-galactosidase. Constructs transfected into Drosophila S2/M3 cells were shown to confer transient ecdysone inducibility on the reporter genes. A single functional ecdysone response element (EcRE) was localized at position −75 relative to the Lsp-2 transcription initiation site. In gel mobility shift assays using fat body nuclear extracts or nuclear receptors synthesized in vitro, a 27-bp sequence harboring the EcRE bound both the Drosophila ecdysone receptor and the Drosophila retinoid-X homologue, Ultraspiracle, in a cooperative manner. Competition experiments indicate that the affinity of the Lsp-2 EcRE for the ecdysone receptor complex is comparable to that of the canonical EcRE of the hsp27 gene and is at least 4-fold greater than that of Fbp1, another fat body-specific Drosophila gene. Our results suggest that structural features of this EcRE determine its ability to induce ecdysone responsiveness at a lower ligand concentration and may form the basis for differential hormone responsiveness within the fat body.

Key words Drosophila melanogaster • Larval serum protein-2 gene • Ecdysone response element • Ecdysone receptor • USP/Drosophila retinoid-X receptor

Introduction

The arthropod steroid hormone 20-hydroxyecdysone (20E) is the main orchestrator of the complex sequence of morphogenetic events underlying metamorphosis in holometabolous insects such as Drosophila melanogaster (reviewed in Riddiford 1993). Ecdysone action, like that of vertebrate steroids, is mediated by specific nuclear receptor binding to target sites of regulated genes. The ecdysone receptor (EcR) was shown to bind to specific ecdysone response elements (EcRE) only as a heterodimer comprising the EcR protein encoded by the ecdysone receptor gene (Koelle et al. 1991) and the Drosophila retinoid-X homologue, Ultraspiracle, in a cooperative manner. Competition experiments indicate that the affinity of the Lsp-2 EcRE for the ecdysone receptor complex is comparable to that of the canonical EcRE of the hsp27 gene and is at least 4-fold greater than that of Fbp1, another fat body-specific Drosophila gene. Our results suggest that structural features of this EcRE determine its ability to induce ecdysone responsiveness at a lower ligand concentration and may form the basis for differential hormone responsiveness within the fat body.

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Introduction

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In vitro analysis of the EcR binding properties of mutated EcREs has demonstrated the requirement for single-base spacing between the half palindromes and the importance within the palindrome of specific positions dictating binding affinity (Antoniewski et al. 1993; Martinez et al. 1991; Ozyhar and Pongs 1993). However, to date there has been no analysis of the role of EcRE structure in determining a tissue-specific
hormone response in the context of the varying concentrations of the receptor ligand, ecdysone, during Drosophila development. Understanding this role requires the characterization of natural EcREs in the cis-regulatory sequences of ecdysone-responsive genes. Examples of such Drosophila genes are the Fat body protein 1 (Fbp1) gene and the Larval serum protein-2 gene (Lsp-2) which encodes the basic subunit of one of the major hexameric serum proteins, LSP-2, of third-instar larvae (Akam et al. 1978). Fbp1 and Lsp-2 were originally identified as two of six genes uniquely expressed in the Drosophila fat body during the third larval instar (Lepesant et al. 1982). Lsp-2 is ecdysone-inducible well before Fbp1, which responds directly to ecdysone during the second half of the third larval instar and has a well-characterized EcRE (Andres et al. 1993; Antoniewski et al. 1993; Nakanishi and Garen 1983). Following induction at the onset of the third instar, Lsp-2 mRNA accumulation peaks at mid-third instar and then declines significantly by the time of puparium formation (Andres et al. 1993; Lepesant et al. 1986). A low-level phase of Lsp-2 expression then sets in by the head-eversion stage of pupal development and is maintained throughout adult life (Benes et al. 1990; Shirras and Bownes 1989). In the ecdysone-deficient mutant l(3)ecd[10] or ec [2] (Garen et al. 1977; Lepesant et al. 1982) the Lsp-2 RNA level is strongly reduced at the restrictive temperature; addition of ecdysone to mutant larvae results in a 10-fold induction of Lsp-2 mRNA synthesis (Nakanishi and Garen 1983; Lepesant et al. 1986).

