

Research Proposals

In depth knowledge of a research area.

Articulate major unanswered questions or gaps in knowledge in a research area.

Describe experimental approaches & data analysis that test specific hypotheses and/or address unanswered questions in a research area.

Proposal organizational format directed at telling the reader what questions/hypotheses you are addressing, why the findings/answers are important and what experimental approaches will be employed to obtain the findings.

Three broad classes of research proposals

1. Hypothesis-driven
2. Hypothesis-generating
3. Methods development

Three essential sections

Specific Aims

Background and Significance

Experimental Design and Methods

Three essential sections

Specific Aims

- A stand-alone description of the problems/ hypotheses that will be examined.
- A listing of what lines of investigation will be used in the study and what will be learned.

Three essential sections

Specific Aims

Background and Significance

- Description of the current state of the field, critically evaluating existing knowledge and gaps that the proposed Aims will fill.
- Address the broader significance of the field and the findings that will arise from your proposed work. **Building a case for why the proposed studies should be done.**

Three essential sections

Specific Aims

Background and Significance

Experimental Design and Methods

- Description of the experimental approaches that will be used to execute each Aim. **The logic behind the experiments, controls and interpretations is more important than details.**
- Briefly describe, if relevant, alternative outcomes and/or approaches.
- At the end of each section, summarize the possible results in relation to advancing the Aim

The Specific Aims has three components

1. Background narrative (like an abstract) that provides a context for the questions that will be addressed.

2. List of the questions /how the questions will be addressed.

3. Discussion of the significance of the results that will be obtained.

(1) SPECIFIC AIMS

Appropriate temporal patterning is essential for embryogenesis and post-embryonic development. Progression through the four larval stages in *C. elegans* is regulated by heterochronic genes (reviewed in Slack and Ruvkun 1997; Ambros 2000). Heterochronic genes encode a diverse set of proteins, mutations in which result in the reiteration or omission of stage-specific programs of cell division, migration and/or differentiation, thereby altering developmental timing. Reiteration of a stage-specific program results in a delayed transition to adulthood (a retarded phenotype) whereas omission of a stage-specific program results in an early transition to adulthood (a precocious phenotype).

Translational repression of two early-acting heterochronic genes, *lin-14* and *lin-28*, is essential for appropriate progression through early larval stages (Ruvkun et al. 1989; Wightman et al. 1991; Moss et al. 1997). This repression generates a temporal gradient of LIN-14 and LIN-28 protein. High, intermediate and low levels of these proteins promote the activation of L1, L2 and L3 stage-specific programs, respectively. One mechanism to downregulate *lin-14* and *lin-28* involves the small temporal RNA (miRNA) product of the *lin-4* gene (Ambros 1989; Lee et al. 1993; Wightman et al. 1993; Moss et al. 1997). However, recent evidence demonstrates the presence of a second mechanism to repress *lin-28* translation, independent of *lin-4*. The role of *lin-4*-independent repression (LIR) of *lin-28* in developmental timing is unknown but may act to modulate LIN-28 protein levels at the L2 to L3 transition. Genetic studies using a GFP-tagged *lin-28* transgene indicate that this novel repression of *lin-28* involves the nuclear hormone receptor, *daf-12* (V. Ambros, personal communication). A working model is that *lin-4*-dependent translational repression initiates the downregulation of *lin-28* in L1 and the LIR pathway maintains downregulation in order to switch from an intermediate to a low level of LIN-28 protein in L2 and L3. Regulation of LIR by *daf-12* may act to coordinate the timecourse of *lin-28* downregulation with other events of larval development, such as the molting cycle. The aims of this proposal are to determine the molecular mechanism of LIR and to identify genes that are essential for this timing mechanism.

General background

Specific background

Novel finding

Model to explain finding

Goal & what will be learned

Listing of the Aims (Here as a list of declarative statements.)

- Aim 1. In order to test the hypothesis that *daf-12* translationally represses *lin-28*, the temporal profile of endogenous *lin-28* mRNA and protein levels in wildtype and *daf-12* mutant animals will be determined.
- Aim 2. Determine if *lin-4*-independent repressor elements (LIREs) in the 3' UTR of *lin-28* mRNA are necessary and sufficient for the downregulation of *lin-28* by the LIR pathway.
- Aim 3. Determine if LIREs in the *lin-28* 3'UTR are required for developmental timing of the L2-L3 transition during larval development.
- Aim 4. Perform genetic screen to identify genes that are required for LIR.

