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# The time of appearance of the *C. elegans let-7* microRNA is transcriptionally controlled utilizing a temporal regulatory element in its promoter

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**Abstract**

MicroRNAs (miRNAs) are a large family of small regulatory RNAs that are poorly understood. The *let-7* miRNA regulates the timing of the developmental switch from larval to adult cell fates during *Caenorhabditis elegans* development. Expression of *let-7* RNA is temporally regulated, with robust expression in the fourth larval and adult stages. Here, we show that, like *let-7* RNA, a transcriptional fusion of the *let-7* promoter to *gfp* is temporally regulated, indicating that *let-7* is transcriptionally controled. Temporal upregulation of *let-7* transcription requires an enhancer element, the temporal regulatory element (TRE), situated about 1200 base pairs upstream of the start of the mature *let-7* RNA. The TRE is both necessary and sufficient for this temporal upregulation. A TRE binding factor (TREB) is able to bind to the TRE, and a 22-base pair inverted repeat within the TRE is necessary and sufficient for this binding. We also find that the nuclear hormone receptor DAF-12 and the RNA binding protein LIN-28 are both required for the correct timing of *let-7* RNA and *let-7::gfp* expression. We speculate that these heterochronic genes regulate *let-7* expression through its TRE.


*Keywords:* Heterochronic gene; miRNA; *let-7*; stRNA; Transcription; *C. elegans*

## Introduction

Development is a four-dimensional process which proceeds along the three spatial axes as well as the temporal axis of time. As with the spatial axes of development, genes govern the temporal axis. Genes that regulate the timing of development are called heterochronic genes (Banerjee and Slack, 2002), one of which, *let-7,* acts late in *C. elegans* postembryonic development. Hypodermal seam cells (lateral skin cells) divide at each larval stage with a stem cell-like pattern and terminally differentiate after the fourth larval (L4) stage, secreting a cuticular structure known as alae. In a *let-7* mutant, seam cells inappropriately reiterate L4 patterns of cell fate at the adult stage (Reinhart et al., 2000). *let-7* is thus required for the normal progression of cell fates during the L4 to adult transition.

*let-7* encodes an untranslated 21-nucleotide RNA. Northern blot analysis has shown that *let-7* is temporally regulated: the 21-nucleotide *let-7* product makes its appearance weakly in the L3 stage and then increases to a stronger presence in the subsequent L4 and adult stages (Reinhart et al., 2000). *let-7* is conserved from nematodes to humans (Pasquinelli et al., 2000), and the temporal regulation of *let-7* during development has also been shown to be conserved in other species, including *Drosophilia* and zebrafish (Pasquinelli et al., 2000). It has been suggested that *let-7* may be a temporal regulator in these species as well.

*let-7* is a founding member of a family of genomically encoded small RNAs called miRNAs (Lagos-Quintana et al., 2001; Lau et al., 2001; Lee and Ambros, 2001). Since *let-7* is temporally regulated, it is referred to as a small temporal RNA (stRNA) (Pasquinelli et al., 2000; Reinhart et al., 2000). *let-7* is not the only heterochronic gene in *C. elegans* that codes for a stRNA; *lin-4,* the most upstream known gene in the heterochronic pathway, also codes for a

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stRNA (Lee et al., 1993). Like *let-7*, *lin-4* is also temporally regulated but its product accumulates during the L1 stage of development (Feinbaum and Ambros, 1999). Like other miRNAs, *let-7* and *lin-4* are both transcribed as 60- to 70-nt pre-RNAs that are predicted to fold into stem loop structures. The pre-RNA is then processed by the RNase DCR-1, such that 21 nucleotides in the proximal arm of the stem are cleaved out and persist to represent the mature *let-7* and *lin-4* stRNAs (Grishok et al., 2001; Hutvagner et al., 2001; Ketting et al., 2001).

