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

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In *Caenorhabditis elegans,* the heterochronic pathway controls the timing of developmental events during the larval stages. A component of this pathway, the *let-7* small regulatory RNA, is expressed at the late stages of development and promotes the transition from larval to adult (L/A) stages. The stage-specificity of *let-7* expression, which is crucial for the proper timing of the worm L/A transition, is conserved in *Drosophila melanogaster* and other invertebrates. In *Drosophila,* pulses of the steroid hormone 20-hydroxyecdysone (ecdysone) control the timing of 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.**

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) and ecdysone-regulated gene expression (reviewed in Thummel, 1996; Richards, 1997). Ecdysone orchestrates a complex, hierarchical gene expression cascade that transforms the larva into a highly motile reproductive adult fly. In *C. elegans,* the heterochronic gene pathway generates the temporal contexts in which the appropriate developmental programs are executed throughout development of the larva to the adult (reviewed in Ambros, 1989, 2000; Slack and Ruvkun, 1997). Although nematodes do not undergo an overt metamorphosis, as do *Drosophila,* the life cycle of the two animals is similar in that both undergo stages of molting development prior to the adult. This common developmental strategy groups them together as ecdysozoans, in recognition that a common ancestor underwent a series of larval stages punctuated by cuticular molts.

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,

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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*

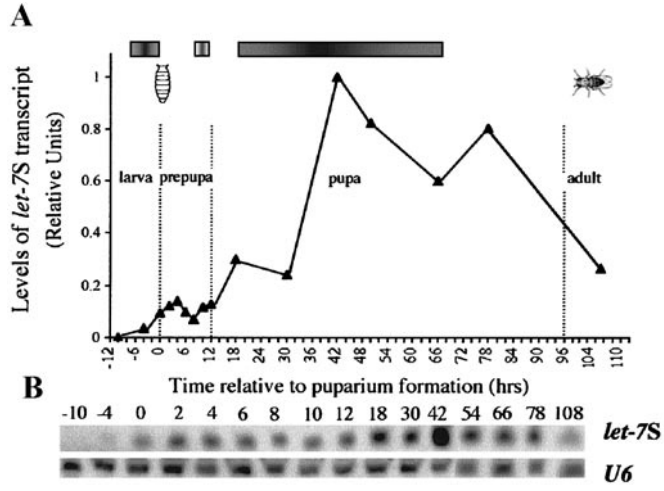

FIG. 1. Developmental profile of *let-7* RNA accumulation in the wild type. (A) Canton-S animals synchronized at egg laying were harvested before and after puparium formation (PF; 0 h). Shaded bars at top represent the ecdysone titer (Richards, 1981; Riddiford, 1993); darker colors indicate higher titers. Vertical lines mark the transition between indicated developmental stages. (B) Northern blot used for the quantification of *let-7* expression; *U6* hybridization was used as loading control and levels of *let-7* RNA are quantified relative to *U6* (see Materials and Methods).

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^6 and ecd^1 (obtained from Bloomington Stock Center, #218) mutants are described elsewhere (Lindsley and Zimm, 1992). The mutation npr^6 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 *l(1)2Bc*, all of which are affected in the npr^6 mutant. npr^6 is maintained in *cis* to a *yellow* (*y*) mutation in a stock with the *FM6-l* balancer chromosome and a duplication, $Dp(1;Y)y^267g$, which is a translocation of 1A-to-2B17-18 to the Y chromosome. The homozygous npr^6 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 puparium formation (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 ecd^1 animals grown at the restrictive temperature (29°C), stage was determined with reference to PF in ecd^1 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-cm^2 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× stock in H_2O) to a final concentration of 5×10^{-6}M. Control cells were treated with an equal volume of H_2O.

