The Expression of the let-7 Small Regulatory RNA Is Controlled by Ecdysone during Metamorphosis in Drosophila melanogaster

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INTRODUCTION

The temporal coordination of cell proliferation, differentiation, and apoptosis during development is essential for the correct morphogenesis of an adult animal. In the fruit fly Drosophila melanogaster and the nematode Caenorhabditis elegans, genetic regulatory circuits control the timing of the transition from larval to adult stages. In Drosophila, larvae pupariate and initiate metamorphosis after the third larval instar. In C. elegans, the adult stage follows immediately after the fourth (final) larval molt. Studies on metamorphosis in Drosophila have revealed a pivotal role for the steroid hormone 20-hydroxyecdysone (ecdysone) in regulating the transition from larval to pupal to adult stages. To test whether let-7 expression is regulated by ecdysone in Drosophila, we used Northern blot analysis to examine the effect of altered ecdysone levels on let-7 expression in mutant animals, organ cultures, and S2 cultured cells. Experiments were conducted to test the role of Broad-Complex (BR-C), an essential component in the ecdysone pathway, in let-7 expression. We show that ecdysone and BR-C are required for let-7 expression, indicating that the ecdysone pathway regulates the temporal expression of let-7 in Drosophila. These results demonstrate an interaction between steroid hormone signaling and the heterochronic pathway in insects. Key Words: heterochronic genes; let-7; ecdysone; metamorphosis.

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Dynamic changes in ecdysone levels regulate progression through the larval stages of holometabolous insects (Riddiford, 1993). During the third (final) instar of Drosophila, a series of low-level ecdysone pulses signal the transition from feeding to wandering, in preparation for pupariation. Following a high-level ecdysone pulse, the white prepupa forms and larval tissues begin to either remodel or histolize. Meanwhile, precursors of adult structures and tissues,
which include the imaginal discs, histoblasts, and imaginal cell nests along the midgut initiate their proliferation and differentiation programs. Some 10–12 h after puparium formation, a second ecdysone pulse leads to head eversion and pupa formation. A broad and high-level peak of ecdysone secretion during the pupal stage triggers the terminal differentiation of the adult structures.

Ecdysone pulses trigger each developmental transition by initiating a program of downstream gene expression. Ecdysone binds a heterodimeric protein receptor, composed of an ecdysone receptor subunit (EcR) and an RXR-like subunit encoded by the ultraspiracle (usp) gene (Thomas et al., 1993; Yao et al., 1993). The ecdysone-receptor complex binds to a cis-acting regulatory element, known as the ecdysone response element (EcRE) in the enhancers of specific target genes, thereby causing an increase in target gene transcription. According to the Ashburner model (Ashburner et al., 1974), postlarval development begins by the hormone-dependent activation of a small set of “early” genes that include Broad-Complex (BR-C), E74, and E75. Each of these genes encodes a complex set of protein isoforms that function as sequence-specific DNA binding proteins and transcriptional regulators (Burtis et al., 1990; Segraves and and Hogness, 1990; Thummel et al., 1990, DiBello et al., 1991). The protein products of early genes activate a second cascade of gene expression, the “late” genes, and inhibit early gene expression by feedback. The outcome of this unfolding genetic cascade is manifest at the cell and tissue levels as biochemical and morphological differentiation.

In C. elegans, a newly hatched larva develops through four larval stages (L1 to L4), punctuated by molts, to a reproductive adult. Blast cells, set aside during embryogenesis, divide during the larval stages and give rise to stage-specific larval features, and the adult-specific reproductive structures (Sulston and Horvitz, 1977). The heterochronic genes lin-4 and let-7 are crucial for promoting the transitions from early to late developmental programs (Lee et al., 1993; Reinhart et al., 2000). lin-4 and let-7 are small regulatory RNAs (22 and 21 nucleotides, respectively), which act as translational repressors by base-pairing with the 3'-UTRs of their target gene mRNAs (Lee et al., 1993; Reinhart et al., 2000). The accumulation of lin-4 RNA at the beginning of the L2 stage downregulates the protein levels of its target genes, lin-14 and lin-28, and permits the coordinated transition from L1 to later programs (Ambros, 1989; Ruvkun and Giusto, 1989; Ambros and Moss, 1994; Ha et al., 1996; Moss et al., 1997; Olsen and Ambros, 1999). Similarly, the accumulation of let-7 RNA at the beginning of the L4 stage downregulates lin-41 (Slack et al., 2000) and possibly lin-57 (A. Rougvie, personal communication) and promotes the larval to adult (L/A) transition from L4 to adult programs (Reinhart et al., 2000).