Recently Andres et al. (1993) observed during the third larval instar four coordinate changes in activity of multiple genes, which may reflect regulation by small hormone peaks in preparation for metamorphosis in Drosophila. As one of only a few cloned Drosophila genes with a unique tissue specificity and a known ecdysone response early in the third instar well before the onset of metamorphosis, Lsp-2 offers an ideal opportunity to characterize the molecular basis for stage-specific responses to varying hormone levels. Here we report the mapping and characterization of a functional EcRE within the 5'-flanking region of Lsp-2. We show that the EcR isoform found in the larval fat body binds to the Lsp-2 EcRE and forms a nucleoprotein complex including the USP protein. The Lsp-2 EcRE appears as a new functional EcR/USP target site whose high affinity for EcR/USP may provide an explanation for the ecdysone responsiveness of Lsp-2 at a low concentration of ligand during the early third larval instar.

Materials and methods

Plasmid constructs

Transfection control plasmids

The actin-β-gal expression vector includes 298 bp of the Drosophila cytoplasmic actin gene promoter plus untranslated RNA leader sequences linked to the Escherichia coli lacZ reporter gene in the pcSpeR vector (Thummel et al. 1988). The X-188-cc-cat expression vector includes the basal Eip28-29 promoter (−188 to +1) linked to the cat gene (Cherbas et al. 1991). Transfection vectors

Lsp-2-cat constructs. The Lsp-2-cat plasmids were constructed by inserting blunt-ended fragments from the 5'-flanking region of the Drosophila Lsp-2 gene (Lepesant et al. 1982; Fig. 1A) into the Smal site of the promoterless plasmid vector pSV0-CAT [2] (Benyajati and Dray 1984). 3.1Lsp-2-cat includes an EcoRI-XhoI fragment of Lsp-2 (Lsp-2 sequences −3100 to +16); 1.5Lsp-2-cat, an XhoI fragment (Lsp-2 sequences −1500 to +16); 0.68Lsp-2-cat contains a ClaI-XhoI fragment (Lsp-2 sequences −667 to +16); and 0.28Lsp-2-cat, a Poulil-XhoI fragment (sequences −267 to +16 of Lsp-2). 0.08Lsp-2-cat was constructed using an HaeIII-XhoI fragment (sequences −65 to +16). All constructs were verified by restriction endonuclease analysis.

Lsp-2-hsp-lacZ fusion genes. To study regulatory sequences both in cell transfection assays and whole-animal, in vivo transformation assays, an hsp-lacZ shuttle vector (pLen) was derived from pHZ50PL (Hiromi and Gehring 1987) by elimination of the rosa gene and P element sequences. pLen includes the Drosophila hsp70 basal promoter (−50 to +271), the lacZ reporter gene and appropriate hsp70 polyadenylation signal sequences, inserted in (Fig. 3A); the original polylinker of pHZ50PL was retained. Blunt-ended fragments of the Lsp-2 5'-flanking region were inserted upstream of the hsp70 promoter into the unique, blunt-ended NorI site of pLen. 0.23Lsp-2-hsp70-lacZ contains a Poulil-XhoI fragment (Lsp-2 sequences −267 to −37). 0.126Lsp-2-hsp70-lacZ carries a HaeIII fragment (Lsp-2 sequences −190 to −66), and 0.076Lsp-2-hsp70-lacZ, a Poulil-HaeIII fragment (−267 to −191). The 0.2Lsp-2-hsp70-lacZ construct was generated by mutagenesis of the 0.23Lsp-2-hsp70-lacZ fusion gene using the primer 5'-GGATGCGACCGGTTGTCTGC-3' and the Unique-Site-Elimination kit (Pharmacia). All constructs were verified by DNA sequence analysis.

Receptor expression vectors

The M1-EcR expression vector was constructed by inserting a blunt-ended XspI-HindIII fragment containing the entire coding region of the EcR-B1 cDNA (pMK1; Talbot et al. 1993) into the HincII site of pHZ50PL (Fig. 1B). M1-USP expression vector contains the entire insert from the cDNA clone pZ7-1 (Henrich 1995), excised with SmaI and EcoRV, and inserted into the HincII site of pRMHa-1.

Cell culture and transfection

Cultured Drosophila S2/M3 cells (Cherbas et al. 1991) were grown at 23°C in M3 medium (Shields and Sang 1977) supplemented with 12.5% heat-inactivated fetal calf serum; Ke167/M3 cells (Cherbas et al. 1991) were grown at 23°C in M3 medium modified after Lindquist et al. (1982), supplemented with 5% heat-denatured fetal calf serum. Cells were transfected either with a calcium phosphate-DNA co-precipitate (DiNocera and Dawid 1983) or with liposomes containing the plasmid DNA and a control for transfection efficiency (1 μg of actin-β-gal DNA, described below, or 1.8 μg of X-188-cc-cat DNA (Cherbas et al. 1991)) in a final 10-ml volume of medium. Following a 3-h incubation with the CaPO4-DNA