

Expression of the 22 nucleotide *let-7* heterochronic RNA throughout the Metazoa: a role in life history evolution?

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SUMMARY The 22 nucleotide *let-7* small temporal RNA has been found consistently in samples from diverse bilateria but not from sponge or cnidarians. Here we further examine the phylogenetic distribution of this regulatory RNA by sampling representatives of diverse metazoan lineages. The 22 nucleotide *let-7* RNA is detectable in triclad and polyclad platyhelminths,

nemertean, and chaetognath but not ctenophore or acael metazoans. These results support recent arguments that acocels are distinct from other acocelomate platyhelminths. We argue that *let-7* is not a bilaterian or triploblast synapomorphy but instead evolved later in metazoan evolution, perhaps in association with complex life history traits.

INTRODUCTION

The *let-7* gene was first identified in the nematode *Caenorhabditis elegans* as an essential regulator of developmental timing (Reinhart et al. 2000). It encodes a 22 nucleotide (nt) RNA that negatively regulates the expression of protein-coding genes that contain regions of complementarity in their 3'-untranslated regions (UTRs) (Reinhart et al. 2000; Slack et al. 2000). Expression of *let-7* RNA is undetectable until the last larval stages in *C. elegans*, where it directs larval to adult cell fate transitions in certain tissues (Reinhart et al. 2000). A major target of *let-7* mediated down-regulation is the *lin-41* protein-coding gene, which contains two complementary sites in its 3'-UTR (Reinhart et al. 2000; Slack et al. 2000). The induction of *let-7* expression in the last larval stage causes decreased LIN-41 protein expression, apparently by a posttranscriptional mechanism (Slack et al. 2000).

The *let-7* RNA is not restricted to nematodes but instead is present in species of diverse animal phyla, including chordates, hemichordates, echinoderms, mollusks, annelids, and arthropods (Pasquinelli et al. 2000). However, more basal metazoans, such as cnidarians and poriferans, as well as plants

and unicellular organisms, fail to exhibit this 22nt RNA (Pasquinelli et al. 2000). Presently, it is unclear if the inability to detect *let-7* RNA expression in more basal metazoans is due to the true absence of this gene in these taxa or to technical issues. Our inability to detect *let-7* RNA expression in unicellular organisms and plants (*Arabidopsis*) appears to be due to the absence of potential orthologs of this RNA gene (Pasquinelli et al. 2001), because sequencing of those genomes is, or is nearly, complete and there are no indications of *let-7*. Both the sequence and the temporal expression pattern of *let-7* RNA appear to be conserved broadly across bilaterians. In fact, the human genome contains at least three identical matches to the *C. elegans* 22nt *let-7* RNA sequence (Pasquinelli et al. 2000; Lagos-Quintana et al. 2001). Additionally, in every case tested, *let-7* RNA is detectable by the adult stage but is absent at earlier developmental stages. In *Drosophila melanogaster*, *let-7* RNA is absent from embryonic and larval stages and first appears during prepupariation in synchrony with the ecdysone pulses that induce metamorphosis (Sempere et al. 2002). Expression of the RNA persists throughout *Drosophila* pupal and adult stages (Pasquinelli et al. 2000; Sempere et al.

2002). Additionally, *let-7* is detected in adult but not in trochophore larvae of a polychaete annelid or in veliger larvae of a mollusk (Pasquinelli et al. 2000). In the zebrafish *Danio rerio*, *let-7* RNA is absent from embryos until some time between 24 and 48 h after fertilization (Pasquinelli et al. 2000), at which time the adult body plan is fully formed. The regulatory mechanism of *let-7* RNA may also be conserved, because potential orthologs of *lin-41* in *Drosophila* and zebrafish contain sites in their 3'-UTRs that could base pair with the 22nt *let-7* RNA (Pasquinelli et al. 2000).

