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Coordinate regulation of small temporal RNAs at the onset of *Drosophila* metamorphosis

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Abstract

The *lin-4* and *let-7* small temporal RNAs play a central role in controlling the timing of *Caenorhabditis elegans* cell fate decisions. *let-7* has been conserved through evolution, and its expression correlates with adult development in bilateral animals, including *Drosophila* [Nature 408 (2000), 86]. The best match for *lin-4* in *Drosophila, miR-125,* is also expressed during pupal and adult stages of *Drosophila* development [Curr. Biol. 12 (2002), 735]. Here, we ask whether the steroid hormone ecdysone induces *let-7* or *miR-125* expression at the onset of metamorphosis, attempting to link a known temporal regulator in *Drosophila* with the heterochronic pathway defined in *C. elegans.* We find that *let-7* and *miR-125* are coordinately expressed in late larvae and prepupae, in synchrony with the high titer ecdysone pulses that initiate metamorphosis. Unexpectedly, however, their expression is neither dependent on the EcR ecdysone receptor nor inducible by ecdysone in cultured larval organs. Although *let-7* and *miR-125* can be induced by ecdysone in Kc tissue culture cells, their expression is significantly delayed relative to that seen in the animal. *let-7* and *miR-125* are encoded adjacent to one another in the genome, and their induction correlates with the transient appearance of an ~500-nt RNA transcribed from this region, providing a mechanism to explain their precise coordinate regulation. We conclude that a common precursor RNA containing both *let-7* and *miR-125* is induced independently of ecdysone in *Drosophila,* raising the possibility of a temporal signal that is distinct from the well-characterized ecdysone–EcR pathway.
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Introduction

Development is organized into discrete temporal stages, each characterized by unique programs of gene expression and differentiation. Genetic studies in *Caenorhabditis elegans* have provided insights into the mechanisms by which this temporal specificity is conferred, revealing a network of heterochronic genes that determine the timing of stage-specific cell fate decisions (Ambros, 2000; Rougvie, 2001). Mutations in heterochronic genes cause temporal transformations in the patterns of cell division such that stage-specific programs are either deleted or reiterated. For example, loss-of-function mutations in the *let-7* heterochronic gene cause larval cell fates to be repeated during adult stages. Conversely, overexpression of *let-7* causes larval cell fates to be deleted and replaced by precocious expression of adult fates during larval stages, thus defining this gene as a key determinant of the larval-to-adult transition (Reinhart et al., 2000). *let-7* encodes an ~70-nucleotide (nt) precursor RNA that is rapidly processed by DCR-1 into a noncoding 22-nt RNA (Reinhart et al., 2000; Grishok et al., 2001; Hutvagner et al., 2001; Ketting et al., 2001). This

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RNA is first detected in the last larval stages and is expressed throughout adulthood, consistent with its role in specifying adult cell fates. *let-7* exerts its regulatory functions by binding to complementary sequences in the 3′-untranslated region (UTR) of another heterochronic gene, *lin-41,* and inhibiting LIN-41 protein expression (Reinhart et al., 2000; Slack et al., 2000). Thus, the timing of *let-7* induction determines when LIN-41 is downregulated to induce the appropriate larval-to-adult cell fate decisions. Genetic studies have indicated that improper regulation of *lin-41* can account for most of the lethal and heterochronic phenotypes observed in *let-7* mutants (Slack et al., 2000). Similar studies of *lin-4* have shown that it also encodes a small temporal RNA and that it plays a central role in regulating early larval cell fates by repressing the translation of *lin-14* and *lin-28* (Lee et al., 1993; Olsen and Ambros, 1999; Seggerson et al., 2002).

Remarkably, *let-7* is not restricted to *C. elegans,* but rather has been highly conserved through evolution in a wide range of animals, including annelid, mollusc, and vertebrate species, as well as the fruit fly *Drosophila melanogaster* (Pasquinelli et al., 2000). More intriguingly, *let-7* expression correlates with the onset of adult differentiation in all bilateral animals tested, raising the possibility that its role as a key determinant of adult differentiation may be conserved through evolution. In addition, homologs of *lin-41* in other species contain conserved *let-7* complementary sequences in their 3′UTRs, suggesting that they may share a similar mechanism of regulation by *let-7* (Pasquinelli et al., 2000). In *Drosophila,* the ~70-nt precursor and 21- to 22-nt mature forms of *let-7* RNA are first detected at the end of larval development, and the mature form remains at high levels through pupal and adult stages (Pasquinelli et al., 2000; Hutvagner et al., 2001), a response that has been attributed to induction by the steroid hormone ecdysone (Sempere et al., 2002). A 22-nt RNA that is similar to *C. elegans lin-4, miR-125,* has also been identified in *Drosophila* and is specifically expressed during pupal and adult stages, raising the possibility that it might be coordinately regulated with *let-7* (Lagos-Quintana et al., 2002).

