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

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

Aim 3. Determine if LIREs in the \textit{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.

Overall Significance of Aims. First identified in the \textit{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 \textit{let-7} miRNA (Pasquinelli et al. 2000). Although, it has been demonstrated that the miRNA \textit{lin-4} regulates \textit{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.
(2) BACKGROUND AND SIGNIFICANCE

Temporal Patterning

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\(^1\)) 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 Ruvkun 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 L2-L3 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-28(\textit{lf})* animals, lateral hypodermal seam cells omit the S2-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 al. 1997).

\(^1\) L1 through L4 refer to chronological larval stages distinguished from each other by molts, while S1 through S4 refer to the corresponding developmental programs as they normally occur in wild type animals (Ambros and Horvitz 1984)
al. 1997). Therefore, both the appropriate expression of lin-28 during L1 and L2 as well as the subsequent downregulation during L3 are critical for the progression through the early larval stages and entry into L3, respectively.

**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 miRNA 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.

**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). Developmental timing is
governed by the LIR pathway in
animals lacking lin-4 and with a
temperature sensitive allele of
lin-14 (lin-4(lf); lin-14(ts)). In
this genotype, lin-28 is
downregulated normally,
preumably by the LIR pathway
alone (Moss et al. 1997).

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

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Phenotype</th>
<th>LIN-28 Protein^</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wildtype</td>
<td>normal</td>
<td>HIGH LOW</td>
<td>Moss, 1997</td>
</tr>
<tr>
<td>lin-4(lf)</td>
<td>retarded</td>
<td>HIGH HIGH</td>
<td>Moss, 1997</td>
</tr>
<tr>
<td>lin-4(lf);lin-14(ts)</td>
<td>normal</td>
<td>HIGH LOW</td>
<td>Moss, 1997</td>
</tr>
<tr>
<td>lin-4(lf);lin-14(ts);daf-12</td>
<td>retarded</td>
<td>HIGH HIGH</td>
<td>Ambros, personal communication</td>
</tr>
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</table>

1 lin-28 protein levels during the L1 and the L4 stage of larval development  
2 lin-14 (n179ts) allele is a temperature sensitive mutation  
3 daf-12(rh61) allele, class 1 mutation (Antebi et al, 2000)
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 (Antebi 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 (Antebi 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* (Antebi et al. 2000) is similar to that of *lin-28(gf)* mutants.

Because *lin-4* is involved in *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 (Reinhart et al. 2000). Also, *let-7* mutant animals do not have elevated *lin-28* expression in a wildtype or a *lin-4(lf);lin14(ts)* genetic background, in which developmental timing depends on LIR (V. Ambros, personal communication).

**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.

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

High, medium and low levels of LIN-14 and LIN-28 protein specify L1, L2, and L3 stage-specific programs, respectively. At the L1-L2 transition, *lin-4* repression of *lin-14* and *lin-28* results in reduced levels of LIN-14 and LIN-28 (to ‘medium’). Feedback between *lin-14* and *lin-28* is necessary for L2-specific events. It is proposed herein that the LIR mechanism, downstream of *daf-12*, represses *lin-28* further to reduce LIN-28 levels (to low or ‘off’), which is necessary for the L2-L3 transition. Model adapted from Moss, 1997.
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(ts)). 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.
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 misexpression 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(lf);lin-14(ts) 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(lf);lin-14(ts) 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.

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.

**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. Germline 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(lf);lin-14(ts)* and *lin-4(lf);lin-14(ts);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(lf);lin-14(ts)* genetic backgrounds. However, in a *lin-4(lf);lin-14(ts);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
performed using Adobe Photoshop and NIH image to subtract background fluorescence, normalize images, and determine the intensity of GFP fluorescence.

Negative controls will include strains with no reporter constructs and strains expressing a reporter construct with the \textit{unc-54} 3’ UTR, which is not temporally regulated during larval development (Wightman et al. 1993; Reinhart et al. 2000). For positive controls, the complete \textit{lin-28} construct (Moss et al. 1997) and a reporter construct under the control of \textit{lin-14} 3’ UTR (will be used, which has been found to be temporally regulated (Wightman et al. 1993).