The listing of Aims or subAims can be either as declarative statements or as a question or a hypothesis.

Aim 3.
Determine if LIREs in the *lin-28* 3'UTR are required for developmental timing of the L2-L3 transition during larval development.

Are the LIREs in the *lin-28* 3'UTR required for developmental timing of the L2-L3 transition during larval development?

I hypothesize that the *lin-28* 3'UTR are required for developmental timing of the L2-L3 transition during larval development.

At least some Aims should have a logical progression

Aim 1. In order to test the hypothesis that *daf-12* translationally represses *lin-28*, the temporal profile of endogenous *lin-28* mRNA and protein levels in wildtype and *daf-12* mutant animals will be determined.



Aim 2. Determine if *lin-4*-independent repressor elements (LIREs) in the 3' UTR of *lin-28* mRNA are necessary and sufficient for the downregulation of *lin-28* by the LIR pathway.



Aim 3. Determine if LIREs in the *lin-28* 3'UTR are required for developmental timing of the L2-L3 transition during larval development.

Aim 4. Perform genetic screen to identify genes that are required for LIR.

An Aim can also stand on its own, but should still be integrated/ highly related to the other Aims in the proposal.

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Aim 4. Perform genetic screen to identify genes that are required for LIR.

Description of the significance of the findings, if the proposed studies are successfully completed.
(Need not be a separate section, can be imbedded into the narrative.)

Overall Significance of Aims. First identified in the *C. elegans* heterochronic pathway, translational repression by miRNAs is emerging as a common mode of regulation in development. Regulation of gene expression by miRNAs may be shared from worms to mammals as indicated by the conservation across phyla of the *let-7* miRNA (Pasquinelli et al. 2000). Although, it has been demonstrated that the miRNA *lin-4* regulates *lin-28* in early larval stages, it remains to be tested whether a second miRNA pathway is involved in the LIR pathway. This proposal will examine this hypothesis and has the potential to identify novel miRNA regulators.

Background and Significance

Move from general to specific.
Use headings to divide sections.
Employ figures & tables to facilitate explanation.

Regulation of embryonic and post-embryonic development requires the coordinated specification of cell fates in time and space. Due to its relatively simple and invariant cell lineages, *C. elegans* post-embryonic development provides an excellent model for the study of developmental timing. In other organisms, hormonal control regulates developmental timing events such as the regulation of the insect molting cycle by ecdysone (Thummel 1996). The identification of *daf-12* as a nuclear hormone receptor with effects on developmental timing has led to the hypothesis that the *daf-12* ligand may be a diffusible signal to coordinate temporal patterning throughout the worm (Antebi et al. 1998; Antebi et al. 2000; Snow and Larsen 2000).

In *C. elegans*, embryogenesis is followed by four stages of larval development distinguished morphologically by molts and the subsequent formation of the fully mature adult. During larval development (stages L1-L4) the appropriate execution of stage-specific programs (S1-S4) is controlled by heterochronic genes, among which the most well described are *lin-4*, *lin-14*, *lin-28* and *lin-29* (Ambros and Horvitz 1984; Ambros 1989; Slack and Ravkin 1997; Ambros 2000). Mutations in heterochronic genes result in the deletion or reiteration of stage-specific programs of cell division, differentiation or migration.

***lin-28* Regulates Developmental Timing at the L3-L4 Transition**

The phenotypes of animals with mutant *lin-28* alleles are consistent with a model that *lin-28* represses the activation of the S3 program. Loss-of-function *lin-28* mutant animals skip the S2 program and precociously express the S3 program in L2 (Ambros and Horvitz 1984). For example, in *lin-29(f)* animals, lateral hypodermal seam cells omit the S3-specific cell division in L2 leading to an abnormal number of seam cells (Ambros and Horvitz 1984). In contrast, gain-of-function (*gf*) *lin-28* mutants repeat the S2 program in L3 and correspondingly have elevated levels of LIN-28 protein at the L4 stage (Moss et al. 1997). A putative RNA-binding protein (Moss et al. 1997), the LIN-28 protein likely represses L3-specific events by binding to and regulating downstream target mRNAs. LIN-28 protein levels are dynamic during larval development, with the strongest expression in late embryos and L1 larvae, reduced expression in L2 larvae and undetectable LIN-28 levels in L3 larvae (Moss et

General background context

More specific background, essential info

Mechanisms to Downregulate *lin-28* During Early Larval Development.