Ecdysone pulses define temporal boundaries in the *Drosophila* life cycle, triggering the major postembryonic developmental transitions, including molting and metamorphosis (Riddiford, 1993). Ecdysone exerts its effects by interacting with a heterodimer of two members of the nuclear receptor superfamily, EcR and the RXR ortholog, USP (Riddiford et al., 2000). This hormone–receptor complex directly induces the expression of primary-response target genes, at least some of which encode transcription factors that transduce and amplify the hormonal signal by coordinating the expression of large batteries of downstream secondary-response late genes (Ashburner et al., 1974; Thummel, 1996; Riddiford et al., 2000). These regulatory cascades specify the appropriate stage- and tissue-specific biological responses to each pulse of ecdysone during development. A high titer pulse of ecdysone in late third instar larvae triggers puparium formation and the onset of metamorphosis, initiating the prepupal stage in development. Prepupal development is terminated by another pulse of ecdysone, approximately 10 h after puparium formation, which triggers adult head eversion, leg and wing elongation, and the prepupal–pupal transition.

Here, we ask whether *let-7* and *miR-125* are coordinately regulated by a known temporal signal in *Drosophila,* the steroid hormone ecdysone. We find that these small temporal RNAs are expressed from a common precursor transcript that is transiently induced at the onset of metamorphosis. Unexpectedly, however, we find that *let-7* and *miR-125* induction is independent of ecdysone signalling in vivo, raising the possibility that their expression is triggered by a distinct temporal regulator.

Materials and methods

Developmental staging and induction of EcR-RNAi

Flies were maintained on cornmeal agar at 25°C. Third instar larvae were staged by using food containing 0.5% bromophenol blue, as described (Andres and Thummel, 1994). Prepupae and pupae were synchronized at puparium formation and aged at 25°C for the appropriate time. Canton S flies were used as wild type. A previously reported transgenic line (w^{1118}; *hs-EcRi-11*) was used to express dsRNA corresponding to the common region of all EcR isoforms (Lam and Thummel, 2000). For heat induction of EcR-RNAi, "dark blue gut" stage wandering third instar larvae (~18 h before puparium formation) from either transgenic or control (w^{1118}) stocks were transferred to 1.5-ml microcentrifuge tubes with perforated caps and incubated in a 37°C water bath for 30 min. Heat-treated larvae were allowed to recover at 25°C until the appropriate stage, after which total RNA was isolated and analyzed by Northern blot hybridization, as described (Karim and Thummel, 1991).

Organ cultures

Organs were dissected from late third instar larvae staged on blue food (~6 h before puparium formation) (Andres and Thummel, 1994), a time when *let-7* is not expressed. These organs were cultured with oxygenated Schneider's *Drosophila* medium (Gibco/Invitrogen) in multiwell Corning dishes, as described (Karim and Thummel, 1991). After a 1-h preincubation, fresh medium was added with either 5 μM 20-hydroxyecdysone (the physiologically active form of ecdysone) (Sigma) or 5 μM 20-hydroxyecdysone and 85 μM cycloheximide (Sigma). Organs were cultured inside an oxygen chamber at 25°C for the appropriate time, after which RNA was isolated and analyzed by Northern blot hybridization, as described (Karim and Thummel, 1991).

20-Hydroxyecdysone induction in cultured cells

Drosophila Kc_{167} cells (kindly provided by L. Cherbas) were maintained in Schneider's medium (Gibco/Invitrogen), 10% heat-inactivated fetal bovine serum (JRH Biosciences), and penicillin–streptomycin (Gibco/Invitrogen) at 24°C. A total of 2.5×10^6 Kc_{167} cells/2.5 ml medium seeded in six-well plates and incubated overnight were replaced with fresh medium containing a final concentration of 1 μM 20-hydroxyecdysone (Sigma). Control wells received fresh medium without hormone. Cells were incubated at 24°C for various times before RNA extraction of pooled duplicate samples with TriZol (Gibco/Invitrogen).