\textit{Identify specific LIREs in the \textit{lin-28} 3’ UTR.} If the above assay indicates that the intact \textit{lin-28} 3’ UTR is sufficient to confer LIR sensitivity to the \textit{col-10-GFP-lin-28(3’UTR)} transgene, then specific 3’UTR regions sufficient for LIR will be identified by repeating the assay with truncated 3’UTR constructs. Mutational analysis will be performed using the \textit{col-10-GFP-lin-28(3’UTR)} construct. Downregulation of \textit{col-10-GFP-lin-28(Δ 3’UTR)} will be determined by examining GFP expression as described above. To test whether specific elements are sufficient for LIR, the putative LIRE will be fused to the \textit{unc-54} 3’ UTR and temporal downregulation of this reporter construct will be determined. The \textit{unc-54} 3’ UTR by itself is not temporally downregulated. For controls, a transgenic line expressing \textit{col-10-GFP-lin-28(3’UTR)} with a complete \textit{lin-28} 3’ UTR as well as a gain-of-function transgenic strain in which the \textit{lin-4} complementary sequences have been deleted from the 3’ UTR of \textit{lin-28} (Moss et al. 1997) will be used.

\textbf{Interpretations:} The working model predicts that the 3’ UTR is sufficient for both the \textit{lin-4}-dependent repression and LIR mechanisms. Therefore, according to the model, the temporal pattern of expression of the \textit{col-10-GFP-lin-28(3’UTR)} reporter construct will recapitulate the expression patterns observed for \textit{lin-28} in wildtype and in mutant strains (see Table 1). In particular, the \textit{col-10-GFP-lin-28(3’UTR)} construct is expected to be downregulated in a wildtype and \textit{lin-4(lf);lin-14(ts)} genetic backgrounds in which the LIR pathway is functional, but not in the LIR-deficient genotype, \textit{lin-4(lf);lin-14(ts);daf-12(rh61)}. If, on the other hand, the 3’ UTR is not sufficient to faithfully reproduce \textit{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 \textit{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 degredation. If
so, then downregulation would be observed with the complete \textit{lin-28} reporter but not with the \textit{col-10-GFP-lin-28(3'UTR)} reporter constructs, which would lack such elements.

It is expected that one or more deletion constructs will be identified that maintain elevated expression at the L4 stage. This would indicate that the constructs are insensitive to LIR. \textit{lin-4}-dependent repression may be able to compensate for the absence of LIREs. If so, it is expected that \textit{col-10-GFP-lin-28(ALIRE)} constructs would be downregulated in wildtype animals but not in a \textit{lin-4(lf);lin-14(ts)} genetic background. Overlapping deletion constructs, or smaller deletions will aid in identifying minimal LIREs. If the entire 3’ UTR of \textit{lin-28} is found sufficient for complete repression but deletion constructs are not found that ablate the LIR pathway for \textit{lin-28} downregulation, then that would indicate a complex element structure or the involvement of multiple elements that are not clustered together. Identification of new genes in \textbf{Aim 4} in the LIR pathway to repress \textit{lin-28} may aid in the characterization of the molecular mechanism of this translational repression.

\textbf{Alternate Methods and Limitations:} Although live-cell imaging with GFP is preferable, if fluorescence levels are too faint, immunofluorescence analysis with an anti-GFP antibody can be performed. This allows for amplification of the GFP signal. It is important to note that relative expression levels will only be compared within an experiment due to inherent variation in the detection methods between experiments. As an alternate method to mutational analysis of the 3' UTR, the genetic screen described in \textbf{Aim 4} will be performed in which gain-of-function mutations in \textit{lin-28} can be identified. This is an unbiased way to identify 3’ UTR elements required for appropriate \textit{lin-28} downregulation. For proof of principle, gain-of-function \textit{lin-14} alleles have been identified in genetic screens which have mutations in the 3’ UTR. These two approaches in parallel increase the likelihood that potential LIREs will be identified.