The phylogenetic distribution and the conserved temporal expression pattern of *let-7* within the Metazoa are quite suggestive. Did this small regulatory RNA have a role in life-history evolution in metazoans? Is *let-7* a synapomorphy for the bilaterally symmetrical animals (Bilateria)? Or perhaps it is a synapomorphy for triploblastic animals that possess ecto-, endo-, and mesodermal layers or the coelomates that have mesodermally lined body cavities (Fig. 1). To help establish if *let-7* is an important trait to trace more broadly in metazoan evolution, we sampled several key metazoan taxa for the presence of *let-7* in adult tissues: the ctenophore *Mnemiopsis leidyi*, three species of acoel flatworms (*Convoluta convoluta*, *Symsagittifera* [*Convoluta*] *roscoffensis*, and *Amphiscolops* sp.), three species of polyclad flatworm (*Prosthiosomum siphunculus*, *Discocelis tigrina*, and *Leptoplana* sp.), a triclad flatworm (*Girardia tigrina*), a nemertean worm (*Cerebratulus lacteus*), and a chaetognath (*Flaccisagitta enflata*).

These animals were chosen for distinct phylogenetic reasons. Ctenophores are the only other extant non-bilaterian diploblastic taxon left to assay (sponges and cnidarians are *let-7* negative). Platyhelminthes flatworms (including acoel flatworms) and nemerteans have historically been categorized as acoelomate triploblasts, although compelling arguments

have been made that acoel flatworms do not belong to the same phylum as polyclads, triclads, and other platyhelminthes (Ruiz-Trillo et al. 1999, 2002), and nemerteans have been shown to be coelomates (Turbeville 1986; Turbeville et al. 1992). The status and origin of the chaetognath body cavity has always been controversial (Willmer 1990; Halanych 1996), and chaetognaths have been difficult to place in the metazoan tree (Ghirardelli 1995; Kapp 2000). They display many deuterostome-like features, but molecular data refute that placement and have suggested either ecdysozoan (Halanych 1996; Peterson and Eernisse 2001) or lophotrochozoan (Haase et al. 2001; Shimotori and Goto 2001; Erber et al. 1998) affinities. It also has been suggested that these animals represent basal protostomes (Giribet et al. 2000), although additional data are clearly needed to place this interesting group.

Here we show that *let-7* is present in chaetognaths, nemerteans, polyclad, and triclad flatworms but undetectable in direct developing ctenophores and acoel flatworms. These data could support previous arguments that acoel flatworms are not aligned with the platyhelminths or other spiral-cleaving lophotrochozoans but are likely to be the most basal extant bilaterians (Ruiz-Trillo et al. 1999, 2002). Thus, *let-7* is not a bilaterian synapomorphy but instead may be associated with true coelomate animals. The role of *let-7* in life history evolution is discussed.

MATERIALS AND METHODS

Animal collection

Adult lobate ctenophores *Mnemiopsis leidyi* were collected off the Marine Fisheries rock jetty or in Eel Pond in Woods Hole, Massachusetts. Embryos were spawned as previously described

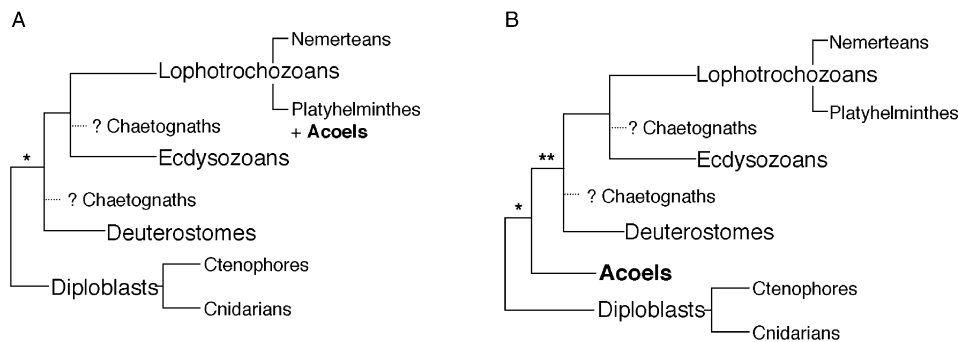


Fig. 1. Alternative metazoan relationships with possible locations of *let-7* appearance (* or **) (A) A prevalent view based on some 18S ribosomal gene sequence analyses and *Hox* gene number, type, and sequences (de Rosa et al. 1999). In this model, Acoels belong to the Platyhelminthes and, because *let-7* appearance (*) may represent a bilaterian synapomorphy, all platyhelminths (acoels included) are predicted to be *let-7* positive. Two opposing placements of Chaetognaths (Willmer 1990; Halanych 1996; Ghirardelli 1995; Kapp 2000) are indicated by dashed lines and question marks. (B) New phylogenetic proposal based on additional 18S ribosomal sequences, myosin type II sequences, and a combined analysis (Ruiz-Trillo et al. 1999, 2002). According to these data, Acoels are not Platyhelminthes but are the most extant basal bilaterians. Appearance of *let-7* may be a bilaterian synapomorphy, which may (*) or may not (**) include the acoels. Hence, Platyhelminthes are predicted to be *let-7* positive, whereas expression in acoels is open.