Northern blot analysis

Total RNA samples were divided into two parts. One was fractionated by formaldehyde agarose gel electrophoresis to detect high molecular weight RNAs, and the other was fractionated by polyacrylamide gel electrophoresis to detect low molecular weight RNAs. RNAs fractionated by formaldehyde agarose gel electrophoresis were transferred to nylon membranes, UV crosslinked, and hybridized with DNA probes, as described (Andres et al., 1993). Probes for the *E74* common region and *rp49* mRNA were made by random priming the appropriate gel-purified DNA fragments (Karim and Thummel, 1991). A 484-bp probe for the *let-7/miR-125* cluster was generated by random-priming of a PCR fragment amplified from genomic DNA using primers flanking the predicted cluster sequence shown in Fig. 5A: forward primer: 5′-TTTCTTCTTGTTTGCCATCATCGTTTC-3′; reverse primer: 5′-AATACCGGAGATCAAAACTTGTGAAAC-3′. *rp49* probe was included in the hybridizations of agarose fractionated RNA samples as an internal control for loading (data not shown). Northern analyses of RNAs separated by polyacrylamide gel electrophoresis were performed as described (Lee et al., 1993), except hybridization and wash steps were performed at 50°C. Oligonucleotides used as Northern probes were *let-7* 5′-AACTATACAACCTACTACCTCACCGGATCC-3′ and *miR-125* 5′-TTAAAAGTCACAAGTTAGGGTCTCAGGGAATCA. 55 rRNA was detected by ethidium bromide staining of polyacrylamide gels prior to transfer.

Results

let-7 and miR-125 are induced in parallel with the E74A early mRNA during the onset of metamorphosis

In order to determine the temporal profile of *let-7* and *miR-125* expression during the early stages of metamorphosis, total RNA was extracted from staged late third instar larvae, prepupae, and early pupae and analyzed by Northern blot hybridization (Fig. 1). As a control, we used a probe to detect *E74A* and *E74B,* the two mRNA isoforms encoded by the *E74* early ecdysone-inducible gene (Burtis et al., 1990). The expression pattern observed corresponds closely to that described earlier, with a burst of *E74B* transcription preceding each burst of *E74A* expression, and with the timing of *E74A* mRNA accumulation reflecting the peaks in ecdysone titer (Karim and Thummel, 1991). Induction of the ~70-nt *let-7* precursor RNA and the final processed 21-and 22-nt RNAs correlates precisely with puparium formation (Fig. 1). Both the precursor and mature forms of *let-7* are first detected at low levels a few hours before puparium formation (−4 h; Fig. 1) and accumulate to higher levels for several hours after puparium formation (0–4 h; Fig. 1). The precursor RNA is then reduced to lower levels in midprepupae, induced again in 10-h prepupae, and remains high through early pupal stages (Fig. 1).

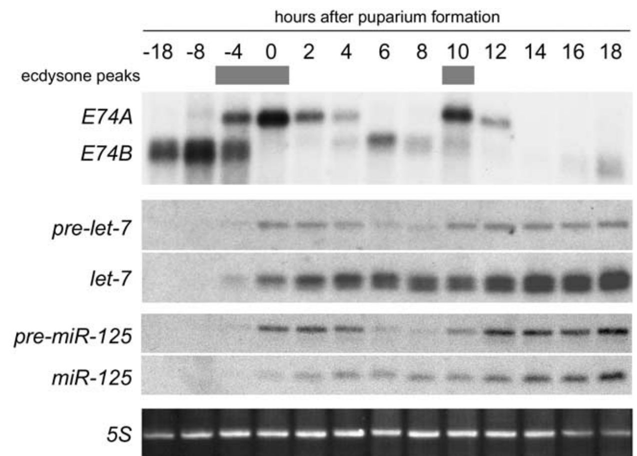


Fig. 1. The temporal profile of *let-7* and *miR-125* expression correlates with ecdysone-triggered responses during the onset of metamorphosis. Total RNA was extracted from staged third instar larvae, prepupae, and pupae, and analyzed by Northern blot hybridization using either formaldehyde agarose gel electrophoresis (for *E74*) or polyacrylamide gel electrophoresis (for *let-7* and *miR-125*). A probe for the common region of *E74* was used to detect both *E74A* and *E74B* mRNAs. Ethidium bromide staining to detect *5S* RNA on the polyacrylamide gels was used as a control for loading. The boxes indicate peaks in ecdysone titer.