The participation of two small regulatory RNAs in the heterochronic pathway raises the question of whether similar regulatory RNAs could be involved in the control of postembryonic development in other animals. Indeed, let-7 is conserved across the bilaterian clade, including flies and humans (Pasquinelli et al., 2000). In invertebrates, let-7 RNA expression coincides with the onset of the L/A transition. Similarly, let-7 expression is upregulated during vertebrate development, although at somewhat different stages depending on the species (Pasquinelli et al., 2000). Studies in human (Pasquinelli et al., 2000) and murine (L.F.S. and V.A., unpublished observations) cell lines demonstrate the presence of high levels of let-7 RNA in mature cell types (e.g., brain and lung) and marginal let-7 expression in immature or totipotent cell types (bone marrow and murine embryonic stem cell). Taken together, these observations suggest a general role for let-7 in the terminal differentiation of bilaterians (Pasquinelli et al., 2000).

In Drosophila, let-7 RNA first appears at the end of the third larval instar, a few hours before puparium formation, and reaches high levels during pupal development (Fig. 1; Pasquinelli et al., 2000; Hutvagner et al., 2001). Given the leading role of ecdysone in the temporal coordination of metamorphosis, we investigated whether the expression of let-7 in Drosophila is dependent on the ecdysone gene pathway. In this report, we demonstrate that ecdysone and the early ecdysone-inducible gene BR-C are required for let-7 expression in intact animals, organ cultures, and S2 cells. These results suggest that hormone-induced expression of let-7 could control developmental stage transitions in animals.
**MATERIALS AND METHODS**

**Drosophila: Strains, Staging, and in Vitro Incubation**

Drosophila stocks were maintained on a standard cornmeal-yeast-agar medium at 25°C. The npr and ecd1 (obtained from Bloomington Stock Center, #218) mutants are described elsewhere (Lindsley and Zimm, 1992). The mutation npr is a lethal non-complementing allele in the BR-C locus (Belyaeva et al., 1981). BR-C is represented by at least three genetic subfunctions: rbp, br, and i(1)2Bc, all of which are affected in the npr mutant. npr is maintained in cis to a yellow (y) mutation in a stock with the FM6-1 balancer chromosome and a duplication, Dp(1;Y)Y67g, which is a translocation of 1A to 2B17-18 to the Y chromosome. The homozygous npr animals were identified by their brownish mouthparts, resulting from the yellow mutation. They develop with a delay of 1 to 2 days and reach their normal size by 130-140 h, then live for several more days and die as third-instar larvae without any sign of pupariation (PF).

Animals were synchronized from egg laying. After hatching, first-instar larvae were hand-collected in groups of 50-70 animals and placed on a standard agar media supplied with the yeast paste. Larvae were allowed to develop at 20 or 25°C or shifted to a restrictive 29°C at an appropriate stage. Developmental landmarks, including hatching, larval molting, pupariation, and eclosion, were used for more precise staging (Zhimulev and Kolesnikov, 1975). For recovery of prepupal, pupal, and adult stages, animals were synchronized at PF and harvested at time intervals thereafter. The time points are approximate: an estimated ±2 h for prepupae, and ±6 h for pupae and adult flies. For nonpupariating ecd1 animals grown at the restrictive temperature (29°C), stage was determined with reference to PF in ecd1 animals grown in parallel at 20°C. In vitro organ culture was essentially performed as described (Andres and Thummel, 1994), except that Schneider’s medium was used.