(Martindale and Henry 1999) and raised to the cydippid “larval” stage before they were concentrated by centrifugation and prepared for RNA isolation. The nemertean *Cerebratulus lacteus* was collected by the Marine Resources Department of the Marine Biological Lab, Woods Hole, Massachusetts. *Flaccisagitta enflata* (Chaetognaths) were collected by plankton tows in Kaneohe Bay on the windward side of Oahu, Hawaii. Adult *F. enflata* were manually separated from other plankton with forceps and prepared for total RNA isolation.

Acoels (*Symsagittifera [Convoluta] roscoffensis*) were collected off the coast of Roscoff, France and quickly frozen for RNA extraction, as were *Convoluta convoluta* and *Amphiscolops* sp., collected at the shores of Nahant, Massachusetts and Panacea, Florida, respectively. Adult polyclads (*Discocelis tigrina*, *Prosthiosomum siphunculus*, and *Leptoplana* sp.) were collected in shallow waters at the Catalan coast 30 km south of Barcelona, whereas adult triclads from an asexual strain of the species *Girardia tigrina* (Ribas et al. 1989) came from laboratory cultures in Barcelona.

RNA preparation and analysis

Total RNA was prepared by homogenization of frozen tissue in RNazol B according to manufacturers recommendations (Tel-Test, Inc., Friendswood, TX). Except where indicated, 20 µg of total RNA was subjected to Northern analysis to probe for expression of *let-7* RNA using conditions identical to those used in Pasquinelli et al. (2000). Specifically, total RNA was separated by electrophoresis in 11% denaturing polyacrylamide gels and transferred to nylon membranes (Zeta-Probe GT, Biorad, Hercules, CA). The 5′ kinase labeled oligo probe for *let-7* (p249: AACTATACAACCTACTACCTACCGGATCC) was hybridized to the blot in 5 × SSC, 7% SDS, 0.02 M sodium phosphate, 1 × Denhardt’s solution for approximately 12 h at 50°C and then washed in 3 × SSC, 5% SDS, 0.025 M sodium phosphate, 10 × Denhardt’s solution at 50°C. Total RNA from mixed stage *C. elegans* was included as a positive control in all Northern analyses.

RESULTS

We previously showed that the 22nt *let-7* RNA is expressed in adult samples from all bilaterally symmetrical animals tested, including examples from the deuterostome, lophotrochozoan, and edysozoan clades (Pasquinelli et al. 2000). In contrast, the RNA was undetectable in samples from animals considered to be more basally derived metazoans, such as members of Cnidaria and Porifera (Pasquinelli et al. 2000). In this present study, we were unable to detect *let-7* in total RNA isolated from adult and cydippid stages of *Mnemiopsis leidyi* comb jellies, which are members of Ctenophora (Fig. 2A). These data are consistent with the notion that *let-7* RNA arose or has been maintained only in bilaterian metazoans.

Considering the conserved expression pattern of *let-7* RNA in adult bilaterians, we predicted that species of nemertean, platyhelminths (Lophotrochozoa), and chaetognath would express *let-7* RNA. A specific *let-7* signal was

readily detectable in RNA prepared from the platyhelminths adult triclad *Girardia tigrina* (Fig. 2B, lane 2). We prepared total RNA from the adult stage of three polyclads, *Prosthiosomum siphunculus*, *Discocelis tigrina*, and *Leptoplana* sp., and probed for the expression of *let-7* RNA. A strong signal corresponding to a single 22nt band was observed in samples from two of the three species, whereas a weaker signal was seen in the *Leptoplana* sample (Fig. 2B, lanes 4–6). It is presently unclear if the difference in this species reflects the amount of *let-7* RNA present in the animals at the time point in adulthood at which they were sampled or if it results from slight sequence variations in the *let-7* RNA that might reduce the efficiency of probe hybridization in the stringent Northern protocol.