Two other miRNAs in *C. elegans, miR-48* and *miR-84,* are similar to *let-7* in sequence and, together with *miR-69,* share similar temporal expression patterns (Lau et al., 2001; D. Bartel personal communication). Potential *Drosophila* orthologs of *miR-48* and *miR-84* were predicted from genome sequence comparisons and RNA secondary structures (Lau et al., 2001), but could not be detected on Northern blots of RNA samples from late third instar larvae through pupae, indicating that they are either not expressed at these stages of development or not detectable under the conditions used (data not shown). Similarly, *miR-69* does not have a homolog in *C. briggsae,* and a related sequence cannot be identified in the *Drosophila* genome. In contrast, *Drosophila miR-125,* which lacks sequence conservation with *let-7* but which is similar to *lin-4,* exhibited an expression pattern virtually identical to that of *let-7* RNA in *Drosophila* (Lagos-Quintana et al., 2002) (Fig. 1). The

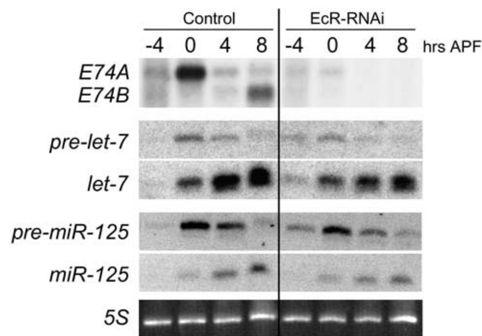


Fig. 2. *EcR*-mediated signaling is not necessary for the onset of *let-7* or *miR-125* expression. Mid-third instar larvae from either control (*w*) or *hs-EcR-RNAi (w; P[hs-EcRi])* stocks, staged based on gut color (−18 h), were subjected to a 30-min heat-treatment and aged to −4, 0, 4, and 8 h relative to puparium formation. Total RNA was extracted from these animals, divided in two, and analyzed by Northern blot hybridization to detect either *E74* mRNA or *let-7* and *miR-125* RNAs. Ethidium bromide staining to detect *5S* RNA on the polyacrylamide gels was used as a control for loading. The increased amount of *let-7* and *miR-125* RNA in the −4-h EcR-RNAi lane relative to the same time point in the control is most likely due to the inherent inaccuracy in staging late third instar larvae (Andres and Thummel, 1994).

observation that precursor forms of both *let-7* and *miR-125* are induced coordinately with *E74A* mRNA, a known ecdysone primary-response gene, suggests that these small temporal RNAs might be induced directly by the ecdysone–receptor complex, as proposed earlier for *let-7* regulation (Sempere et al., 2002).

EcR-mediated signaling is not necessary for let-7 or miR-125 induction

Because null mutations in the EcR ecdysone receptor lead to death during embryogenesis (Bender et al., 1997), we used heat-inducible expression of an inverted repeat derived from the *EcR* common region to inactivate all EcR isoforms by RNAi at the onset of metamorphosis (Lam and Thummel, 2000). Control and *hs-EcRi* late third instar larvae were collected at ~18 h before puparium formation and subjected to a single heat treatment at 37°C for 30 min, after which animals were allowed to recover at 25°C, and RNA samples were collected from time points surrounding puparium formation. These RNA samples were analyzed by Northern blot hybridization in order to determine whether *EcR* activity is required for *let-7* or *miR-125* induction at puparium formation (Fig. 2).

Under the conditions used, all animals arrested development at the larval–prepupal transition, as stationary untanned elongated prepupae with everted anterior spiracles (see Fig. 3C in Lam and Thummel, 2000), indicative of a strong block in *EcR* function. EcR protein levels are known to be significantly reduced under these conditions (Fig. 2B in Lam and Thummel, 2000). Consistent with this external phenotype, ecdysone-induced *E74A* and *E74B* expression is almost entirely abolished in these animals (Fig. 2). In contrast, the timing of *let-7* and *miR-125* induction is unaffected by this reduction in *EcR* function, and the levels of both precursor and stable RNAs are only slightly reduced. We conclude that *let-7* and *miR-125* are induced independently of the ecdysone–EcR signaling pathway.

