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Specific transcriptional responses to juvenile hormone and ecdysone in *Drosophila*

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Abstract

Previous studies have shown that ecdysone (E), and its immediate downstream product 20-hydroxyecdysone (20E), can have different biological functions in insects, suggesting that E acts as a distinct hormone. Here, we use *Drosophila* larval organ culture in combination with microarray technology to identify genes that are transcriptionally regulated by E, but which show little or no response to 20E. These genes are coordinately expressed for a brief temporal interval at the onset of metamorphosis, suggesting that E acts together with 20E to direct puparium formation. We also show that *E74B*, *pepck*, and *CG14949* can be induced by juvenile hormone III (JH III) in organ culture, and that *CG14949* can be induced by JH independently of protein synthesis. In contrast, *E74A* and *E75A* show no response to JH in this system. These studies demonstrate that larval organ culture can be used to identify *Drosophila* genes that are regulated by hormones other than 20E, and provide a basis for studying crosstalk between multiple hormone signaling pathways.

Keywords: Ecdysone; Juvenile hormone; Metamorphosis; Gene regulation

1. Introduction

Like all holometabolous insects, *Drosophila melanogaster* has two primary lipophilic hormones, the steroid hormone 20-hydroxyecdysone (20E) and the sesquiterpenoid juvenile hormone (JH) (Riddiford, 1993). 20E arises from its immediate precursor, ecdysone (E), which is released in bursts from the *Drosophila* prothoracic gland in response to neuropeptide signaling. Pulses of 20E during the first and second larval instars trigger molting of the cuticle while a high titer pulse of 20E at the end of the third instar triggers puparium formation, initiating metamorphosis and the prepupal stage of development. 20E exerts its effects by binding to a heterodimer of two nuclear receptors, the Ecdysone Receptor (EcR) and Ultraspiracle (USP) (Koelle et al., 1991; Thomas et al., 1993; Yao et al., 1993). This activated receptor complex reprograms gene expression in target cells, directing the appropriate stage-specific developmental programs during the fly life cycle.

It has long been known that E is modified by peripheral tissues into 20E, although only recently has the 20-monooxygenase been discovered that catalyzes this reaction (Petryk et al., 2003). E is widely believed to be inactive, with little or no effect on classic EcR-mediated responses in *Drosophila* (Richards, 1978; Baker et al., 2000; Hu et al., 2003). Several studies in other insects, however, have invoked developmental functions for E, including effects on DNA synthesis in cultured wing discs of *Galleria mellonella* and molting in *Chironomus tentans* (Oberlander, 1972; Clever et al., 1973). More recently, the high E titer during early *Manduca* metamorphosis has been shown to direct neuroblast proliferation and optic lobe development, establishing a critical role for this hormone that is distinct from functions regulated by 20E (Champlin and Truman, 1998). In addition, *Aedes aegypti* EcR, unlike its *Drosophila* counterpart, can be efficiently activated by both E and 20E (Wang et al., 2000). These observations raise the interesting possibility that E could act as a hormone, much like 20E, although there is no molecular data to support such a role for this ecdysteroid.

Studies in Coleoptera and Lepidoptera have shown that JH defines the nature of the biological responses triggered

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by 20E during development, leading to the status quo model for JH action. In the presence of high JH titers, pulses of 20E lead to larval–larval molts whereas, in the absence of JH, 20E initiates maturational responses and metamorphosis (reviewed by Riddiford, 1996; Gilbert et al., 2000). In contrast, much less is known about roles for JH in *Drosophila* pre-adult development. Ectopic application of a JH analog during prepupal stages results in lethality with defects in abdominal, nervous system, and muscular development (Riddiford and Ashburner, 1991; Restifo and Wilson, 1998). The defects in abdominal development are accompanied by changes in cuticle gene expression that are mediated by sustained expression of the Broad-Complex transcription factors (Zhou and Riddiford, 2002). No normal developmental functions, however, have been shown for JH during *Drosophila* larval or prepupal stages. Similarly, relatively little is known about its molecular mechanisms of action. Two candidate JH receptors have been proposed in the literature, USP and Methoprene-tolerant (Met) (Jones and Sharp, 1997; Ashok et al., 1998; Miura et al., 2005), although biological evidence for these acting as JH receptors is lacking (Restifo and Wilson, 1998; Gilbert et al., 2000; King-Jones and Thummel, 2005; Palanker et al., 2006). Rather, the identification of several genes that are transcriptionally controlled by JH provides a foundation for understanding its molecular mechanism of action (reviewed by Berger and Dubrovsky, 2005).