**Cell Culture and Ecdysone Treatment**

Drosophila S2 cells were cultured in Schneider’s medium (Gibco) supplemented with 10% fetal bovine serum at 25°C. Ecdysone treatment was as follows: cells were plated in 25-cm² flasks containing 4 ml of medium and allowed to grow for 48 h when they reach 80% of confluence. 20-hydroxyecdysone (Sigma) was then added (1000 M) to a final concentration of 5 x 10⁻¹⁰ M. Control cells were treated with an equal volume of H₂O.

**RNA Interference**

A fragment of 700 bp corresponding to the core region of BR-C isoforms (DiBello et al., 1991; Bayer et al., 1996) was PCR amplified with T7/BR-C-sense and T3/BR-Cantisense primers from Z4 cDNA clone 2B1 (Bayer et al., 1996). A control fragment of 550 bp was PCR amplified with T7/mocksense and T3/mockantisense from C. elegans genomic DNA. Primers contained T3 or T7 promoter sites at their 5' ends. PCR products flanked by T3/T7 promoter sites were gel-extracted with Qiagen Gel Extraction kit (Qiagen) and used to synthesize sense (T7 RNA pol) and antisense RNA (T3 RNA pol) with T3/T7 MEGAscript kit (Ambion). Sense and antisense RNAs were annealed by heating at 95°C for 5 min, followed by 65°C for 30 min, and cooling down slowly to room temperature. The formation of dsRNA was confirmed by 1% agarose electrophoresis. dsRNAs were stored at –20°C. S2 cells were transfected with 40 µg of dsRNA essentially as described (Clemens et al., 2000).

**Northern Blot Analysis**

Total RNA was extracted from animals, tissues, or S2 cells by using the Trizol method (Gibco). Animals were homogenized in 2 mL of Trizol with a dounce glass homogenizer prior to extraction. Total RNA, 10 µg per lane, in formamide loading buffer (Ambion) was heated at 90°C for 3 min, and electrophoretically separated through a 12% denaturing urea-polyacrylamide gel (8 x 6 x 0.2 cm) at 125 V for 2 h in 1X TBE at 25°C. RNA was electrotransferred to a Zetaprobe membrane (Biorad) at 80 V for 1 h or 0.5 X TBE at 4°C. RNA was cross-linked to the membrane by UV irradiation (1200 mJ; Stratagene UV Stratalink), subsequently the membrane was baked at 80°C for 30 min. let-7 and U6 antisense StarFire (Integrated DNA Technologies) radioabeled probes were prepared by incorporation of [α-32P]ATP 6000 Ci/mmol as recommended by the vendor. For let-7 probe, membranes were hybridized for 24 h at 42°C in 7% SDS, 0.2 M NaPO₄, pH 7.2, and washed twice with 2X SSPE 0.1% SDS, and once with 1X SSPE 0.1% SDS, and 0.5X SSPE 0.1% SDS at 42°C. For U6 probe, membranes were hybridized overnight at 42°C in 7% SDS 0.05 M NaPO₄, pH 7.2, and washed twice with 1X SSPE 0.1% SDS, and once with 0.5X SSPE 0.1% SDS and 0.1X SSPE 0.1% SDS at 42°C. The radioactive signals of let-7S and U6 transcripts were quantified by using a PhosphorImager (Molecular Dynamics). The relative levels of let-7 transcript were represented as the ratio of let-7 and U6 radioactive signals normalized to a 0–1 scale.

**Dissection of Prepupae and Adult Flies**

Tanning prepupae 0–4 h after puparium formation and adult female flies were dissected in Ringer’s solution (Sullivan et al., 2000). Brains, fat bodies, imaginal discs, salivary glands, and Malphigian tubules were dissected from prepupae. Ovaries, carrying some developing eggs, and the remaining carcasses were dissected from female flies. Tissues were sorted and placed with a pipette into 100% ethanol. RNA was extracted as described above with the addition of 10 µg of glycogen as carrier.