There are three inputs which contribute to the downregulation mechanism of *lin-28*: *lin-4* dependent repression, positive feedback by *lin-14*, and the LIR pathway. Northern analysis demonstrates no change in *lin-28* mRNA levels during larval development (E. Moss, personal communication), indicating the dependence on translational control to regulate LIN-28 protein levels. Specifically, the 3' UTR of the *lin-28* mRNA is emerging as a critical regulatory region. The expression of a *lin-28::GFP* transgene under the control of the 3' UTR of the *unc-54* gene is not repressed by *lin-4* or the LIR pathway and does not depend on *lin-14*. This demonstrates that the 3' UTR of *lin-28* is required for all three regulatory mechanisms. Moreover, regulation of *lin-28* by the mRNA *lin-4* is mediated by specific elements in the *lin-28* 3' UTR. A *lin-28* allele in which a *lin-4* complementary 3' UTR sequence was deleted confers a gain-of-function phenotype. This proposal will focus on the LIR pathway for *lin-28* translational repression.

Importance of *lin-28* 3'UTR
What proposal will focus on

The LIR Pathway is Regulated by *daf-12*.

The following evidence supports the model that *daf-12* is upstream of *lin-28* and regulates execution of the S3 specific program. 1. Genetic analysis demonstrates the presence of the LIR pathway to downregulate *lin-28* and indicates the involvement of *daf-12* in this pathway (Table 1).

Genotype	Phenotype	LIN-28 Protein		Reference
		L1	L4	
Wildtype	normal	HIGH	LOW	Moss, 1997
<i>lin-4(lf)</i>	enacted	HIGH	HIGH	Moss, 1997
<i>lin-4(lf);lin-14(ts)</i> ¹	normal	HIGH	LOW	Moss, 1997
<i>lin-4(lf);lin-14(ts);daf-12¹</i>	enacted	HIGH	HIGH	Ashibe, Ambros, personal communication

¹ *lin-28* protein levels during the L1 and L4 stage of larval development
² *lin-14* Gal790 allele is a temperature sensitive mutation
³ *daf-12(D464)* allele class 1 mutation (Ashibe et al. 2000)

Another pathway that needs to be explored

However, this LIR-dependent downregulation of *lin-28* does not occur in triple mutant animals which

also have a mutation in *daf-12*, i.e., *lin-4(lf); lin-14(ts); daf-12(rh61)*, thus demonstrating *daf-12* action in the LIR pathway (V. Ambros, personal communication). 2. *daf-12(rh61);lin-28(lf)* double mutant animals have a precocious phenotype equivalent to *lin-28(lf)* alone. Thus, *lin-28* is epistatic to *daf-12* in the heterochronic gene pathway (Ashibe et al. 1998), consistent with *daf-12* playing a role in the regulation of *lin-28* levels. 3. Mutations in *lin-4* and *lin-14* enhance the heterochronic phenotype in *daf-12* mutants (Ashibe et al. 1998). This suggests *daf-12* acts in a separate pathway independent of *lin-4* and *lin-14* to control developmental timing, potentially through *lin-28* regulation. 4. Further, the retarded phenotype caused by a class 1 allele of *daf-12* (Ashibe et al. 2000) is similar to that of *lin-28(lf)* mutants.

Importance of *daf-12*

Because *lin-4* is involved in the *lin-28* repression, it could be hypothesized that the miRNA *let-7* acts in the LIR pathway. However, this is unlikely. *let-7* is not expressed in the L1 to L3 stages when the LIR mechanism acts (Reinhardt et al. 2000). Also, *let-7* mutant animals do not have elevated *lin-28* expression in a wildtype or a *lin-4(lf);lin-14(ts)* genetic background, in which developmental timing depends on LIR (V. Ambros, personal communication).