In this study, we exploit the use of larval organ culture and microarray technology to identify genes regulated by E or JH. We show that E can regulate genes in *Drosophila* that are distinct from those controlled by 20E. These genes are coordinately induced or repressed at puparium formation, suggesting that E contributes to the onset of metamorphosis in parallel with 20E. We also identify three genes that are induced by JH in larval organ culture and characterize their regulation by JH, as well as how this response can be modified by 20E. This study provides a basis for understanding the regulatory functions of E and JH in *Drosophila*.

2. Experimental procedures

2.1. Organ culture

For the E microarray study, third instar larvae at approximately 10 h prior to puparium formation were staged by the addition of 0.05% bromophenol blue to the food and selecting animals with partial blue guts, as described previously (Andres and Thummel, 1994). Eight animals were dissected in a nine-well glass dish (Corning) and cultured in approximately 0.1 ml oxygenated Schneiders *Drosophila* medium (Invitrogen) at 25 °C for the times indicated below. Each well contains the integument and all internal organs from the dissected animals. Cultures were incubated under a constant flow of oxygen in a styrofoam box. Following an initial incubation of 1 h, the medium was removed and replaced with either fresh Schneiders *Drosophila* Medium (no hormone) or medium plus 5×10^{-6} M ecdysone (E) (Sigma-Aldrich) for 6 h at 25 °C. This is the same concentration that has been used for studies of 20E responses in cultured larval organs, which is two-fold higher than the amount required for a 50% maximal early puff response in the salivary gland polytene chromosomes and comparable to the peak 20E titers seen *in vivo* (Ashburner, 1973; Karim and Thummel, 1991). The commercial source of E used in our studies was found to be 93% pure by RP-HPLC, as determined by Dr. J. Warren (University of North Carolina, Chapel Hill). We used highly purified E, kindly provided by Dr. Warren, for all validation Northern blot hybridization studies reported here. Similarly, purified (10*R*)-JH III was used for Northern blot studies along with (10*S*)-JH III as a negative control, both kindly provided by Dr. W. Goodman (University of Wisconsin, Madison). Organs were collected and RNA was extracted as described below. Glass culture plates were treated with a 20% solution of PEG 20,000, rinsed at least six times, and autoclaved, to prevent JH binding to the glass. All experiments were done in triplicate and harvested separately for array analysis. For Northern blot analysis, third instar larvae were synchronized at the second-to-third instar larval molt and aged for either 28 or 38 h (approximately −20 or −10 h relative to puparium formation, respectively). Eight animals were processed as above for organ culture and treated with no hormone, 5×10^{-6} M (10*R*)-JH III, 5×10^{-6} M 20-hydroxyecdysone, 5×10^{-6} M ecdysone, 8.5×10^{-5} M cycloheximide (Sigma-Aldrich), 5×10^{-6} M (10*S*)-juvenile hormone, 5×10^{-6} M farnesol (Sigma-Aldrich), or 5×10^{-6} M methyl linoleate (Sigma-Aldrich). JH and 20E were added simultaneously in all cases where both hormones were used in combination.

2.2. Microarray and cluster analysis

Total RNA was isolated from organ cultures using TriPure (Roche) and purified on RNAeasy columns (Qiagen). All samples were prepared independently in triplicate to facilitate statistical analysis. Probe labeling, hybridization to Affymetrix GeneChip *Drosophila*® Genome 1.0 Arrays (Affymetrix), and scanning were performed by the University of Maryland Biotechnology Institute Microarray Core Facility. Raw data were normalized using dChip1.2 and replicate arrays were combined for expression analysis (Li and Wong, 2001). Expression analysis using dChip1.2 was based on the PM-only model, and expression-level differences between control and experimental were analyzed by assigning a fold-change value and filtering for outliers. Data sets were analyzed using Microsoft Access selecting those genes with a change in expression level of $\geqslant$1.5-fold. Comparisons made between the E and 20E data sets were performed using Microsoft Access. The 20E gene set for this comparison was derived from both the 20E-regulated genes and 20E primary-response genes described by Beckstead et al.

(2005). Cluster analysis was performed using dChip1.2. Comparisons were made between our microarray data sets and previously published microarray data sets using Microsoft Access.

2.3. Northern blot hybridizations

Total RNA was isolated from cultured larval organs using TriPure (Roche), fractionated by formaldehyde gel electrophoresis, transferred to nylon membranes, and probed with radioactively labeled probes, as described (Andres et al., 1993). To facilitate direct comparison, blots were probed, washed, and exposed at the same time. Probes were generated by PCR from genomic DNA using the following pairs of primers: *dro5*F AACCGACAACATGCAGATCA, *dro5*R TTCCGGCAATTTAGAGCAAC, *pepckF* ATGAACGCAAAGTCCTCGAC, *pepckR* GGCTGCTCCAATGAAGACTC, *CG7906*F AGTTGACTCAGCATACGG, *CG7906-3′* TGGCACAAATGGACAGTG, *CG7924-5′* AGGTTGTGCCAAATGCAG, *CG7924*R TCGGCACACATCCTCATC, *nmdmc*F CTCCGACCAGCTTAAACTGG, *nmdmc*R ACTAGCACACGAGCTCAAGG, *CG11956*F TACATATGGTGCGAGTTCCAGGAG, *CG11956*R CAAGCTTATCCTGCAAGGACCTAC, *CG14949* F ACCAGTTTTGAGAGCCAGGA, *CG14949R* CTAATCCTCGGGTTCCGTCT, *kr-h1*F GTGCGAGAAGACATTCATCCAGTC, *kr-h1*R TTGAAGTGCTCGATCAGCACGGTG, *black*F CACCAGCACCAGGAACAGTA, *black*R ACTCGGGCGAGTGTCTGTAG.