Ecdysone is not sufficient to induce let-7 or miR-125 transcription in organ culture

Organs were dissected from staged late third instar larvae and cultured for various times in an oxygen chamber by using media containing either 20-hydroxyecdysone (20E; the physiologically active form of ecdysone) or 20E and cycloheximide. Both *E74* and *let-7* are widely expressed in all tissues examined, including organs that were present in these mixed cultures (Thummel et al., 1990; Sempere et al., 2002). Addition of the protein synthesis inhibitor cycloheximide in the presence of ecdysone stabilizes direct targets of ecdysone signaling (early primary-response gene expression) and blocks induction of indirect targets (late secondary-response gene expression). Total RNA was isolated from these cultures at 0, 2, 4, and 8 h after hormone addition, and the levels of *E74*, *let-7,* and *miR-125* RNAs were determined by Northern blot hybridization (Fig. 3). This time course spans the periods of maximal early (4 h) and late (6–8 h) responses to ecdysone (Ashburner, 1972). As expected, *E74A* is induced by the earliest time point examined, 2 h after 20E addition, peaks at 4 h, and begins to be downregulated by 8 h (Thummel et al., 1990). This repression fails to occur in the presence of 20E and cycloheximide, leading to superinduction of *E74A* mRNA. In contrast, no significant *let-7* or *miR-125* RNA is detectable under these conditions, indicating that 20E is not sufficient to induce their transcription.

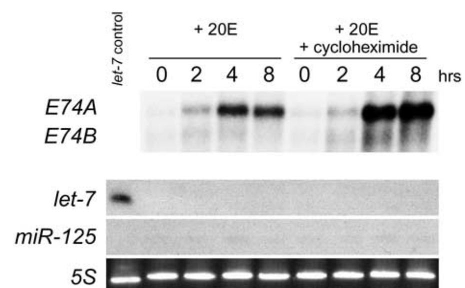


Fig. 3. Ecdysone is not sufficient to induce *let-7* or *miR-125* expression in organ culture. Larval organs were dissected from late third instar larvae and cultured in media containing either 5 μM 20-hydroxyecdysone (+20E) or 5 μM 20-hydroxyecdysone and 85 μM cycloheximide (+20E +cycloheximide). Total RNA was extracted from these organs, divided in two, and analyzed by Northern blot hybridization to detect either *E74* mRNA or *let-7* and *miR-125* RNAs. An RNA sample from mixed stage *C. elegans* was used as control for detection of *let-7* RNA (*let-7* control). Ethidium bromide staining to detect *5S* RNA on the polyacrylamide gels was used as a control for loading. *E74B* mRNA peaks between 0 and 2 h after 20E addition, and thus only low levels are detectable at later times for this transcript (Karim and Thummel, 1991).

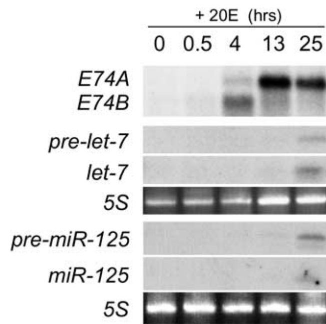


Fig. 4. *let-7* and *miR-125* RNAs are induced in Kc cells as a delayed response to 20E. Kc cells were cultured for different times in the presence of 1 μM 20E. Total RNA was extracted from individual cultures, divided in two, and analyzed by Northern blot hybridization to detect either *E74* mRNA or *let-7* and *miR-125* RNAs. Ethidium bromide staining to detect *5S* RNA on the polyacrylamide gels was used as a control for loading.

let-7 and miR-125 regulation in Kc cells is distinct from that seen in vivo

Several *Drosophila* tissue culture cell lines, derived from dissociated embryos, display well-characterized molecular and morphological responses to 20E (Cherbas et al., 1980). Upon addition of 20E, Kc cells rapidly and directly induce primary-response gene expression. The cells initiate morphological changes ~12 h later, extending bipolar microtubule-rich extensions to form spindle-shaped cells (Cherbas et al., 1980, 1991). Over the course of the next few days, the cells arrest in the G_2 phase of the cell cycle, aggregate, and finally die. We examined the temporal profile of *let-7* and *miR-125* expression in 20E-treated Kc cells in an effort to extend our observations in the animal.

E74B expression predominates at 4 h after hormone addition and is replaced by the *E74A* isoform which continues to be expressed throughout the duration of the experiment, reflecting the normal ecdysone-induced isoform switch seen in vivo (Fig. 4). In contrast, both the precursor and processed forms of *let-7* and *miR-125* RNAs are at the borderline of detection by 13 h after hormone addition and are not readily detectable until 25 h after hormone addition, after which they continue to accumulate to high levels (Fig. 4, and data not shown). Similar results have been reported for *let-7* expression in S2 tissue culture cells (Sempere et al., 2002). This significant delay is in sharp contrast to the precise temporal coordination of *E74A, let-7,* and *miR-125* induction seen in the animal (Fig. 1), suggesting that these miRNAs are induced by a distinct regulatory pathway in Kc tissue culture cells.