\textbf{Aim 3. Determine if LIREs in the \textit{lin-28} 3'UTR are required for developmental timing during larval development.}

\textbf{Rationale:} If a specific LIRE is identified from mutational analysis performed in \textbf{Aim 2}, then my strategy will be to test the function of this element in the regulation of endogenous \textit{lin-28} expression. One question that will be addressed is whether \textit{lin-4} and LIR represent entirely independent pathways. If LIRE sequences are found (in the above experiments) to be distinct from the \textit{lin-4} complementary
elements, then I will address whether the LIR pathway is required for repression of lin-28 by lin-4. Note that LIR is defined as an activity that can repress lin-28 in the absence of lin-4, but we do not know if lin-4 can repress lin-28 in the absence of LIR activity. If the LIR pathway is required for lin-28 repression in normal development, then the omission of LIRE(s) from the lin-28 3’UTR is expected to confer a lin-28 gain-of-function heterochronic phenotype, equivalent to the daf-12(rh61) retarded phenotype.

**Experimental plan and Controls:** The strategy is similar to **Aim 2**. Transgenic strains will be made which express lin-28::GFP with putative LIREs deleted from the 3’ UTR of lin-28. Modifications will be introduced into the rescuing lin-28::GFP construct (pVT#218, Moss et al. 1997), in which GFP has been inserted into the last codon leaving the coding and regulatory regions intact. Standard molecular biology techniques will be used to generate constructs in which putative LIREs are deleted from the 3’ UTR of lin-28::GFP. Germline transformation in lin-28 null animals will be performed as in **Aim 2**. For confirmation, sequences identified as putative LIREs will be deleted from a lin-28 construct not fused to GFP.

Transgenic lines will be generated for each construct and expression will be verified by examining GFP fluorescence in L1-stage larvae. Southern blot analysis will be performed to confirm the presence and determine the abundance of transgenes. Downregulation of lin-28 will be determined in L3 and L4 animals, when LIN-28 is normally undetectable in wildtype animals. Protein levels can be confirmed by western blot analysis. The formation of lateral alae and the number of hypodermal seam cells in L4-stage animals and adults will be scored to assess precocious and retarded heterochronic phenotypes, respectively. Arrays will be crossed into the daf-12(rh61), lin-4(lf);lin-14(ts), and lin-4(lf);lin-14(ts);daf-12(rh61) genetic backgrounds to analyze the effect of the deletion on lin-4-dependent and lin-4-independent repression of lin-28.

For controls, a transgenic line expressing lin-28::GFP with a complete 3’ UTR as well as a gain-of-function line in which the lin-4 complementary sequences have been deleted from the 3’ UTR of lin-28::GFP (Moss et al. 1997) will be used.

**Interpretations:** LIREs are predicted to be necessary for the appropriate downregulation of lin-28 and for the progression through the L2 and L3 stages in wildtype animals. The removal of LIRE(s) from the lin-28 3’ UTR is expected to give a retarded heterochronic phenotype equivalent the phenotype of
daf-12(rh61) mutants. If a lin-28 (ΔLIRE)::GFP expressing strain reiterates the S2 program, this would provide strong evidence supporting the hypothesis that the LIR pathway is required for the L2-L3 transition. However, if lin-4 is functionally redundant with LIR, then lin-4 may be able to compensate and sufficiently downregulate lin-28 such that no heterochronic phenotype is observed in lin-28 (ΔLIRE)::GFP animals. This possibility would be addressed by crossing the array into a lin-4(lf);lin-14(ts) strain in which the potentially redundant lin-4 pathway is absent. If a retarded phenotype is observed in a lin-4(+) background, then I will conclude that repression by lin-4 may also require factors associated with LIR activity. If this phenotype is only observed in a lin-4(lf); lin-14(ts) background, I will conclude that that lin-4 can act independently of the LIR.

Alternate Methods and Limitations: are the same as those described in Aim 2.

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

**Rationale:** To identify genes involved in the LIR pathway for lin-28 downregulation, I will perform a screen for heterochronic mutants in a lin-4(lf);lin-14(ts) genetic background where normal developmental timing is governed by the LIR pathway. A strain with a col-19::GFP transgene specifically expressed in the hypodermis of adult animals and not in larvae or embryo will be used. This transgene is regulated by the activity of heterochronic gene pathway and therefore is a useful tool to efficiently identify precocious or retarded mutants (Abrahante et al. 1998). This screen may identify known translational repressors or novel genes, including miRNA-encoding genes, and/or novel alleles of lin-28 that confer a gain-of-function phenotype may be identified.