3. Results

3.1. Identification of genes regulated by E

Organs were dissected from third instar larvae staged at approximately 10 h before pupariation, and cultured in the presence of either no hormone or 5×10^{-6} M ecdysone (E), following protocols used for earlier studies of 20E-regulated transcription (Ashburner, 1972; Beckstead et al., 2005). RNA was extracted from these tissues, labeled, and hybridized to Affymetrix *Drosophila* 1.0 Genome Arrays. Data was analyzed using dChip1.2 (Li and Wong, 2001). For statistical comparisons, RNA from three independent organ culture experiments was used for each data point in the microarray study. Comparison of the control and E-treated data sets led to the identification of 267 E-regulated genes that exhibited a change in expression level of 1.5-fold or greater. Given that E can be converted to 20E by *Drosophila* larval organs (Somme-Martin et al., 1988; Petryk et al., 2003), we compared this data set to the 376 genes that exhibit a change in expression level of 1.5-fold or greater when treated by either 20E alone or 20E plus cycloheximide (Beckstead et al., 2005). This resulted in the identification of 55 genes that appear to be responding specifically to E and not to 20E (Fig. 1A; Table 1).

If these 55 genes correspond to part of an E-specific genetic program, then we might expect to see them

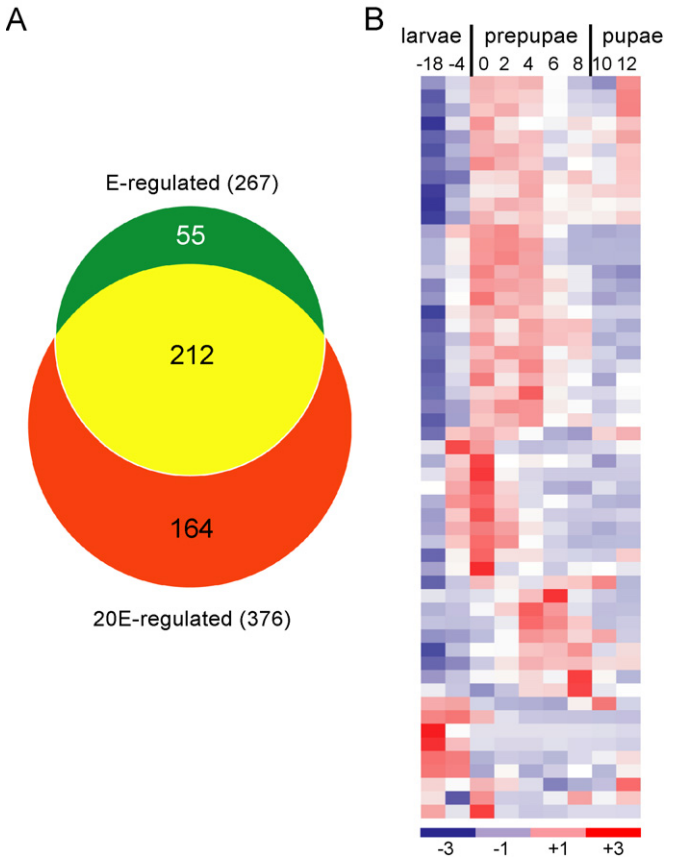

Fig. 1. Ecdysone regulates a distinct set of genes from those controlled by 20-hydroxyecdysone. (A) Venn diagram depicting the overlap between genes regulated by ecdysone (E) and those regulated by 20E. The set of 376 20E-regulated genes was derived from both 20E-regulated genes and 20E primary-response genes from Beckstead et al. (2005), as described in Experimental Procedures. (B) Cluster analysis of microarray data derived from RNA samples isolated from staged wild-type animals (Beckstead et al., 2005). The colors for each time point represent the change in expression level relative to the average expression levels across all time points for that gene, with dark blue indicating the lowest level of expression and red indicating the highest level, as depicted on the bottom. The numbers at the top indicate hours relative to pupariation.