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-C*antisense primers from Z4 cDNA clone 28-I (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 Qiaquick 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 was resuspended in RNAse-free H_2O. 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 × 6 × 0.2 cm) at 125 V for 2 h in 1× TBE at 25°C. RNA was electrophoretically transferred to a Zetaprobe membrane (Biorad) at 80 V for 1 h in 0.5× TBE at 4°C. RNA was cross-linked to the membrane by UV irradiation (1200 mJ; Stratagene UV Stratalinker), subsequently the membrane was baked at 80°C for 30 min. *let-7* and *U6* antisense StarFire (Integrated DNA Technologies) radiolabeled probes were prepared by incorporation of [α-^{32}P]dATP 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 Na_2PO_4, pH 7.2, and washed twice with 2× SSPE 0.1% SDS, and once with 1× SSPE 0.1% SDS, and 0.5× SSPE 0.1% SDS at 42°C. For *U6* probe, membranes were hybridized overnight at 42°C in 7% SDS 0.05 M Na_2PO_4, pH 7.2, and washed twice with 1× SSPE 0.1% SDS, and once with 0.5× SSPE 0.1% SDS and 0.1× 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), ACTATACAACCTACTACCTCA; *U6* antisense StarFire (IDT), GCAGGGGCCATGCTAATCTTCTCTGTATTG; T7/*BR-C*sense: GAATTAATACGACTCACTATAGGGAGAGATCCACAGCCAGAACCAGACAC; T3/*BR-C*antisense, GCTCGGAATTAACCCTCACTAAAGGGACAGGCTGTTGTCCGAGCCGCGC; T7/mocksense, GTAATACGACTCACTATAGGGCTGTGTCTCAACTATCTACCATTCC; T3/mockantisense, CGAAATTAACCCTCACTAAAGGGTTACCCGGAACTCATGATAGC. (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-7*S, short) and approximately 70 (*let-7*L, long) nucleotides. *let-7*S is the biologically active isoform (Reinhart *et al.*, 2000), which results from the processing of the precursor *let-7*L (Grishok *et al.*, 2001; Hutvagner *et al.*, 2001). Unless otherwise noted, we will use "*let-7* RNA" in reference to *let-7*S. *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

ecd^1 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, *ecd*^{1} 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), *ecd*^{1} 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 *ecd*^{1} animals. *ecd*^{1} 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 *ecd*^{1} animals grow more slowly and less synchronously than the wild type. The majority of *ecd*^{1} animals that were upshifted in the early L3 stage remained as larvae (98%, 197 out of 200), and these *ecd*^{1} retarded larvae were harvested at various times relative to PF of *ecd*^{1} animals maintained at 20°C. *ecd*^{1} 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 *ecd*^{1} 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 *ecd*^{1} animals (Fig. 2). This correlation between PF and *let-7* expression in *ecd*^{1} 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.

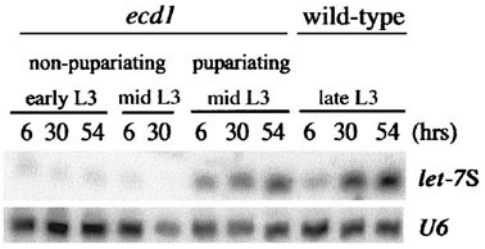

FIG. 2. *let-7* RNA accumulation in *ecd*^{1} homozygous animals upshifted during the third larval stage. *ecd*^{1} animals reared at 20°C were transferred to 29°C during early and mid-L3 stage and harvested at time intervals after puparium formation (PF; 0 h). The time of PF for *ecd*^{1} animals upshifted in the early L3 was defined by the time of PF of *ecd*^{1} animals grown in parallel at 20°C. *ecd*^{1} animals upshifted in mid L3 were harvested in two separated populations (nonpupariating and pupariating). The profile of *let-7* RNA accumulation in wild-type animals upshifted during the late L3 is shown for comparison.

In *ecd*^{1} 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 *ecd*^{1} 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 *ecd*^{1} 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

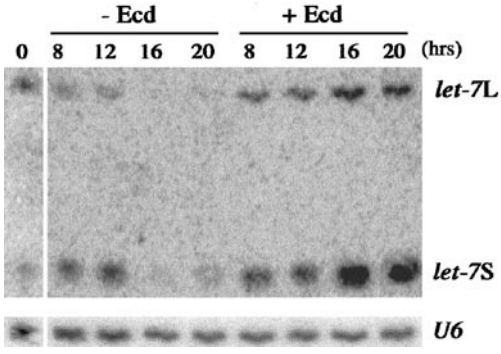

FIG. 3. Induction of *let-7* expression by ecdysone in organ cultures from late third instar larvae. Late L3 larvae were dissected and organs were cultured in Schneider's medium with (+Ecd) or without (−Ecd) 5 μM ecdysone for the periods of time shown. Total RNA was harvested and *let-7* RNA was detected by Northern blot, with *U6* as loading control (see Materials and Methods).