**Oligonucleotides**

Sequences from 5’ to 3’: let-7 antisense StarFire (IDT), ACTATACAACCTACTAACCTCA; U 6 antisense StarFire (IDT), GCAGGGGCCATGCTAATCTTCTGTATTG; T7/BR-C-sense: GAATTAAGCTGAATACGGACACACAGAAGACAGCAGACG; T3/BR-Cantisense: GCCCTGGAATTACCTCTAAGGGAGCTGTTGACTCCAGCGGCCTG; T7/mocksense, GTAATACGACTCACTATAGGGCCGTGTCCTCAACTCTACTACC; T3/mockantisense: GCAATATTACCCCACT5AAAGGTGTTACCGGAATCGATAGC. (Note: The underlined sequences correspond to the T3/T7 promoter sites.)
RESULTS

The Expression of let-7 RNA Is Developmentally Regulated in Drosophila

The gene products of let-7 are two noncoding small RNAs of 21 (let-7S, short) and approximately 70 (let-7L, long) nucleotides. let-7S is the biologically active isoform (Renn-hart et al., 2000), which results from the processing of the precursor let-7L (Grishok et al., 2001; Huttner et al., 2001). Unless otherwise noted, we will use "let-7 RNA" in reference to let-7S. let-7 RNA is first detected in late third instar (L3) larvae (Pasquinelli et al., 2000) around the time when a pulse of the steroid hormone 20-hydroxyecdysone (ecdysone) triggers puparium formation (PF) and the onset of metamorphosis. To explore the potential influence of ecdysone on let-7 RNA expression, we determined the profile of let-7 expression during a period from late third larval instar, through prepupal development (a period marked by the sequential expression of ecdysone-inducible genes; Thummel, 1996; Richards, 1997), to the adult stage. let-7 RNA was first detected at low levels around 4 h before PF (Fig. 1). This stage coincides with a short period of high ecdysone titer that triggers PF (Richards, 1981; Riddiford, 1993). let-7 RNA remained at low levels during prepupal development, and then rapidly accumulated to high levels throughout pupal development reaching a maximum of expression during the second day of pupal life. This rise in let-7 RNA parallels a prolonged pulse of high-level ecdysone secretion (Richards, 1981; Riddiford, 1993). This correlation between the profile of let-7 expression and the time course of changes in ecdysone titer suggests that ecdysone could induce let-7 transcription.

Ecdysone Is Required for Expression of let-7 RNA

ecd1 is a temperature-sensitive mutation (Garen et al., 1977) that impairs the biosynthesis of ecdysone at restrictive temperature (29°C). To test whether reduced ecdysone at the end of the L3 stage affects the expression of let-7 RNA, ecd1 animals were transferred to 29°C at various times during the L3 stage to interfere with the generation of this ecdysone pulse. At the permissive temperature (20°C), ecd1 animals pupariate around 230 h after egg laying (AEL). This time of PF was used as a reference for defining the duration of the L3 stage of ecd1 animals. ecd1 animals were synchronized at egg laying and reared at 20°C until they were transferred to 29°C in the early and mid L3 stage. These developmental stages are approximate, because ecd1 animals grow more slowly and less synchronously than the wild type. The majority of ecd1 animals that were upshifted in the early L3 stage remained as larvae (98%, 197 out of 200), and these ecd1 retarded larvae were harvested at various times relative to PF of ecd1 animals maintained at 20°C. ecd1 animals upshifted in the mid-L3 stage produced a mixture of pupariating (40%) and nonpupariating (60%) individuals, and these were harvested separately. Wild-type animals reared at 20°C were upshifted as late L3 larvae for comparison. Using Northern analysis of total RNA, we found that let-7 RNA was marginally expressed in ecd1 animals (Fig. 2) that failed to pupariate due to the absence of ecdysone. In contrast, let-7 was expressed at much higher levels in pupariating ecd1 animals (Fig. 2). This correlation between PF and let-7 expression in ecd1 animals suggests that these two events are triggered by the same pulse of ecdysone. This could reflect that the induction of let-7 is mediated by the ecdysone signaling pathway. Alternatively, let-7 expression could be activated by another developmental signal associated with PF and/or progression through metamorphosis. This issue will be addressed in the next section.