coordinately regulated when E titers peak during development. Such a time occurs late in the third instar, when an increase in E titer precedes the high-titer pulse of 20E that triggers puparium formation (Parvy et al., 2005; Warren et al., 2006). To examine the temporal profiles of E-regulated genes at this time, we used data from an earlier microarray study that determined transcript levels in w^{1118} animals staged at −18, −4, 0, 2, 4, 6, 8, 10, and 12 h relative to pupariation (Beckstead et al., 2005). Interestingly, this study revealed that each of the 55 E-regulated genes is expressed for a brief temporal window at the onset of metamorphosis (Fig. 1B). The majority of genes are induced at puparium formation and down-regulated in 4–6 h prepupae. A second set of genes are specifically induced in mid-prepupae, immediately preceding the late prepupal 20E peak that occurred at 10 h after pupariation in these animals (Beckstead et al., 2005). The remaining genes are down-regulated in late third instar larvae.

Table 1
E-regulated genes

Gene	Fold change	Molecular function
dro5	5.35	Drosomycin
CG7906	3.49	n/a
CG7924	2.41	n/a
CG14957	1.93	n/a
Su(Tpl)	1.87	Transcription elongation factor
mob1	1.81	n/a
malvolio	1.69	Cation transporter
CG11686	1.69	n/a
nmdmc	1.69	NAD-dependent methylenetetrahydrofolate dehydrogenase
regena	1.68	Transcription factor
CG12224	1.67	Oxidoreductase activity
smi35A	1.66	Serine/threonine kinase
CG14478	1.65	n/a
creg	1.64	Transcriptional repressor
snap	1.64	Soluble NSF attachment protein
croquemort	1.63	Scavenger receptor
CG8054	1.62	n/a
adenosine 3	1.62	Phosphoribosylamine-glycine ligase
CG7606	1.6	Uncertain
NPC2	1.57	Receptor binding
emc	1.57	Transcription co-repressor
Vap-33-1	1.57	Structural molecule
Hsc70Cb	1.57	Chaperone binding
CG15248	1.56	Uncertain
egghead	1.56	Beta-1,4-mannosyltransferase
CG1965	1.56	Transcription regulator
CG7806	1.56	Xenobiotic-transporting ATPase
CG9317	1.55	Organic cationic transporter
CG1837	1.55	Protein disulfide isomerase
CG6145	1.54	n/a
CG11169	1.54	n/a
rab-11	1.53	Small GTPase
fu12	1.53	1-acylglycerol-3-phosphate *O*-acyltransferase
CG2082	1.53	n/a
CG7696	1.53	Transcription regulator
smi21F	1.53	n/a
CG5455	1.52	Catalytic activity
Stat92E	1.51	Transcription factor
CG3823	1.51	Vitamin E binding
CG12965	1.51	Transcription factor
CG1246	1.51	n/a
rap	1.5	n/a
CG17110	1.5	Metallopeptidase
CG12004	1.5	n/a
CG3223	1.5	n/a
CG6169	1.5	n/a
bowl	1.5	Transcription factor
CG10114	−1.52	Helena element
new glue 3	−1.55	Structural molecule
PGRP-SA	−1.56	Peptidoglycan receptor
CG13187	−1.57	Protein binding
nemy	−1.58	glutaminase
GST-E1	−1.62	Glutathione transferase
acp26Ab	−1.74	Hormone
GST-D3	−1.77	Glutathione transferase

Taken together, these observations suggest that E activates a genetic program that operates in parallel to that of 20E at the onset of metamorphosis.

Northern blot hybridizations were used to validate the microarray results and confirm the specificity of these genes for responding to E as opposed to 20E. Organs were dissected from larvae staged at either 10 or 20 h prior to puparium formation, and treated with no hormone (control), E, or 20E. RNA was then extracted from these tissues and analyzed on Northern blots using probes to detect several E-regulated genes (Fig. 2, lanes 1,4,5,6,9,10). As a control, we examined the expression of two well-characterized 20E primary-response transcripts, *E74A* and *E75A*, both of which are induced by 20E at 10 h before pupariation, as expected (Segraves and Hogness, 1990; Karim and Thummel, 1991) (Fig. 2, lanes 6, 9). A similar response is seen with E, albeit somewhat weaker, consistent with the expectation that E can be converted to 20E by the cultured larval organs (Fig. 2, lanes 6, 10). Interestingly, this response is only seen in −10 h animals, indicating that the −20 h animals have not yet gained competency to respond to 20E, consistent with earlier work (Richards et al., 1999). We next examined the regulation of *CG7924*, *nmdmc*, *dro5*, *CG7906* and *CG12224*, five genes that display high levels of induction by E alone in our microarray study (Table 1). As predicted, these genes are induced by E but