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-7*S and its precursor form (*let-7*L) 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-7*S and *let-7*L 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 npr^6 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. npr^6 animals remain as larvae for about 5–10 days after the normal time of pupariation and then die. npr^6/npr^6 and $npr^6/+$ animals were synchronized and harvested at various times relative to the time of PF (of the $npr^6/+$ animals). *let-7* RNA was detected at very low levels in npr^6/npr^6 animals, compared with $npr^6/+$ 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 npr^6/npr^6 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

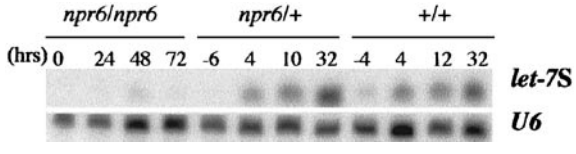

FIG. 4. Developmental profile of *let-7* RNA accumulation in npr^6 mutants. $npr^6/+$ animals were harvested at the late L3 and at time intervals during prepupal and pupal stages. npr^6/npr^6 animals were harvested relative to the time of puparium formation (0 h) in $npr^6/+$. *let-7* RNA was detected by Northern blot. The profile of *let-7* RNA in the wild type is shown for comparison.

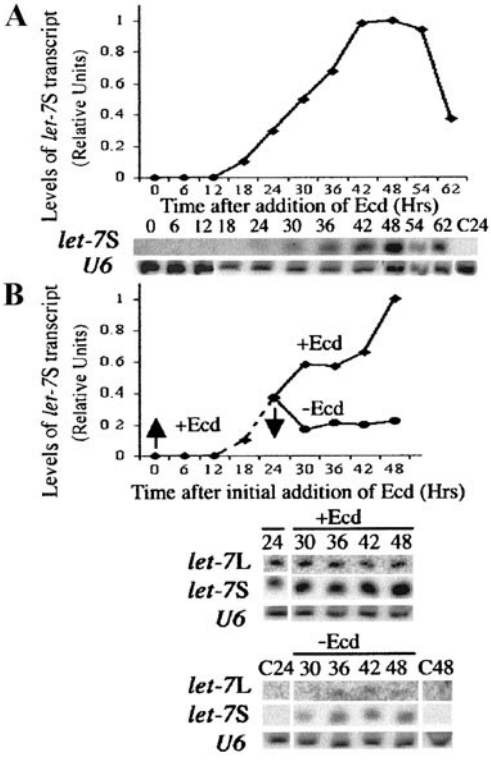

FIG. 5. Time course of *let-7* expression in S2 cells treated with ecdysone. (A) S2 cells were incubated in medium with 5 μM ecdysone, harvested every 6 h, and total RNA was analyzed by Northern blot. *let-7* RNA is not detected in untreated cells (C24) harvested at 24 h relative to addition of ecdysone. (B) S2 cells were incubated with 5 μM ecdysone for 24 h. At this time point, ecdysone was removed from the medium (−Ecd) and cells were harvested every 6 h. In parallel, cells were maintained in ecdysone (+Ecd) for the length of the experiment and harvested at the same time points. *let-7* RNA is not detected in untreated cells harvested at 24 (C24) or 48 h (C48) relative to initial addition of ecdysone to treated cells. Dashed line shows the profile of *let-7* expression obtained in (A).

medium, compared with the cells maintained with ecdysone (Fig. 5B). The levels of *let-7*S and *let-7*L coordinately decreased. These results indicate that ecdysone is required for both the initiation and maintenance of *let-7* transcription in S2 cells.

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

We found that *BR-C* is involved in relaying the ecdysone 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 ecdysone. In parallel, nontransfected cells were also treated with 5 μM ecdysone. S2 cells were harvested for 32, 40, and 48 h after the addition of ecdysone, 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 ecdysone-dependent *let-7* expression in S2 cells. Since levels of *let-7*S and *let-7*L RNA coordinately decreased when *BR-C* activity was reduced by RNAi, *BR-C* likely affects transcription of *let-7* in response to ecdysone. The residual *let-7* expression in *BR*-C RNAi cells could be due to the ineffecient uptake of the dsRNA or the incomplete inhibition of *BR-C* activity. Alternatively, other components of the ecdysone pathway could contribute to *let-7* expression, consistent with the residual *let-7* expression observed in *npr*^{6} mutant animals (Fig. 4).