In ecd1 pupae maintained at 29°C for more than 6 h after PF, levels of let-7 RNA were reduced compared with the wild type (compare lanes 7–8 to 10–11 in Fig. 2), suggesting that a prolonged pulse of high ecdysone titer throughout pupal development may be required to sustain let-7 expression. A similar reduction of let-7 RNA levels was also observed in ecd1 pupae transferred at different times after PF (data not shown).

Ecdysone Is Required for Expression of let-7 RNA in Organ Cultures of Third Instar Larvae

We inferred from the absence of let-7 RNA in nonpupariating ecd1 mutants that ecdysone is required for let-7 expression. To test this supposition, organ cultures from third instar larvae were incubated with ecdysone. Late L3 larvae were dissected to expose the internal organs to the medium (Andres and Thummel, 1994). After washes to remove ecdysone circulating in the hemolymph, organs were cultured in Schneider's medium with or without 5 μM ecdysone, for various lengths of time (0–20 h). Levels of let-7 RNA were examined by Northern analysis of total RNA recovered from these harvested organs. Let-7 was...
already expressed at relatively low levels at the time of dissection (0 h), presumably due to the rising titer of ecdysone (Richards, 1981). let-7 expression remained at this low level in organs incubated without ecdysone during the first 12 h (Fig. 3), and levels of let-7S and its precursor form (let-7L) decreased substantially at later times. In contrast, levels of let-7 RNAs increased after 12 h in organs incubated with ecdysone. This increased accumulation likely results from increased transcription since levels of let-7S and let-7L increased coordinately.

The Early Ecdysone-Inducible Gene Broad-Complex Is Required for Expression of let-7 RNA

The early gene, Broad-Complex (BR-C), is located at the top of the regulatory hierarchy in the ecdysone pathway and plays an essential role in regulating the expression of downstream targets (Belyaeva et al., 1981; Zhimulev et al., 1982). BR-C encodes four isoforms of a zinc finger transcription factor (Z1-Z4) (DiBello et al., 1991; Bayer et al., 1996) that not only control directly the expression of late genes, but that are also required for full expression of other early genes (Karim et al., 1993; von Kalm et al., 1994; Urness and Thummel, 1995; Crossgrove et al., 1996; Dubrovsky et al., 1996, 2001). To test whether BR-C is involved in mediating the response of let-7 expression to ecdysone pulses, we determined the levels of let-7 RNA in animals homozygous for a BR-C null mutation. Homozygous npr6 animals are deficient in all four BR-C isoforms (Z1–Z4), rendering them unresponsive to the ecdysone pulse at the end of the L3, and hence they fail to pupariate. npr6 animals remain as larvae for about 5–10 days after the normal time of pupariation and then die. npr6/npr6 and npr6/+ animals were synchronized and harvested at various times relative to the time of PF of the npr6/+ animals. let-7 RNA was detected at very low levels in npr6/npr6 animals, compared with npr6/+ siblings, indicating that BR-C is required in vivo to activate let-7 expression in response to ecdysone (Fig. 4). Interestingly, there was a slight increase of let-7 expression in npr6/+ animals 48 h after "PF," possibly corresponding to a rise of ecdysone titer. This suggests that other components of the ecdysone pathway could play a secondary role in let-7 expression, independent of BR-C.

Ecdysone Is Required for Initiation and Maintenance of let-7 RNA Expression in S2 Cells

The above in vivo experiments suggest that ecdysone signaling is required for let-7 expression, and that there is at least one intermediate player between ecdysone and let-7 expression, the BR-C transcription factor. Next we asked whether ecdysone directly activates let-7, that is, does ecdysone act on a tissue and triggers the expression of let-7 in that tissue, instead of initiating a signaling cascade resulting in the expression of let-7 in another tissue? To address this, we examined the ability of ecdysone to initiate let-7 expression in cultured S2 cells, which are known to be responsive to ecdysone (Cherbas and Cherbas, 1981, 1998; Vitek and Berger, 1984). S2 cells were incubated in the presence of ecdysone at a final concentration of 5 µM for 6–62 h, and harvested every 6 h. let-7 RNA was first detected at approximately 18 h after incubation with ecdysone, increased in level from 24 to 42 h, and reached a plateau at 42–54 h, and decreased thereafter (Fig. 5A). let-7 RNA was not detected in untreated S2 cells (Fig. 5; data not shown). This result indicates that ecdysone induces let-7 expression directly in cells to which it is applied. The long delay between the ecdysone primary action and let-7 activation in S2 cells is consistent with the participation of intermediate regulators.