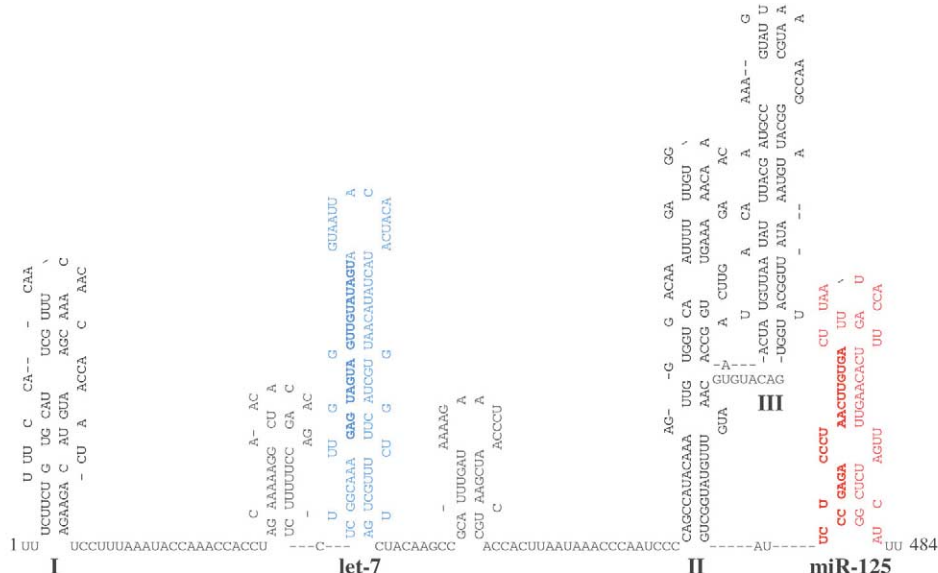
let-7 and miR-125 reside within a miRNA gene cluster and appear to be expressed from a common precursor RNA

Mapping of the *miR-125* sequence to the *Drosophila* genome revealed that it is encoded adjacent to *let-7,* with their precursor sequences located ~300 bp from one another at position 36F in the polytene chromosomes (Fig. 5A). This close physical proximity combined with the precise temporal coordination of *let-7* and *miR-125* expression raises the interesting possibility that they might be expressed from a common precursor RNA. To test this possibility, a fragment encompassing this region was used as a probe for Northern blot hybridization using RNA samples from staged *Drosophila* as well as RNA samples from Kc cells treated with 20E. An ~500-nt RNA can be detected in synchrony with the appearance of *let-7* and *miR-125* RNA in late third instar larvae, early pupae, and Kc cells treated with 20E for 25 h (Fig. 5B). This expression, however, is transient, as the ~500-nt RNA is not detected at later stages during *Drosophila* development. The size and temporal expression pattern of this RNA is consistent with the proposal that it acts as an initial common precursor for the synthesis of both *let-7* and *miR-125.* Moreover, the transient expression of this precursor indicates that the *let-7/miR-125* gene cluster is only expressed during early *Drosophila* metamorphosis, while the more stable 21- to 22-nt products persist through adult stages.

Fig. 5A depicts the predicted folding structure (*mfold* 3.0 by Zuker and Turner) of a 484-nt region encompassing both *let-7* and *miR-125.* Three other stem-loop structures are possible within this region; however, it remains unclear whether these sequences are processed into stable miRNAs.

Discussion

The timing of *let-7* induction plays a central role in the *C. elegans* heterochronic pathway, controlling the switch from larval to adult cell fates (Reinhart et al., 2000). This RNA is not restricted to nematodes, but rather is widely conserved through animal evolution (Pasquinelli et al., 2000). In addition, *let-7* is expressed in an adult-specific manner in all bilateral animals tested, suggesting that it may play a conserved role in controlling late temporal transformations across animal phylogeny. In this paper, we attempt to link a known temporal regulator of *Drosophila* adult differentiation, the steroid hormone ecdysone, with the expression of *let-7* and *miR-125.* We show that *let-7* and *miR-125* are induced in late third instar larvae and prepupae in a temporal pattern that mirrors that of a known ecdysone primary-response mRNA, *E74A* (Fig. 1). In spite of this tight temporal correlation, however, our data argue against a role for ecdysone signaling in controlling the timing of *let-7* and *miR-125* induction in *Drosophila.* We see little effect on their induction when *EcR* function is blocked by RNAi (Fig. 2), and we see no induction of *let-7* or *miR-125* by the physiologically active form of the hormone, 20E, in cultured larval organs under conditions where *E74A* is abundantly expressed (Fig. 3). *let-7* and *miR-125* induction by 20E in Kc cells is delayed by at least 10 h relative to that of the primary-response *E74A* mRNA (Fig. 4 and 5). This