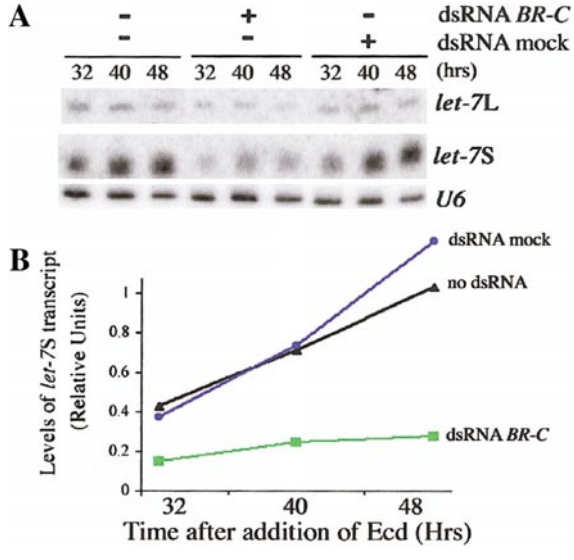

FIG. 6. Profile of *let-7* RNA accumulation in S2 cells RNAi against *Broad-Complex*. (A) S2 cells were transfected with 40 μg of dsRNA corresponding to a conserved region of all *BR-C* mRNA isoforms or a nonspecific dsRNA (mock) from *C. elegans*. Then cells were treated with 5 μM ecdysone, harvested 32, 40, and 48 h later, and levels of *let-7* RNA were analyzed by Northern blot. (B) Levels of *let-7* RNA in the different treatments shown in (A) (green line for dsRNA *BR-C*; blue line for dsRNA mock) are relative to those in nontransfected S2 cells treated with ecdysone (black line for no dsRNA).

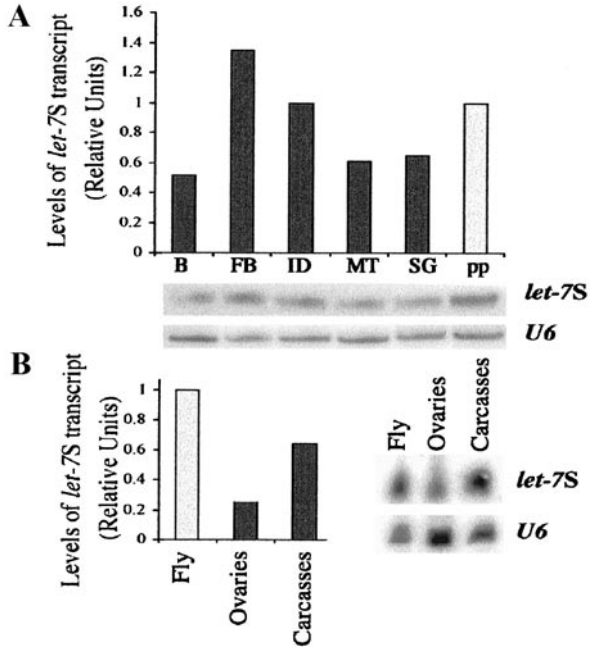

FIG. 7. Expression of *let-7* RNA in different tissues from early prepupae and adult animals. (A) Brains (B), imaginal discs (ID), fat bodies (FB), Malphigian tubules (MT), and salivary glands (SG) were dissected from early prepupa 0–4 h after puparium formation (PF). Total RNA was prepared and analyzed by Northern blot. Levels of *let-7* RNA in different tissues are relative to those in whole prepupa (PP) 0–4 h after PF. (B) Ovaries, carrying some developing eggs, were dissected from adult females. Levels of *let-7* RNA in ovaries and carcasses (tissues remaining after ovaries dissection) are relative to those in whole flies.

let-7 RNA Is Widely Expressed in Tissues That Respond to Ecdysone Signals during Metamorphosis

Finally, we determined the tissue-specificity of *let-7* expression in prepupae and adult animals in order to correlate expression domains with function. We dissected tanning prepupae (0–4 h after PF) to obtain a variety of tissues: brains, imaginal discs, fat bodies, salivary glands, and Malphigian tubules. Levels of *let-7* RNA in these tissues were quantified by Northern analysis. *let-7* RNA was detected in all tissues examined, though at relatively higher levels in fat bodies and imaginal discs (Fig. 7A). The widespread expression of *let-7* RNA suggests that *let-7* could mediate diverse metamorphic processes, such as the terminal differentiation of imaginal discs and apoptosis of salivary glands and fat bodies. Levels of *let-7* RNA in adult ovaries and carcasses (tissues remaining after ovaries dissection) were quantified by Northern analysis. *let-7* is expressed in both somatic and gonadal tissues in the adult (Fig. 7B).

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-7*L (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 dipterans

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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