To test whether ecdysone is required to sustain let-7 expression in S2 cells, we carried out a pulse-chase experiment. S2 cells were incubated for 24 h in the presence of 5 µM ecdysone, and then divided into two cultures. From one culture, the medium was removed and replaced by an ecdysone-free medium. The other culture was kept in 5 µM ecdysone for the remainder of the experiment. Northern blot analysis of total RNA showed a decrease in let-7 RNA levels in S2 cells after removal of ecdysone from the
medium, compared with the cells maintained with ecdyson (Fig. 5B). The levels of let-7S and let-7L coordinately decreased. These results indicate that ecdyson is required for both the initiation and maintenance of let-7 transcription in S2 cells.

**Broad-Complex Mediates the Ecdyson-Dependent Regulation of let-7 RNA Expression in S2 Cells**

We found that BR-C is involved in relaying the ecdyson signal to trigger let-7 expression in vivo. To determine whether a similar BR-C-dependent pathway mediates let-7 activation in S2 cells, we inhibited BR-C activity by RNA interference (RNAi) (Fire et al., 1998; Clemens et al., 2000). S2 cells were transfected with a dsRNA corresponding to a conserved sequence in all four BR-C transcript isoforms (DiBello et al., 1991, Bayer et al., 1996). Control cells were transfected with a nonspecific dsRNA (from an unrelated C. elegans sequence). After 30 min of incubation with dsRNA, cells were treated with 5 μM ecdyson. In parallel, nontransfected cells were also treated with 5 μM ecdyson. S2 cells were harvested for 32, 40, and 48 h after the addition of ecdyson, and total RNA was analyzed by Northern blotting. The profile of let-7 expression was very similar in nontransfected cells and in cells transfected with nonspecific dsRNA (Fig. 6). However, let-7 expression was dramatically reduced in cells transfected with dsRNA against BR-C (Fig. 6). This indicates that BR-C is required for ecdyson-dependent let-7 expression in S2 cells. Since levels of let-7S and let-7L RNA coordinately decreased when BR-C activity was reduced by RNAi, BR-C likely affects transcription of let-7 in response to ecdyson. The residual let-7 expression in BR-C RNAi cells could be due to the inefficient uptake of the dsRNA or the incomplete inhibition of BR-C activity. Alternatively, other components of the ecdyson pathway could contribute to let-7 expression, consistent with the residual let-7 expression observed in npr mutant animals (Fig. 4).
DISCUSSION

We have presented several lines of evidence that both ecdysone and the early ecdysone-inducible gene BR-C are required for the expression of let-7 RNA in Drosophila. This indicates that the ecdysone pathway regulates the temporal expression of let-7 in the fly. Previously identified ecdysone-inducible genes include a series of transcriptional factors that are organized in a hierarchical network to control metamorphic processes (Thummel, 1996; Richards, 1997). In C. elegans, let-7 is a translational repressor (Slack et al., 2000), and so Drosophila let-7 may mediate aspects of the hormonal control of metamorphosis by regulating gene expression post-transcriptionally.