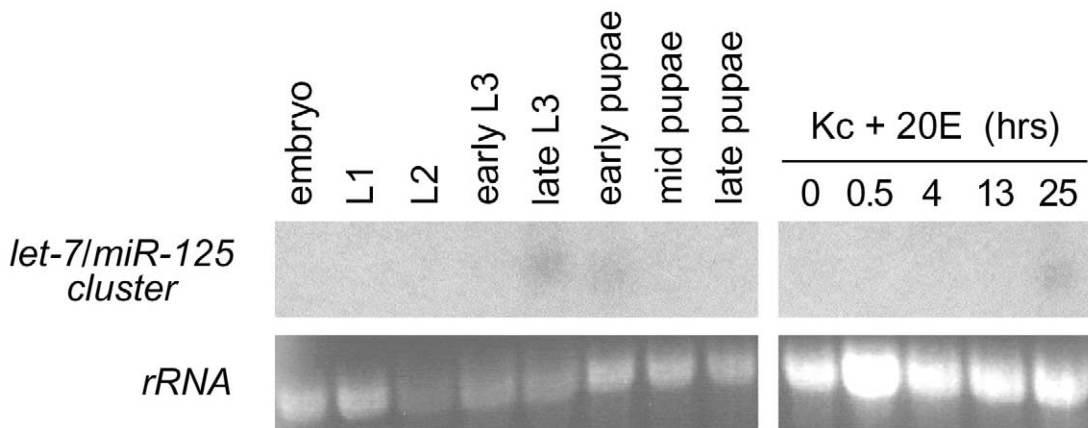


Fig. 5. The *let-7/miR-125* gene cluster can be expressed from a common precursor RNA. (A) Predicted folding structure of a 484-nt region encompassing the *let-7* and *miR-125* coding regions. I, II, and III indicate other possible stem-loop structures within this sequence. (B) Equal amounts of total RNA from staged *Drosophila* embryos, larvae, and pupae were fractionated by formaldehyde agarose gel electrophoresis and analyzed by Northern blot hybridization using a probe derived from sequences encompassing the region shown in (A) (see Materials and methods). These samples of RNA have been used previously to detect *let-7* expression (Pasquinelli et al., 2000; Hutvagner et al., 2001). Samples of RNA from Kc cells treated with 20E, described in Fig. 4, were also included. Ethidium bromide staining to detect *5S* RNA on the polyacrylamide gels was used as a control for loading. RNA levels are relatively low for the sample isolated from second instar larvae (L2).

delay suggests that these microRNAs are expressed as either a secondary-response to the hormone or as an indirect consequence of the 20E-induced differentiation program in these cells. Regardless, this pattern of expression is distinct from the precise coordinate expression of *E74A, let-7,* and *miR-125* seen in vivo (Fig. 1), indicating that a different mechanism is responsible for miRNA induction in the animal.

Sempere et al. (2002) recently published a study of *let-7* regulation in *Drosophila,* but arrived at a different conclusion from ours. Much of their data, however, is consistent with the results presented in this study. Sempere et al. present a time course of *let-7* induction by 20E in S2 tissue culture cells that is very similar to that reported here in Kc cells, although they do not use a primary-response gene as a temporal marker for direct induction by the ecdysone-receptor complex. As they point out, their results with the *ecd1* ecdysone-deficient mutant and *npr6* mutant are difficult to interpret because the lack of *let-7* expression in these mutants could simply be attributed to their developmental arrest as third instar larvae and inability to initiate puparium formation. The most significant contradiction with our data

is their results with cultured larval organs, which show increased levels of *let-7* RNA in the presence of 20E (Fig. 3 in Sempere et al., 2002). Their experiment is, however, difficult to interpret. The 0-h time point has *let-7* RNA present, as do the 8- and 12-h time points in the absence of 20E, indicating that the organs used for this study were taken at a time when *let-7* is already expressed. It is possible that 20E could be stabilizing or maintaining *let-7* expression in these organs. The authors provide no marker for a known primary-response to ecdysone, so the timing of their induction cannot be interpreted. The authors also take their earliest collection (8 h) at the time when primary-response genes are starting to be repressed, several hours after their induction (Ashburner, 1972; Karim and Thummel, 1991).