Argue against alternative possibilities

Model for LIR pathway.

As stated in the Specific Aims, a working model is that *lin-4*-dependent translational repression initiates *lin-28* downregulation resulting in a switch from high to intermediate levels of LIN-28 protein in L1. Then in L2 and L3, the LIR pathway maintains downregulation resulting in a switch from an intermediate to a low level of LIN-28 protein (Figure 1). The aims in this proposal will test the function and molecular mechanism of the LIR pathway to repress *lin-28* in the early larval stages.

Model to be tested in this proposal

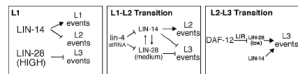


Figure 1. Working model of translational repression of *lin-28* by *lin-4* and *daf-12*.

In the Experimental Section, each Aim or subAim should have four components

1. Rationale for the experiment
2. Experimental plan and controls
3. Interpretations
4. Alternative approaches and limitations

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Ok to use these as heading. But more informative to have headings telling the reader what is the experiment, question or hypotheses that is being examined in the next section.

AIM 1. Test the hypothesis that *daf-12* translationally represses *lin-28*.

Rationale: The first goal is to determine if mutations in *daf-12* alter *lin-28* mRNA and protein levels during larval development. In animals carrying the *rh61* allele of *daf-12*, *lin-28* GFP levels are elevated, indicating *daf-12* involvement in the LIR pathway. However, it has not yet been demonstrated that *daf-12* mutants have elevated levels of *lin-28* mRNA or endogenous LIN-28 protein. This Aim will test whether the expression of the *lin-28* GFP transgene accurately reflects the regulation of the *lin-28* gene. Furthermore, these experiments will determine if *daf-12* affects *lin-28* transcription or translation. The strategy is to determine both *lin-28* mRNA levels and endogenous LIN-28 protein levels at various time points during larval development in wildtype and *daf-12(rh61)* animals.

Experimental plan and Controls: To determine if LIN-28 protein levels are affected in *daf-12* mutant animals, northern blot and western blot analysis will be performed on lysates from wildtype and *daf-12(rh61)* staged larvae. Additionally, effects of the LIR pathway on endogenous *lin-28* mRNA and protein levels will be examined using a strain in which both the *lin-4*-dependent repression and the *lin-14* positive feedback are absent (i.e., *lin-4(lf);lin-14(1)*). In this genotype, the down-regulation of *lin-28* GFP depends on the LIR, and is affected by *daf-12* mutations (V. Ambros, personal communication). L1-stage larvae will be synchronized by hatching in the absence of food. Following the addition of food (*E. coli*), larvae will be collected at increasing times during larval development. Stages of larvae will be determined with Nomarski differential interference contrast (DIC) microscopy as described in Olsen and Ambros, 1999. Multiple time points within each larval stage will be taken.

Northern analysis will be performed as described in Feinbaum and Ambros 1999. RNA will be prepared from staged lysates, separated by electrophoresis, and transferred to Zetaprobe membrane (BioRad). Membranes will be cross-linked and incubated with a radiolabelled antisense *lin-28* probe. Following hybridization and washing, membranes will be exposed to film and quantified with a PhosphorImager (Molecular Dynamics). To control for the amount of RNA loaded per lane, membranes will be stripped and re-probed with an antisense U6 probe. Lysates from worms which lack *lin-28* (i.e., null alleles) will be used as a negative control.

Logic for what will be done

General outline of experiment (limit the detail)

Controls

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Typos!!
Should be *lin-4(lf);lin-14(1)*

For western analysis, proteins will be separated by SDS-PAGE and transferred to PVDF membrane. Blots will be probed with anti-LIN-28 (to be obtained from E. Moss). Bands will be detected and quantified using enhanced chemi-luminescence and the Molecular Dynamics imaging system with Imagequant software. To control for the amount of protein loaded per lane, blots will also be probed with anti- β tubulin which will allow for normalization and comparison of bands between larval stages. Non-specific IgG (Sigma) will be used as a negative control antibody. Lysates from worms which lack *lin-28* (i.e., null alleles) will be used as an additional negative control for anti-LIN-28 specificity.