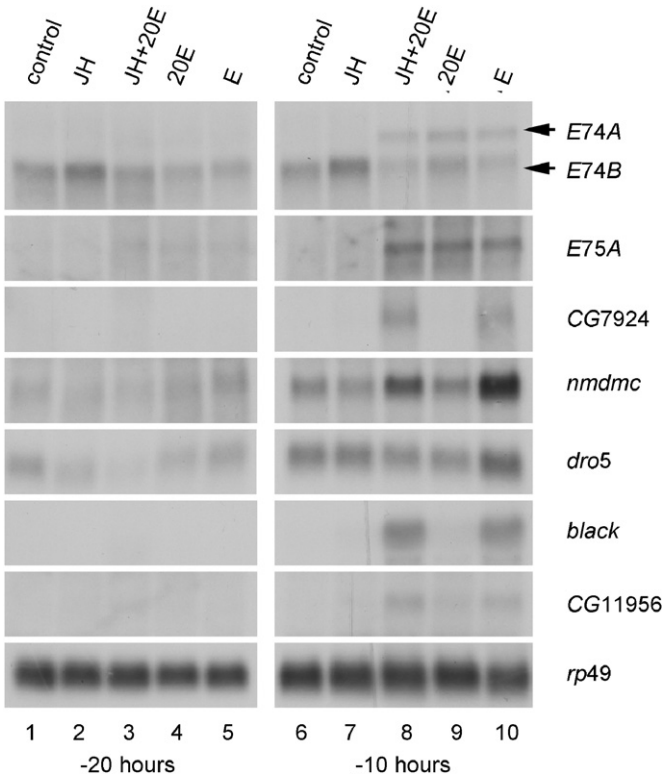

Fig. 2. Validation of transcriptional responses to E and JH. Northern blot analysis of RNA samples isolated from cultured third instar larval organs treated with either no hormone (control), (10R)-juvenile hormone III (JH), JH and 20-hydroxyecdysone (JH + 20E), 20E alone, or ecdysone (E) for 6 h. Organ cultures were established from third instar larvae staged at either ~20 h or ~10 h prior to pupariation. Lane numbers are shown at the bottom. The blots were hybridized to detect transcripts as shown, with *rp49* mRNA used as a control for loading and transfer.

not by 20E (Fig. 2), with the exception of *CG12224* which does not respond to either E or 20E (data not shown). The observation that we can validate the E-specific regulation for four of these five genes indicates that most genes identified by our microarray study respond specifically to this hormone. We also examined two genes that show a higher level of induction by E than 20E in the microarray studies, *black* and *CG11956*. These genes show a very weak response to 20E and much more efficient induction by E, suggesting that at least some of the genes in this class are also likely to represent specific responses to E (Fig. 2 and data not shown). As with *E74* and *E75A*, the E-regulated genes are only induced in organs cultured from −10 h larvae, suggesting that they also require competence for their response to hormone.

3.2. Many E-regulated genes are also regulated by JH in combination with 20E

In the course of doing this study, we made the unexpected discovery that E-regulated genes can also respond to JH in the presence of 20E. As shown in Fig. 2, the Northern blots used for validating the E-regulated genes also contain samples of RNA isolated from third instar larval organs cultured with either JH III alone or JH III and 20E. We used JH III in these experiments because it has been shown to have potent effects on both genetic and biological responses in *Drosophila* (reviewed in Riddiford, 1993; Berger and Dubrovsky, 2005). *CG7924*, *nmdmc*, *black* and *CG11956* are all E-inducible genes that are up-regulated in organ cultures that are treated with JH and 20E, but not JH or 20E alone (Fig. 2, lanes 6–9). Moreover, the magnitude of this up-regulation is similar to that seen in response to E (Fig. 2, lane 10). Interestingly, *dro5* shows no significant response to JH and 20E, indicating that not all E-regulated genes are also controlled by JH and 20E. As with the E and 20E response, JH and 20E only has effects in organs that were harvested at 10 h prior to pupariation (Fig. 2).

Studies in other insects have shown that ecdysone is capable of stimulating JH release from the corpora allata (Gu and Chow, 2003). This raises the possibility that the similar transcriptional responses we observe to treatment with E or 20E plus JH might be due to the production of JH by the ring gland in the larval organs cultured with E. According to this model, E would be converted to 20E in the organ culture system and then either E or 20E might stimulate the release of JH. To test this possibility, we dissected organs from third instar larvae staged at approximately 10 h before puparium formation and removed the brain and its associated ring gland from one set of organs (Fig. 3, right panels). Given that JH in *Drosophila* arises from the corpora allata in the ring gland, we reasoned that the removal of the brain and ring gland would prevent any endogenous JH response in those tissues. These two sets of organs were cultured with either no hormone, E alone, or JH III and 20E, after which RNA