Some miRNAs map within close proximity to one another, forming apparent gene clusters in *C. elegans, Drosophila,* and humans (Lagos-Quintana et al., 2001; Lau et al., 2001; Mourelatos et al., 2002). We find a similar arrangement for *let-7* and *miR-125* in *Drosophila,* which map within ~300 bp of one another on the left arm of the second chromosome. In addition, we detect a precursor RNA that could encode both miRNAs, providing a means of explaining their tight temporal coordination. This observation is consistent with the recent identification in HeLa cells of relatively long primary transcripts (pri-miRNAs) that contain multiple ~70-nt stem-loop microRNA precursors, and indicates that similar precursors can be expressed in a developing organism (Lee et al., 2002). Although expression of some miRNAs may be regulated by multiple processing steps, the low abundance and transient appearance of the *let-7/miR-125* pri-miRNA and pre-miRNA forms indicate that transcription of the primary transcript could be largely responsible for the precise temporal appearance of the mature miRNAs.

Interestingly, *miR-125* provides the best match in the *Drosophila* genome to a second small heterochronic RNA in *C. elegans, lin-4* (Lagos-Quintana et al., 2002). The two mismatches between *lin-4* and *miR-125,* however, are sufficient to render *miR-125* undetectable on Northern blots of *Drosophila* RNA using *lin-4* sequences as a probe (data not shown). Moreover, it is likely that these sequence differences have an impact on the specificity of *miR-125* interactions with possible target sequences in 3′UTRs. The functional significance of this sequence similarity thus remains to be determined.

Our studies leave us with the question of what induces *let-7* and *miR-125* expression at the onset of *Drosophila* metamorphosis. The timing of the upregulation of these miRNAs in staged animals argues that it is responding to a temporal signal that occurs in parallel with the well-characterized 20E/EcR/USP signaling pathway (Fig. 1). Because of this tight correlation, and the widespread expression of *let-7* RNA in *Drosophila* (Sempere et al., 2002), the most likely candidate regulator would be a hormone that acts through a receptor other than EcR. This conclusion is consistent with several recent studies that have provided indirect evidence of other hormone signaling pathways in *Drosophila.*

Many genes are coordinately upregulated in mid-third instar larvae, when the *Adh* gene switches from its larval to adult promoter, and the larval salivary gland switches its genetic program from the *ng/Pig* genes to the *Sgs* glue genes (Benyajati et al., 1983; Andres and Cherbas, 1992; Andres et al., 1993; Mougneau et al., 1993). The temporal signal for this mid-third instar transition remains undefined, but could comprise a 20E/EcR-independent pathway (Andres and Cherbas, 1992; Andres et al., 1993). Similarly, the *E74A* and *E75A* early mRNAs are coordinately induced at some times during development when the ecdysteroid titer is thought to be low, arguing that another temporal signal may be responsible for this response (A. Sullivan and C.S.T., unpublished results; Segraves, 1988; Thummel et al., 1990). Finally, α-ecdysone, the precursor to 20E, is sufficient to drive furrow progression in *Manduca* eye primordia as well as stimulate optic lobe neuroblast proliferation, suggesting that this hormone, which is a poor activator of the EcR/USP complex, can act as temporal signal in this lepidopteran insect (Riddiford, 1993; Champlin and Truman, 1998a, b). Further studies should help to elucidate the roles of other ecdysteroids during insect development and provide a foundation for better understanding the temporal regulation of *let-7* and *miR-125* in *Drosophila.*

It is interesting to note that the DAF-12 orphan nuclear receptor is required for the proper timing of *let-7* induction in *C. elegans* (Johnson et al., 2003; S. Lee and G.R., personal communication). Genetic evidence suggests that DAF-12 is regulated by a steroid hormone under the control of the *daf-9* cytochrome P450 gene (Gerisch et al., 2001; Jia et al., 2002). Perhaps more interesting from the perspective of this study, the *Drosophila* genome encodes a ortholog of DAF-12: DHR96 (Fisk and Thummel, 1995). It is possible that functional studies of *DHR96* in *Drosophila* will shed light on the regulation of *let-7/miR-125* gene cluster in this insect model system.

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