Interpretations: These experiments will determine if *daf-12* regulates, directly or indirectly, *lin-28* mRNA or protein levels. It is important to note that because *lin-28* mRNA levels remain constant through larval development (E. Moss, personal communication), *daf-12* regulation of *lin-28* transcription is not expected. Northern analysis will serve as a control to verify that potential changes in LIN-28 protein levels between wildtype and *daf-12(rh61)* animals are due to translational control. If LIN-28 protein levels remain elevated in L2 and L3 in *daf-12(rh61)* relative to wildtype animals, then this will support a two-step translational repression model for regulation of *lin-28* in which both *lin-4*-dependent repression and LIR are required. Elements in *lin-28* required for regulation by the LIR pathway will be examined in Aim 2. If no misregulation is observed in lysates from *daf-12(rh61)* relative to wildtype lysates, it is possible that functional redundancy with the *lin-4* pathway is sufficient to appropriately downregulate *lin-28*. Analysis of lysates from *lin-14(f);lin-14(c)* animals will address this possibility.

Alternate Methods and Limitations: Due to some heterogeneity of developmental timing, each lysate will represent an average of protein levels in pooled individual animals at slightly different stages. This heterogeneity may prevent the detection in LIN-28 protein levels in lysates from *daf-12(rh61)* or *lin-14(f);lin-14(c)* animals, if such changes are subtle relative to wildtype. To address this possibility, the sensitivity of the detection protocol will be optimized in order to use as few individual animals as possible. In addition, shorter time intervals within the L2 stage can be examined.

Expected finding leads to experiments in Aim 2.

Approach if alternative result

Aim 2. Identify elements in the 3' UTR of *lin-28* that are necessary and sufficient for *daf-12* translational repression.

Rationale: The 3' UTR of *lin-28* has been determined to be necessary to direct the temporal downregulation of *lin-28*. Deletion of *lin-4* complementary elements in the 3' UTR results in a gain-of-function *lin-28* allele that remains elevated at the L4 stage (Moss et al. 1997). Furthermore, *lin-28::GFP* under the regulation of the 3' UTR of the *unc-54* gene is not temporally downregulated (Moss and Ambros, personal communication). These results suggest that all the known negative regulatory inputs to *lin-28* act via the 3' UTR, but this supposition has not been directly tested for the LIR pathway. Therefore, it will be determined whether the *lin-28* 3' UTR is sufficient to direct the downregulation of a GFP reporter gene by the LIR pathway, and what elements within the 3' UTR are required.

Relevant background

Question to be addressed

How, in general, it will be addressed

Experimental plan and Controls:

Determine if the *lin-28* 3' UTR is sufficient for downregulation by the LIR pathway. Standard molecular biology techniques will be used to generate reporter constructs, consisting of a *col-10* promoter (Hong et al. 2000)-driven GFP coding region under the regulation of either the *lin-28* 3' UTR (Moss et al. 1997) or control (see below) 3' UTR sequences. The *col-10* promoter directs specific expression in the lateral hypodermal cells, thus aiding in the analysis of expression levels. Constructs will be confirmed by sequencing. Germ-line transformation will be performed by microinjecting reporter constructs along with a marker, which allows for the selection of transformants (e.g., *rol-6* (*rol1006*)), into wildtype animals. Arrays will be crossed into *lin-4(f);lin-14(c)* and *lin-4(f);lin-14(c);daf-12(rh61)* genetic backgrounds in order to analyze the sensitivity of reporter constructs to the LIR pathway. As outlined in Table 1, a LIR-dependent construct is expected to be downregulated by the L4 stage in wildtype and *lin-4(f);lin-14(c)* genetic backgrounds. However, in a *lin-4(f);lin-14(c);daf-12(rh61)* genetic background, in which the LIR pathway is absent, a LIR-dependent construct is expected to remain elevated in the L4 stage. Southern blotting with DNA from transgenic strains will be performed to confirm the presence and abundance of the transgenes.