We also detected *let-7* RNA signals in RNA prepared from the chaetognath, *Flaccisagitta enflata* and the nemertean, *Cerebratulus lacteus*. A doublet was seen in RNA prepared from the adult chaetognath (Fig. 2C, lane 2). This doublet pattern resembles that observed in *Drosophila melanogaster*, where it is likely that the two bands arise from a single RNA species that undergoes differential processing to produce the 22 and 21nt forms of *let-7*, because the *Drosophila* genome harbors only one *let-7* sequence (Pasquinelli et al. 2000; Hutvagner et al. 2001). Alternatively, the *let-7* RNA doublet observed in the chaetognath sample could result from expression of distinct *let-7* genes encoding divergent precursors that still give rise to identical 22 or 21nt mature RNAs. For example, the human genome contains three separate *let-7* genes, as well as several nearly identical *let-7* genes (designated *let-7b-i*), that contribute to the multiple approximately 22nt bands that are often detected in samples of human RNA (Pasquinelli et al. 2000; Lagos-Quintana et al. 2001, 2002). An exceptionally strong *let-7* signal was detected in RNA from the adult nemertean *C. lacteus* (Fig. 2C, lane 3). In addition to the 22nt form of *let-7*, a longer approximately 70nt band, which likely corresponds to the precursor form, was also readily detectable in the nemertean. Under standard conditions, the precursor form of *let-7* is virtually undetectable in the adult stage of most other species (Pasquinelli et al. 2000; Grishok et al. 2001). Presently, the significance of the prominent expression of the precursor and mature *let-7* RNA forms in the adult *Cerebratulus lacteus* is unknown.

In contrast to all other animals considered bilaterians that have been tested to date, a *let-7* signal was undetectable in adult acoels. RNA from the adult acoel species, *Convoluta convoluta* and *Amphiscolops* sp., failed to produce a *let-7* signal in standard Northern assays (Fig. 2D, lanes 2–3). The RNA in these preparations was intact, as indicated by ethidium bromide staining (e.g., see the 5S rRNA bands); additionally, nuclear genes, including *Hox* genes, have been cloned from cDNA made from the same acoel RNA samples, making RNA degradation an unlikely problem. A