*let-7* has been shown to negatively regulate the heterochronic gene, *lin-41* (Slack et al., 2000). It is believed that the stRNA product of *let-7* binds to complementary sites in the 3′ untranslated region (3′ UTR) of the *lin-41* mRNA and prevents translation of the message. A LIN-41::GFP fusion protein is expressed in all stages, from late embryogenesis into adulthood, in many tissues, but is downregulated in the hypodermal seam cells in the beginning of the L4 stage coincident with *let-7* upregulation (Slack et al., 2000). Fusion of the *lin-41* 3′UTR downstream of a *lacZ* reporter construct is sufficient to cause downregulation of *lacZ*, resulting in the *let-7*-dependent reduction of $\beta$-galactosidase in the L4 and adult stages (Slack et al., 2000). Interestingly, in addition to *lin-41*, the 3′ UTRs of several other members of the heterochronic pathway contain *let-7* complementary sites (Reinhart et al., 2000). The other stRNA, *lin-4*, negatively regulates *lin-14* (Lee et al., 1993; Wightman et al., 1993) and *lin-28* (Moss et al., 1997). The *lin-4* stRNA inhibits the production of LIN-14 protein through binding to seven *lin-4* complimentary sites in the *lin-14* 3′UTR and preventing the translation of the message (Olsen and Ambros, 1999).

*lin-4* and *let-7* RNAs first accumulate during the first and fourth larval stages, respectively. They act as timing switches to initiate a cascade of developmental changes in the somatic cells between the L1 and L2 stages (*lin-4*), and the L4 and adult stages (*let-7*) (Feinbaum and Ambros, 1999; Reinhart et al., 2000). Since the appearance of these stRNAs correlates with temporal transitions in cell fates, we can distill the timing of temporal transitions down to the timing of appearance of the stRNAs. However, the mechanism of temporal regulation of *lin-4* and *let-7* is not understood. In this report, we investigate the regulation of *let-7*. Here, we show that the time of *let-7* appearance is transcriptionally controlled, requiring genes upstream of *let-7* in the heterochronic pathway and a temporal regulatory element (TRE) that we have defined in the *let-7* promoter, which is bound by a factor called TREB.

## Materials and methods

### *General methods*

All plasmid and PCR constructs were injected into N2 animals at a concentration of 50 ng/$\mu$l along with the injection marker pRF4 at 100 ng/$\mu$l, except for the *let-7* rescue constructs that were injected at 5 ng/$\mu$l with the injection marker *goa-1::gfp* (pKP13) fusion gene at 85 ng/$\mu$l (Reinhart et al., 2000). Northern blotting was performed as described previously (Reinhart et al., 2000). At least 20 animals were scored for each construct unless noted otherwise. Staging of animals was by gonadal development, except for *daf-12* mutants, which was by vulval development.

### *Plasmid constructs*

pSJ11 was made by amplifying 1.8 kb of genomic sequence (base pairs −1762 to −1) upstream of the mature *let-7* sequence from pBS+let-7, (a plasmid containing the entire *let-7* rescue fragment) (Reinhart et al., 2000) and adding an *Age*I site to the 3′ end using the polymerase chain reaction (PCR) with primers LET7SMJ2 and LET7SMJ3 (for primer sequences, see Table 1). This PCR product was digested with *Age*I and then cloned into the pPD95.70 vector (Fire Lab) digested with *Sma*I and *Age*I. pSJ12 was made by digesting pSJ11 with *Nhe*I and *Hin*cII removing the first 594 bp between −1762 and −1169 bp upstream of the mature *let-7* sequence, filling with Klenow (NEB) and self-ligation. pSJ13 was made by digesting pSJ11 with *Hin*cII and *Eco*RV and religation resulting in the deletion of the first 327 bp between −1762 and −1436 bp upstream of the mature *let-7* sequence. pSJ14 was made by digesting pSJ11 with *Eco*RV and *Nhe*I, resulting in a 266-bp internal deletion between −1436 and −1169 bp upstream of the mature *let-7* sequence, filling with Klenow and self-ligation. pSJ15 was made by digesting pSJ11 with *Eco*RV and *Bst*EII resulting in a 146-bp internal deletion between −1436 and −1290 bp upstream of the mature *let-7* sequence, filling with Klenow and self-ligation. pSJ16 was made by digesting pSJ11 with *Nhe*I and *Bst*EII resulting in a 116-bp internal deletion between −1285 and −1169 bp upstream of the mature *let-7* sequence, filling with Klenow and self-ligation. ΔNarI was made by digesting pSJ11 with *Nar*I to remove a 104-bp fragment followed by religation with T4 DNA ligase (NEB).