Our primary evidence that let-7 expression is triggered by the ecdysone pathway comes from two lines of experiments. First, mutant animals defective in ecdysone biosynthesis (Fig. 2) or in BR-C activity (Fig. 4) displayed reduced or absent let-7 RNA levels. Second, sustained expression of let-7 RNA in organ culture required the application of exogenous ecdysone (Fig. 3). These experiments did not rule out the possibility that let-7 expression could be a collateral consequence of ecdysone signals, for example, as a consequence of pupariation or progression through metamorphosis. However, if this were the case, then we would not expect ecdysone-induced let-7 expression in S2 cells, since S2 cells do not undergo morphogenesis. The finding that ecdysone induced let-7 expression in S2 cells (Fig. 5) strongly suggests that ecdysone pathway triggers let-7 expression within the cells exposed to the hormone, and independently of overt metamorphosis. The requirement of BR-C activity in animals (Fig. 4) and S2 cells (Fig. 6) for let-7 RNA induction indicates that BR-C is an intermediate player between the ecdysone signal and activation of let-7, and that the let-7 response to ecdysone exhibited in S2 cells likely reflects the same process as in vivo. One difference between the response to ecdysone in S2 cells and animals is that, in S2 cells, let-7 expression begins about 24 h after the addition of ecdysone, while in animals, let-7 expression begins about 4 h after the pulse of ecdysone at the end of the third larval stage. Components of the pathway mediating let-7 activation by ecdysone in S2 cells may be relatively limiting compared to intact animals, and perhaps the concentration of ecdysone applied to S2 cells may not be as effective as that in vivo to activate let-7 expression. Although we cannot rule out a role for RNA stability or processing in the induction of let-7 by ecdysone, the simplest interpretation is that let-7 induction occurs at the transcriptional level, since levels of let-7 RNA and its precursor let-7L (Grishok et al., 2001; Hutvagner et al., 2001) increased or decreased coordinately, depending on the status of ecdysone signaling.

The timing of let-7 expression is conserved in invertebrates, in that let-7 RNA accumulates toward the end of larval development in flies, worms, and mollusks, coinciding with the specification of adult programs (Pasquinelli et al., 2000). Although our results indicate that in diptera...
let-7 is induced by ecdysone, let-7 expression could be coupled to other signals in other animals. In C. elegans, the timing of let-7 expression is controlled partly by upstream components of the heterochronic pathway in conjunction with other regulatory signals (L.F.S. and V.A., unpublished observations). daf-12, an orphan nuclear hormone receptor (NHR) (Antebi et al., 2000), is an upstream component of the heterochronic pathway implicated in the regulation of let-7 expression. DHR96, the closest ortholog of daf-12 in Drosophila, also encodes an orphan NHR (Fisk and Thummel, 1995). DHR96 is one of the eight NHRs whose expression is regulated by ecdysone during metamorphosis (Thummel, 1995). daf-12 and dhr96 could represent an evolutionary conserved point of convergence in C. elegans and Drosophila let-7 regulatory pathways. Whether C. elegans utilizes other components of the ecdysone pathway during its development remains an open question. More than 200 NHRs have been predicted in C. elegans (Sluder and Maina, 2001), and some of these are clear orthologs of Drosophila NHRs involved in metamorphosis, suggesting that they could play similar roles in C. elegans.

The increase in let-7 RNA at pupariation in response to ecdysone/BR-C activity has suggested a potential role for let-7 in Drosophila metamorphosis. In C. elegans, let-7 promotes the larval to adult transition by downregulating lin-41 protein levels. There are several complementary sites to let-7 in the 3′-UTR of lin-41 mRNA to which let-7 could bind to repress translation (Slack et al., 2000). lin-41 codes for a RBCC protein (Slack et al., 2000) and is a founding member of the NHL domain family (Slack and Ruvkun, 1998). In Drosophila, there are three orthologs of lin-41: dappled, brat, and mei-P26. These lin-41 orthologs appear to be involved somehow in growth suppression in flies; mutations in dappled and brat result in melanotic tumors (Rodriguez et al., 1996; Arama et al., 2000; Sonoda and Wharton, 2001), and mutations in mei-P26 result in ovarian tumors (Page et al., 2000), dappled and brat are expressed at the end of the third instar in fat bodies and ring gland (brain), and brain and wing imaginal discs, respectively (Rodriguez et al., 1996; Arama et al., 2000; Sonoda and Wharton, 2001), mei-P26 is expressed in the germ line (Page et al., 2000). These expression patterns overlap with that of let-7 RNA (Fig. 7), suggesting that the lin-41 orthologs could be let-7 targets. Indeed, sequences in the 3′-UTR of dappled, brat, and mei-P26 resemble let-7 complementary sites to which let-7 could bind. Future work is required to determine whether let-7 is a regulator of these and/or other genes and to assess the implications of this regulation in metamorphic processes, such as apoptosis, differentiation, and morphogenesis.

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