**Experimental plan.**

**Mutagenesis.** lin-4(lf);lin-14(ts);col-19::GFP animals will be mutagenized with EMS (ethane methylsulfonate). Subsequently, F1 progeny of mutagenized hermaphrodites will be screened for dominant mutations and F2 progeny will be screened for recessive mutations.

**Identification of Mutants.** Initial screening will be performed with a dissecting epifluorescent microscope to visualize GFP expression in L3 larvae (to identify precocious mutants) or the absence of GFP expression in adult animals (to identify retarded mutants). Since the purpose of this screen is to select for mutations that result in the inappropriate regulation of lin-28, animals will be analyzed for
phenotypes resembling lin-28 gain-of-function or loss-of-function mutants. Accordingly, for secondary screening, mutants will be analyzed with DIC microscopy for seam cell defects as well as for semi-Dpy and egg laying defective phenotypes, characteristic of lin-28 mutants. Alternatively, mutants with supernumery seam cells due to abnormal hypodermal cell proliferation, characteristic of lin-28(gf) mutations, will be selected. Mutants will be outcrossed to test for a phenotype in the absence of col-19::GFP and mutations in lin-4 and lin-14.

Genetic Analysis of Newly Isolated Mutants. To facilitate the mapping of mutated genes, mutants will be crossed with C. elegans isolate from Hawaii, CG4856, as described by Wicks et al. (Wicks et al. 2001). This approach takes advantage of single nucleotide polymorphisms which alter restriction enzyme sites (“snip-SNPs”) in CB4856 relative to the wildtype parent strain used for all other experiments herein, C. elegans var. Bristol N2. Lysates will be prepared from F1 or F2 animals that display the mutant phenotype (homozygous mutants) or that are wildtype (homozygous wildtype or heterozygous). Multiple PCR reactions with the two samples will performed using primers designed to amplify sequences which flank snip-SNPs--one for each arm of the chromosomes and one for the center regions (Wicks et al. 2001). The relative levels of the CB4856–derived product and the N2–derived product will allow for the determination of map position. For example, a SNP allele from CB4856 that is closely linked to the wild type allele of a mutation being mapped will be underrepresented in (or absent from) the PCR products from mutant animals. Once linkage to a chromosomal region is established, more precise mapping will be performed using primers corresponding to additional snip-SNPs in the region.

After snip-SNP mapping, has narrowed down the location of a mutation a chromosomal region, complementation analysis will be performed to test for new alleles of known genes. New gene will be identified by two approaches: cosmid rescue will be performed using cosmid DNA from the gene, and for regions where a battery of RNAi feeding strains are available (Fraser et al. 2000) these will be used. The mutant phenotype can be replicated by RNAi with a candidate gene.

Initial Functional Analysis of Newly Isolated Mutants. First, inferences can be made from BLAST analysis of the predicted coding region. Second, temporal and spatial expression patterns will be examined. The coding sequence of the newly isolated gene will be fused to a GFP reporter and this construct will be microinjected into mutant worms to test for rescuing activity. Fluorescence analysis during early larval development will be performed to determine temporal and spatial patterns of expression. Third, epistatic analysis will be performed to analyze the genetic relationship between the
newly isolated gene and other relevant genes in the heterochronic gene pathway, such as lin-4, lin-14, lin-28 and daf-12. Additionally, if temperature-sensitive mutations are identified, temperature shift analysis will be performed to determine when the putative lin-28 regulator acts.

**Interpretations:** It is predicted that additional genes involved in the LIR pathway will be identified. In support of this prediction, the effects on developmental timing and lin-28 expression of the rh61 non-null allele of daf-12 have been proposed to be due to the interference with the activity of daf-12-interacting proteins (Antebi et al 2000). Such interacting proteins may be identified in this screen. Potential candidate genes are novel miRNAs and translational repressors containing putative RNA binding domains, such as members of the STAR subfamily (Preiss and Hentze 1999). If newly isolated candidate genes lack an RNA binding motif, then it is expected that multi-protein complexes may be required, as is the case for Nanos and Pumilio-dependent repression of the hunchback mRNA in Drosophila. Because both lin-28 and daf-12 are expressed in phenotypically-affected lineages, it is predicted that putative lin-28 regulators will similarly expressed (e.g., hypodermal seam cells, muscle and neurons). Further, it is expected that expression of putative lin-28 regulators will be detected after the mid to late L1 stage with strong expression in the L2 stage. If expression is detected in late embryos or early L1s, then that would suggest the presence of factors which prevent the early downregulation of lin-28.