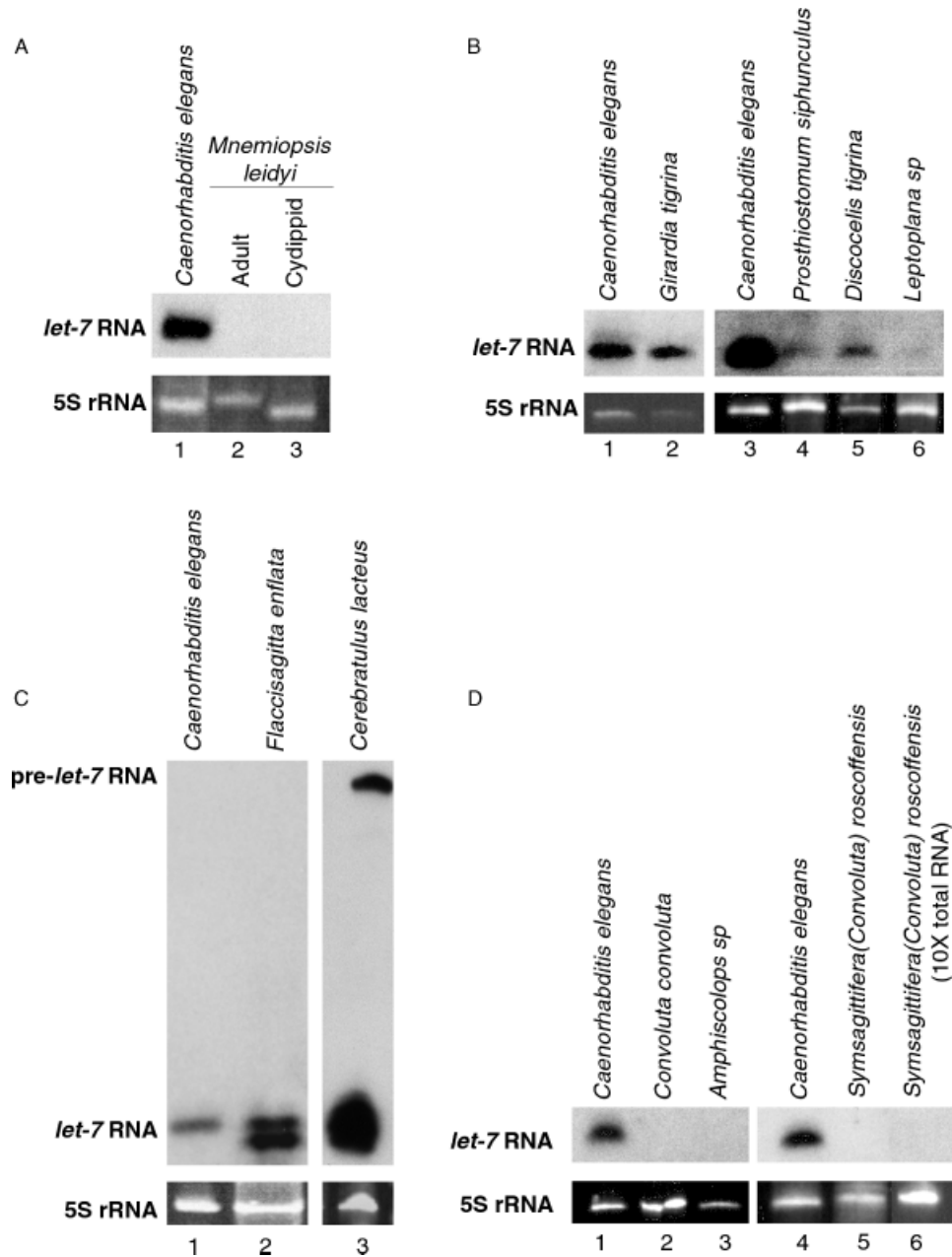


Fig. 2. Detection of the 22 nucleotide *let-7* RNA by Northern analyses. (A) Northern blot of total RNA from mixed stage *Caenorhabditis elegans* (lane 1) and *Mnemiopsis leidyi* ctenophores at the adult (lane 2) and cydippid (lane 3) stages probed for *let-7* RNA. Ethidium bromide staining of 5S rRNA serves as a control for the loading and quality of RNA in each sample. (B) Total RNA from mixed stage *C. elegans* (lanes 1 and 3), adult triclad *Girardia tigrina* (lane 2), and adult polyclads *Prosthiosomum siphuncululus* (lane 4), *Discocelis tigrina* (lane 5), and *Leptoplana* sp (lane 6) were analyzed for the expression of *let-7* RNA as described in A. (C) Total RNA from mixed stage *C. elegans* (lane 1), adult chaetognath *Flaccisagitta enflata* (lane 2), and adult *Cerebratulus lacteus* (lane 3) were probed for the expression of *let-7* RNA as described in A. (D) Total RNA from mixed stage *C. elegans* (lanes 1 and 4) and the adult acoels *Convoluta convoluta* (lane 2) and *Amphiscolops* sp (lane 3) and *Symsagittifera* (*Convoluta*) *roscoffensis* (lane 5) were analyzed for the expression of *let-7* RNA as described in A. A 10-fold greater concentration of total RNA was analyzed for *Symsagittifera* (*Convoluta*) *roscoffensis* in lane 6.

let-7 signal was also undetectable in the adult acoel *Symsagittifera* (*Convoluta*) *roscoffensis*, even when a 10-fold greater concentration of total RNA was assayed (Fig. 2D, lanes 5–6).

DISCUSSION

The previous cross-species analysis of expression on *let-7* RNA revealed a striking division between bilaterians and the