To analyze the reporter gene constructs in wildtype and the different genetic backgrounds, larvae at the L1 and L4 stages (see Aim 1) will be collected and fluorescence microscopy will be used to visualize GFP expression patterns. Relative GFP levels of reporter constructs will be analyzed between stages from CCD images taken with equivalent exposure times (Hong et al. 2000). To obtain a picture in the linear range of detection, multiple exposures will be taken. Image analysis will be

Heading indicates question to be addressed in this section

Experimental plan and Controls:

Determine if the lin-28 3' UTR is sufficient for downregulation by the LIR pathway. Standard molecular biology techniques will be used to generate reporter constructs, consisting of a *col-10* promoter (Hong et al. 2000)-driven GFP coding region under the regulation of either the *lin-28* 3' UTR (Moss et al. 1997) or control (see below) 3' UTR sequences. The *col-10* promoter directs specific expression in the lateral hypodermal cells, thus aiding in the analysis of expression levels. Constructs will be confirmed by sequencing. Germine transformation will be performed by microinjecting reporter constructs along with a marker, which allows for the selection of transformants (e.g., *rol-6 (su1006)*), into wildtype animals. Arrays will be crossed into *lin-4(f);lin-14(t)* and *lin-4(f);lin-14(t);daf-12(rh61)* genetic backgrounds in order to analyze the sensitivity of reporter constructs to the LIR pathway. As outlined in Table 1, a LIR-dependent construct is expected to be downregulated by the L4 stage in wildtype and *lin-4(f);lin-14(t)* genetic backgrounds. However, in a *lin-4(f);lin-14(t);daf-12(rh61)* genetic background, in which the LIR pathway is absent, a LIR-dependent construct is expected to remain elevated in the L4 stage. Southern blotting with DNA from transgenic strains will be performed to confirm the presence and abundance of the transgenes.

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Presents overall approach, but not lost in experimental details

Interpretations: The working model predicts that the 3' UTR is sufficient for both the *lin-4*-dependent expression and LIR mechanisms. Therefore, according to the model, the temporal pattern of expression of the *col-10-GFP:lin-28 3'UTR* reporter construct will recapitulate the expression patterns observed for *lin-28* in wildtype and in mutant strains (see Table 1). In particular, the *col-10-GFP:lin-28 3'UTR* construct is expected to be downregulated in a wildtype and *lin-4(f);lin-14(t)* genetic backgrounds in which the LIR pathway is functional, but not in the LIR-deficient genotype, *lin-4(f);lin-14(t);daf-12(rh61)*. If, on the other hand, the 3' UTR is not sufficient to faithfully reproduce *lin-28* expression, then these data would indicate that other elements in either the 5' UTR or in the LIN-28 protein are responsible for LIR. For example, downregulation of *lin-28* may require access of the affected nascent polypeptide to protein degradation machinery, and hence may require motifs akin to the "destruction box" for ubiquitin-mediated proteolysis for targeted LIN-28 protein degradation. If

Expected result, given the model

Reasonable possibility if expected result not found

Experimental plan and Controls:

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Anticipates potential pitfall

Illustrates what is necessary to obtain reliable data

General Tips

1. Look at successful proposals.
2. Have a good idea.
3. Know the literature, issues, questions/controversies in the area.
4. Instead of just feedback, try feed forward, where you discuss your ideas with others before beginning the writing process.
5. Place the work in a broader perspective, indicating significance.
6. Use clear and concise writing style.
7. Proofread - zero tolerance for typos, formatting & citation errors
8. Critique your own proposal.
9. Have other critique your proposal.
10. Prepare your proposal early.

Plagiarism

Two useful websites that define plagiarism and provide tips on how to avoid it in your writing.

<http://www.indiana.edu/~wts/pamphlets/plagiarism.shtml>

<http://www.unc.edu/depts/wcweb/handouts/plagiarism.html>

- For Advanced Genetics, proposal should have
- hypothesis that will be tested
 - some component of genetics

Next Monday, in Small Group Discussion Sections

Long Chain FA Proposal
Neurotrophic Factors Proposal

- For each component of the proposals, what are the positives and negatives in the authors execution of the section.
- Is the writing clear as to what the author is proposing?
- Are you convinced it is a significant problem?
- Do the experiments address the issues/questions?
- Are you convinced that the author can execute the proposed studies?