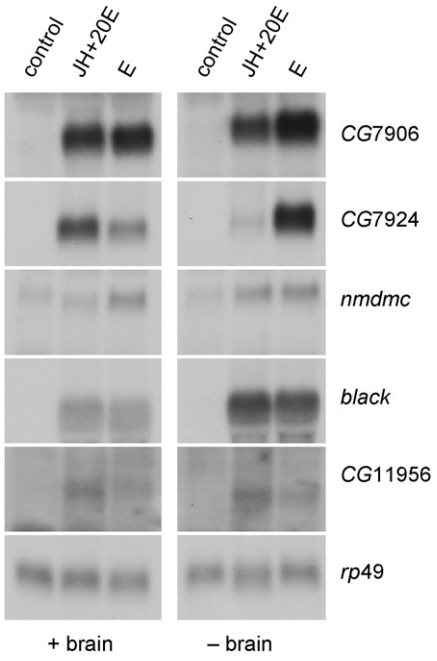

Fig. 3. The brain and ring gland are not required for transcriptional responses to ecdysone. Northern blot analysis of RNA samples isolated from organ cultures derived from −10 h third instar larvae treated with no hormone (control), (10*R*)-juvenile hormone III and 20-hydroxyecdysone (JH + 20E), or ecdysone alone (E). One set of cultures had the brain and ring gland removed prior to the addition of hormone (– brain). The blots were hybridized to detect transcripts as shown, with *rp49* mRNA used as a control for loading and transfer.

was extracted and analyzed by Northern blot hybridization for the expression of E-regulated genes (Fig. 3). *CG7906*, *CG7924*, *nmdmc*, *black*, and *CG11956* are all induced by E or JH plus 20E, as shown previously. We can see some variability in the level of *CG7924* transcription which is typical for this gene, which appears to show more stage specificity than other E-regulated genes (data not shown). Removal of the brain and ring gland, however, had no significant effect on the response to either E alone or JH and 20E, indicating that the ability of E to upregulate this set of genes is not dependent upon the production of JH by the ring gland.

3.3. Identification of JH-regulated genes

In addition to the response to JH and 20E, we also noted that some genes, such as *E74B*, can be induced by JH III alone in organs dissected from either −20 h or −10 h third instar larvae (Fig. 1, lanes 1,2,6,7). This raised the possibility that we might be able to identify JH-regulated genes by microarray analysis of RNA isolated from cultured larval organs treated with JH. Conducting these studies using either methoprene or JH III, however, resulted in a high degree of variation between the JH-treated samples and a low change in expression level between the JH-treated samples and the controls, primarily below 1.5-fold (data not shown). Moreover, most responses could not be validated

by Northern blot hybridization. Two JH-regulated genes did, however, emerge from these studies: *pepck*, which encodes an enzyme involved in gluconeogenesis, and *CG14949*, which encodes a novel protein of unknown function. To characterize their regulation in more detail, organs were dissected from −20 h third instar larvae and treated with either the protein synthesis inhibitor cycloheximide, JH III alone, JH III and 20E, JH III and cycloheximide, or JH III and 20E and cycloheximide. Three negative controls were also included in this study—the biologically inactive JH III 10S isomer, farnesol, and methyl linoleate. RNA was extracted from these organs and analyzed by Northern blot hybridization to detect *E74B*, *pepck*, *CG14949*, and *E75A* mRNA (Fig. 4). *E75A* was included in this study because it can be directly induced by JH III in *Drosophila* S2 tissue culture cells and is up-regulated by methoprene in ovaries from *apterous* mutant females (Dubrovsky et al., 2004). *E74B*, *pepck*, and *CG14949* are all up-regulated by treatment with JH alone (Fig. 4, compare lanes 1 and 3). Both *E74B* and *pepck* show approximately the same response to cycloheximide alone and cycloheximide plus JH, although the levels of *pepck* RNA may be increased ~2-fold by JH relative to the control (Fig. 4, lanes 2, 5). In contrast, *CG14949* transcription is significantly increased upon treatment with JH and cycloheximide relative to the cycloheximide alone control, suggesting that it is a direct target for JH regulation. Interestingly, the JH induction of *E74B* and *pepck* is suppressed by adding 20E (Fig. 4, lanes 3, 4) and, for *pepck*, this down-regulation also occurs in the presence of cycloheximide (Fig. 4, compare lanes 5 and 6). This observation suggests that the response of JH-regulated genes can be suppressed by 20E. Finally, *E75A* is only responsive to 20E, indicating that its JH regulation cannot be recapitulated under the conditions of larval organ culture. All four transcripts failed to respond to any of the negative control compounds, as expected (Fig. 4, lanes 7–9).