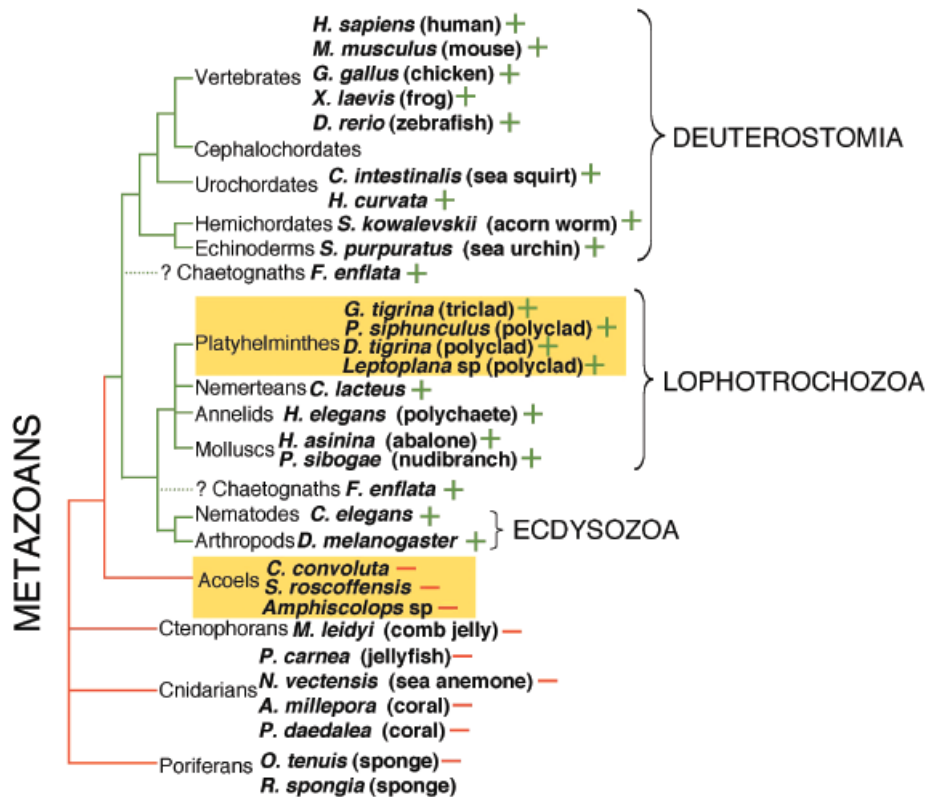


Fig. 3. An abbreviated phylogeny tree depicting species that do (green +) and do not (red -) express detectable *let-7* RNA based on this study and Pasquinelli et al. (2000). Two opposing placements of Chaetognaths (Willmer 1990; Ghirardelli 1995; Halanych 1996; Kapp 2000) are indicated by dashed lines and question marks. The expression of *let-7* RNA in representative triclads and polyclads but not in acoels (shaded in yellow) supports previous work suggesting the polyphyly of Platyhelminthes (Ruiz-Trillo et al. 1999, 2002).

most basal metazoans (Fig. 3) (Pasquinelli et al. 2000). The absence of detectable *let-7* RNA in ctenophores, cnidarians, and sponges further supports the possibility that *let-7* RNA arose in association with a bilaterally symmetrical triploblastic basal bilaterian. If acoels represent the earliest branching bilaterians, then the absence of *let-7* RNA in this group indicates that *let-7* may not be a bilaterian triploblastic synapomorphy but instead may have arisen sometime after the advent of these characters.

Presently, it is unclear whether the inability to detect *let-7* RNA in acoels or the most basal metazoans, such as ctenophores, reflects the absence of this small RNA gene or a divergence in sequence that prohibits hybridization to the common *let-7* probe. The sequences flanking the identical 22nt *let-7* RNA diverge extensively among different species, making it impossible to identify *let-7*-like genes by standard PCR cloning techniques. Genomic sequence data would make it possible to search for sequences similar to *let-7*, and the candidates could be tested for expression as 22nt RNAs by standard Northern analyses.

It is likely that acoels, ctenophores, cnidarians, and poriferans express 22nt RNA genes but that they lack a sequence identical to *let-7*. The inability to detect *let-7* RNA in samples from unicellular organisms and plants whose genomes have been sequenced correlates with the absence of sequence similarity to this 22nt RNA gene (Pasquinelli et al. 2000). Presently, we do not know if that will also be the case for the most basally derived metazoans or whether their genomes will harbor *let-7*-like genes that either are not expressed or have significantly diverged. An ancient precursor of the *let-7* gene may have been common to the earliest animal lineages, but selective pressure maintained expression or sequence divergence that resulted in the conserved *let-7* RNA expression pattern apparently shared by only the most recently diverging metazoan lineages.