**Alternate Methods and Limitations:** A inherent limitation of this approach is that redundant pathways may prevent the identification of genes by forward genetic screens. Another potential pitfall is the possibility that alleles that result in a desired phenotype could be rare. For example, if a particular phenotype requires a missense change or a special kind of regulatory mutation then it may be infeasible to screen enough animals required for the identification of such a gene. However, one advantage of this screen is that dominant mutations can be identified. The ability to screen more F1s for dominant mutations increases the probability that a rare mutation will be found.

If no new genes are identified in the LIR pathway, then that would raise the possibility that daf-12 may regulate lin-28 translation directly. To address this, direct binding of DAF-12 to elements in the 3’ UTR of the lin-28 mRNA would be tested. The effect on lin-28 3’ UTR binding of the presence or absence of the daf-12 ligand binding domain would also be analyzed. However, these experiments are beyond the scope of this proposal.
STATISTICAL METHODS. For the comparisons of LIN-28 protein levels in wildtype and daf-12(rh61) mutant lysates at various stages describe in AIM 1, replicates will be analysed by standard ANOVA and t-tests to determine statistical significance of potential differences. Similar analysis will be performed to compare GFP reporter gene expression levels in experiments in AIM 2 and 3.

HAZARDOUS MATERIAL. All biological, chemical or radioactive hazardous material will be handled following standard safety guidelines, as regulated by the Dartmouth Office of Environmental Health and Safety.

RESEARCH TIMELINE. The experiments outlined in the first Aim is expected to be completed within the first year after which the experiments outlined in the second and third Aim will be initiated. These experiments are expected to require 1-2 years for completion. The genetic screen in Aim 4 will be performed concurrently with the experiments of the first three Aims.

(4) LITERATURE CITATIONS


30c. RESPECTIVE CONTRIBUTIONS.

The ideas of this research proposal came from early discussions that I had with Dr. Ambros. Because of my inexperience in genetics, I particularly relied on his input regarding the approach for the genetic screen in Aim 3. Otherwise, the research design, methods and presentation were generated independently by me, with feedback provided from Dr. Ambros. It was designed with the purpose of building on previous experience with protein analysis (Aim 1) and extending my training with molecular biology (Aim 2) and genetics (Aim 3) to address an important question in the field of developmental timing.

30d. SELECTION OF SPONSOR AND INSTITUTION.

I chose to do post-doctoral research with Dr. Ambros because of my interests in temporal patterning and to broaden my training in developmental biology using *C. elegans* as a model organism. I became interested in the heterochronic gene pathway following a research seminar during my graduate studies. Although much is known regarding patterning along the spatial axes during embryogenesis, patterning on the temporal axis presented a new and exciting area of research. The molecular mechanisms of developmental timing in *C. elegans* post-embryonic development are beginning to be elucidated but many fundamental questions remain. The recent identification of heterochronic gene orthologs in mammals, including humans, indicates that conserved mechanisms from worms to humans are likely to be involved in temporal patterning, as is true for spatial patterning mechanisms.

Dr. Ambros and his lab have contributed seminal work in the field of developmental timing and has been responsible for the identification and characterization of many novel genes in the heterochronic gene pathway. This work has resulted in a high-impact publication record. Dr. Ambros is a leader and pioneer in this field, with an excellent track record of mentoring post-doctoral fellows, making him an ideal choice for a sponsor.

The environment at Dartmouth Medical School is highly conducive to my training. Core imaging and molecular biology laboratories will provide the necessary resources to perform the research outlined in this proposal. Additionally, the newly established Department of Genetics will bring together leading researchers studying diverse systems from yeast to mice. In addition to interacting with colleagues at Dartmouth, the Ambros lab participates in regular Boston area meetings of *C. elegans* researchers as well as annual regional or international meetings. I believe that working
with Dr. Ambros at Dartmouth provides an exceptional opportunity to develop the skills of an academic scientist.