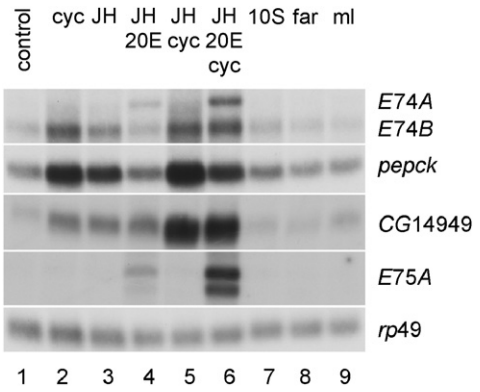

Fig. 4. Juvenile hormone regulates gene expression in larval organ culture. Northern blot analysis of RNA samples isolated from organ cultures derived from third instar larvae at ~20 h prior to pupariation, treated with either no hormone (control), cycloheximide (cyc), (10*R*)-juvenile hormone III (JH), JH III and 20-hydroxyecdysone (JH, 20E), JH III and cycloheximide (JH, cyc), JH III and 20E and cycloheximide (JH, 20E, cyc), (10*S*)-juvenile hormone III (10*S*), farnesol (far), and methyl linoleate (ml). The latter three are negative controls for (10*R*)-JH III. Lane numbers are shown at the bottom. The blots were hybridized to detect transcripts as shown, with *rp49* mRNA used as a control for loading and transfer.

4. Discussion

This paper describes the identification of *Drosophila* genes regulated by E or JH using microarrays in combination with RNA isolated from cultured larval organs. We show that E regulates a set of genes that are distinct from those controlled by 20E. In addition, we identify three classes of genes that are regulated by JH: genes that are induced by JH alone, genes induced by JH in combination with 20E, and genes in which their JH induction is blocked by the addition of 20E. This study provides a foundation for future efforts aimed at identifying receptors for these hormones as well as a more complete understanding of the biological and molecular targets of 20E and JH signaling.

We identified 267 genes that change their expression in response to E in cultured larval organs. By comparing this list with those genes shown previously to be regulated by 20E (Beckstead et al., 2005), we identified three sets of genes: (1) those regulated by E but not 20E, (2) those that are regulated by either E or 20E, and (3) those specifically regulated by 20E (Fig. 1A). The set of 55 E-specific genes provides molecular evidence that E can act as a hormone in *Drosophila* independently of 20E. Our effort to validate this list by Northern blot hybridization confirmed that four of five genes tested are regulated by E and not 20E—*CG7924*, *nmdmc*, *dro5*, and *CG7906*—suggesting that most of the genes in this list are E specific. The predicted functions of these 55 genes include transcriptional control, enzymes, and transporters (Table 1). Interestingly, two of the validated E-inducible genes, *CG7906* and *CG7924*, map next to one another on the genome and encode related proteins that carry a Kazal-type serine protease inhibitor motif, although their functions remain undefined. The simple structure of these genes and their close physical association suggest that they are coordinately regulated by E, and provide a good basis for future efforts to identify E-response elements in the genome.

The second set of 212 genes that are regulated by E or 20E most likely include 20E-specific genes that are responding to 20E produced from the E added to the organ cultures. Interestingly, two genes that show a preferential response to E in the microarray studies, *black* and *CG11956*, display a significantly better induction by E than 20E on Northern blots (Fig. 2), indicating that these 212 genes include additional E-regulated genes. Finally, 164 20E-regulated genes did not respond to the conversion of E to 20E in organ culture (Fig. 1A). It is possible that these genes require a greater concentration of 20E than was achieved by the conversion of E to 20E during the incubation period.

Upon examining the temporal profiles of the 55 E-regulated genes, we found that most are induced between 4 and 18 h prior to puparium formation, along with a

smaller set of genes that are down-regulated at this time (Fig. 1B). The timing of response suggests that these genes may be responding to the increased E titer that precedes the pulse of 20E (Parvy et al., 2005; Warren et al., 2006), and that E and 20E act together to control the appropriate changes in gene expression that direct entry into metamophosis. Recent genetic studies of the ecdysone biosynthetic pathway in *Drosophila* provide some support for this model. In their characterization of the *neverland* gene, Yoshiyama et al. (2006) reported that 7-dehydrocholesterol rescues the lethality of *neverland* RNAi more effectively than 20E, implying that one or more compounds upstream from 20E are essential for proper development. Similarly, preliminary studies indicate that *phantom* (*phm*), *disembodied* (*dib*), and *shadow* (*sad*) mutants, which block specific steps in E biosynthesis (Warren et al., 2002, 2004), are more effectively rescued by feeding E than 20E (H. Ono, M. O'Connor, personal communication). We are attempting to confirm and extend these studies by constructing transgenic lines that will provide temporal control over *dib* and *shade* (*shd*) inactivation. The *dib* gene encodes the enzyme that catalyzes the conversion of 2,22-dideoxyecdysone to 2-deoxyecdysone, while Shd directs the conversion of E to 20E. By inactivating *dib* or *shd* at specific times during development, we should be able to observe the consequences of removing both E and 20E (through inactivation of *dib*) or just removing 20E (through inactivation of *shd*). These tools should allow us to determine whether E provides functions distinct from those of 20E alone.