The *let-7* RNA is actually a founding member of a large family of approximately 22nt RNA genes identified in *C. elegans*, *Drosophila*, and vertebrates, generally referred to as microRNAs (miRNAs) (Lagos-Quintana et al. 2001, 2002; Lau et al. 2001; Lee and Ambros 2001; Mourelatos

et al. 2002). Recently, miRNAs were also discovered in Arabidopsis (Llave et al. 2002; Park et al. 2002; Reinhart et al. 2002), showing that such RNA genes are not restricted to animals. Remarkably, *let-7* RNA shows the broadest conservation of exact sequence of any miRNA thus far reported. The 22nt RNA product is identical in nematodes and humans and is likely to also be the same or very similar in all other organisms that express it, because a *let-7* signal is readily detectable by stringent Northern analyses (Pasquinelli et al. 2000). In contrast, no other miRNA reported to date maintains absolute nucleotide identity across the entire sequence of the mature form in the *C. elegans*, *D. melanogaster*, and *Homo sapiens* genomes. The strict conservation of *let-7* RNA sequence may have been maintained to regulate multiple conserved target genes via base pairing interactions. A nucleotide change in the *let-7* RNA sequence may not be tolerated if it alters regulation of more than one gene, which would reduce the likelihood of compensatory nucleotide changes to restore base pairing. This suggests that targets of *let-7* should also show a matching distribution throughout the Metazoa. The identification and functional analyses of these targets will be of ultimate importance for reconstructing the ancestral role of the *let-7* stRNA gene.

The expression of *let-7* RNA in Rhabditophora (polyclads and triclads), but not acoels, further supports the proposal that these organisms belong to distinct phyla. Historically, flatworms (Platyhelminthes, including acoels) have been considered a monophyletic group representing the most basal bilaterian clade (see references in Willmer 1990), primarily because they are nonsegmented, simple shaped, and do not possess a coelom, anus, and circulatory or skeletal systems. Lack of a coelom was the primary argument to consider nemertines as basal bilaterians, but they were subsequently shown to be coelomates (Turbeville 1986). However, no morphological synapomorphies for platyhelminths have ever been found (Smith et al. 1986), rendering uncertain their monophyly, whereas recent molecular studies (Carranza et al. 1997; Balavoine 1998; Littlewood et al. 1999) have shown the bulk of platyhelminths (the so-called Rhabditophora) to be a derived group belonging to the Lophotrochozoa. As for nemertines, recent embryological (e.g., cell lineage and fate mapping; Henry and Martindale 1998), molecular, and ultrastructural (Turbeville 1986; Turbeville et al. 1992) studies have convincingly shown they possess reduced coelomic cavities (e.g., blood vessels and rhynchocoel) and also belong to the Lophotrochozoa.

Meanwhile, the position of the acoels based on morphological features has remained contentious. Recent molecular (Ruiz-Trillo et al. 1999, 2002) and embryological (reviewed by Henry et al. 2000) work has argued that acoels are (a) a distinct clade of animals from other lophotrochozoan flatworms and (b) that these animals represent the most basal

extant group of triploblastic bilaterians. The finding that *let-7* is not detectable in acoels as it is in other platyhelminths and that basal metazoans such as sponges, ctenophores, and cnidarians also do not detectably express this gene are one character in support of that basal placement. The finding of *let-7* in a nemertean agrees with its placement as a lophotrochozoan and, along with the platyhelminths results, argues that the absence of *let-7* expression is not associated with the reduction of the coelomate condition. Finally, the fact that chaetognaths possess significant features of both protostomes and deuterostomes does not help distinguish whether they lie close to the division of these two major clades; the presence of *let-7* in these animals, but not acoels, suggests that *let-7* appeared subsequent to the divergence of these two bilaterian groups.

If *let-7* is a synapomorphy for bilaterian groups except acoels, what is its function? Ctenophores, cnidarians, sponges, and acoels are all “direct developers” without a feeding larval stage. Could *let-7* be associated with the invention of feeding larvae, an increase in lifespan, or with inhibiting a delay in reproductive ability? For example, the mollusk *Phestilla sibogae* undergoes a larval veliger period that can last from 1 day to 7 weeks in the plankton. The lifespan of the adult is unaffected by the length of the larval period (Miller and Hadfield 1990). The *let-7* RNA is detectable in adults of *P. sibogae* but not in veliger larvae (Pasquinelli et al. 2000). Perhaps *let-7* expression is a mechanism to regulate the onset of adulthood in animals, displaying complex life history patterns. Alternatively, *let-7* may specify the adult program, namely to activate the terminal differentiation of organs, tissues, and specific cell types. Like most basal metazoans, and despite being bilaterians, acoels do not bear true organs (including gonads), their mesoderm being limited to a thin rim of muscle cells and parenchyma. Further elucidation of *let-7* targets throughout the bilaterian groups is essential for a detailed understanding of the role of this small temporal RNA in metazoan life history evolution.

Acknowledgments

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