The ability of E to regulate a distinct set of genes from 20E raises that question of what receptor might mediate this response. A good candidate is the DHR38 nuclear receptor which can be activated by several ecdysteroids that are distinct from those that activate EcR, including E (Baker et al., 2003). Curiously, E does not manifest this effect through direct binding to the receptor and thus other factors must cooperate with DHR38 to transduce an E signal.

Previous studies have identified several JH-regulated genes in *Drosophila* (for a recent review, see Berger and Dubrovsky, 2005). These include *dRNaseZ*, *minidiscs*, *JHI-26*, and *E75A*, all of which are induced by JH III in *Drosophila* S2 tissue culture cells (Dubrovsky et al., 2000, 2002, 2004), as well as the *Drosophila* juvenile hormone esterase gene (Kethidi et al., 2005). In this study, we use cultured larval organs to identify genes regulated by JH and characterize the role of 20E in their transcriptional control. This approach bypasses the need for the hormone to penetrate the cuticle and assays the response of multiple tissues to hormone treatment. Microarray analysis of RNA samples isolated from third instar larval tissues treated with JH III, however, resulted in many false positives that could not be validated by Northern blot hybridization. This result, combined with the observation that *E74B*, *pepck*, and *CG14949* can be clearly induced by JH III in cultured larval organs (Fig. 4), suggests that JH III alone may not have widespread effects on gene expression at this stage. This is consistent with biological studies which have failed to reveal critical functions for JH during *Drosophila* larval development (Riddiford, 1993). Alternatively, JH III may be metabolized under our organ culture conditions into less effective or inactive compounds. Another possibility is that a more significant transcriptional effect might be seen in response to JHB_3, which is the major form of JH produced by the *Drosophila* ring gland at larval wandering (Richard et al., 1989). Further studies are required to determine what role, if any, JH might play in regulating normal transcriptional responses during larval stages.

Extensive studies by Riddiford and co-workers have shown that JH modifies the 20E-regulation of a number of key transcription factor-encoding genes in *Manduca*: *EcR*, *USP*, *E74*, *E75*, and the *Broad-Complex* (for a review, see Riddiford et al., 2003). Similar regulatory interactions between JH and 20E have been seen in other insects, providing a molecular context for understanding the status quo action of JH (Berger and Dubrovsky, 2005). We find that a known *Drosophila* 20E primary-response gene, *E74B*, can be upregulated by JH (Fig. 4), a response opposite that seen in *Manduca* (Stilwell et al., 2003). Another well-characterized 20E primary-response gene, *E75A*, shows no response to JH in cultured larval organs, in contrast to its robust JH induction in S2 tissue culture cells (Dubrovsky et al., 2004). Our result is similar to the lack of an effect of JH or JH mimics on *E75A* transcription in *Manduca* GV1 cells or *Manduca* epidermis (Keshan et al., 2006). Given that *E75A* can be induced by JH in the ovaries of *apterous*^{4} mutants, it is possible that third instar larval organs are not competent to mediate this transcriptional response.

Several genes we identified in this study provide unique insights into the interactions between JH and 20E. We identified five genes that are unresponsive to JH or 20E alone, but specifically induced by these two hormones in combination: *CG7906*, *CG7924*, *nmdmc*, *black*, and *CG11956* (Figs. 2 and 3). To our knowledge, this is the first such description of genes that require both JH and 20E for their expression. Curiously, these same genes are induced by E but not 20E. The observation that *dro5* can be up-regulated by E, but shows no response to JH and 20E, suggests that these are distinct modes of regulation (Fig. 2). Further studies will be required to determine the basis for this unexpected correlation between transcriptional responses to E and responses to 20E and JH.

Our studies have also shown that 20E can block the JH induction of *E74B* and *pepck* (Fig. 4), providing evidence that 20E can modify the JH regulation of at least some genes. The same effect is seen for *pepck* in the presence of the protein synthesis inhibitor cycloheximide, suggesting that 20E can directly prevent JH induction, possibly through EcR. This observation is the opposite of the well-characterized effects of JH on 20E signaling, where JH modifies responses to 20E, and suggests that the interplay

between these two hormones is more complex than previously expected.

The identification of E-regulated genes, JH-regulated genes, and genes regulated by 20E in combination with JH provides a basis for future studies aimed at defining the crosstalk between hormone signaling pathways in insects. For example, studies of the effect of JH on *E74B*, *pepck*, and *CG14949* transcription in organs dissected from *usp* or *met* mutants could provide an opportunity to examine their possible role in mediating JH responses. Similarly, the identification of *CG14949* as a primary target for transcriptional induction by JH may facilitate the identification of JH response elements, providing new insights into the molecular mechanisms of JH action.

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