pI16 was made by amplifying the 116-bp TRE region for the *let-7* promoter located between −1284 and −1169 bp upstream of the mature *let-7* sequence by PCR using pSJ11 as template with primers 16H3UP and 16S1DN. The PCR product was digested with *Sal*I and *Hin*dIII and cloned into the vector pPD97.78 (Fire Lab) digested with *Sal*I and *Hin*dIII. pI1616 was made by amplifying the same TRE region by PCR using pSJ11 as template with primers 16S1UP and 16X1DN followed by digestion with *Sal*I and *Xba*I. This product was cloned into pI16 digested with *Sal*I and *Xba*I resulting in a plasmid with two tandem copies of the TRE separated by 14 bp.

p2S1 was made by amplifying the 282-bp region of the

Table 1
Oligonucleotide sequences

| Name | Sequence (5′–3′) |
|---|---|
| LET7SMJ2 | GGTACCCTCCCTCTTTTAAGCCTG |
| LET7SMJ3 | CAACAAACCGGTCCGGATCCACAGTGTAGACCGTCC |
| 16H3UP | CCCAAGCTTGGGCGCAATAAAATACCATCAAAAGTG |
| 16S1DN | ACGCGTCGACGTCGCACTGCGTGACACCCGATTAAATTC |
| 16S1UP | ACGCGTCGACGTCGCGCAATAAAATACCATCAAAAGTG |
| 16X1DN | GCTCTAGAGCCACTGCGTGACACCCGATTAAATTC |
| 2S1DN | ACGCGTCGACACAGCAAAAAACGGCTGCACGAATG |
| 1S1DN | ACGCGTCGACAAAAAATGGGTCTATATTAATTGCTC |
| PSJ11UP | CCTGCAGGTCGACTCTAGACCATC |
| GFP3UTR | CTATACAACCTACTACCTCACTATTTGTATAGTTCATCCATGCCATGTG |
| PSKDOWN | CCGCGGTGGCGGCCGCTCTAG |
| 3UTRGFP | TGGATGAACTATACAAATAGTGAGGTAGTAGGTTGTATAGTTTGGAATATTAC |
| LET7SMJ5 | TGAAAACTAAAAACACTAACAAAGAATTG |
| LET7SMJ6 | GCTAGCCGTTGCACAGGTCATTG |
| LET7SMJ7 | GACGTAGTAGTGGGTATATCTTTC |
| LET7SMJ8 | CAACACCCATACCTTTCTTATCA |
| LET7SMJ9 | GAACCGCGAGCCACCAAGTTGCTC |
| GFP2C | GGAAACAGTTATGTTTGGTATATTGGG |
| LET7SJ11 | AACTTGGTGGCTCGCGGTTCAAAAAATGGGTCTATATTAATTGC |
| LET7SJ10 | TTAATATAGACCCATTTTTTGAACCGCGAGCCACCAAGTTGCTC |
| LET7IRDN | TTTCGAATCGGATAAAAAAGTTGATGGTATTTTATTGCGGTGAC |
| LET7IRUP | CCGCAATAAAATACCATCAACTTTTTTATCCGATTCGAAAGAGAG |
| TREUP | CGCAATAAAATACCATCAAAAGTG |
| TREIRUP | CGCAATAAAATACCATCAACTTTTTTATC |
| TREDN | CACTGCGTGACACCCGATTAAATTC |
| IRUP | AAGTGGAACAAAAGTTCCATTT |
| IRDN | AAATGGAACTTTTGTTCCACTT |
| RANDUP | CATCTACGCGAATTTAATCGGG |
| RANDDN | CCCGATTAAATTCGCGTAGATG |

*let-7* promoter between −1284 and −1003 bp upstream of the mature *let-7* sequence by PCR using pSK+let-7 (a plasmid containing the entire *let-7* rescue fragment) (Reinhart et al., 2000) as template with primers 16H3UP and 2S1DN followed by digestion with *Sal*I and *Hin*dIII and cloning into pPD97.78 (Fire Lab) digested with *Sal*I and *Hin*dIII. p1S1 was made the same way except primer 1S1DN was used in place of 2S1DN amplifying the 671-bp region from −1284 to −614 bp upstream of the *let-7* coding region.

pΔTRE was made by digesting pSK+let-7 sequentially with *Nhe*I and *Bst*EII followed by Klenow filling and self-ligation resulting in the removal of 118 bp spanning the TRE.

### *PCR constructs*

PCR3 was made by using overlap extension PCR. Briefly, the *let-7* genomic region along with the GFP coding sequence contained in pSJ11 was amplified by PCR using primers PSJ11UP and GFP3UTR. In pSJ11, the PSJ11UP primer is immediately upstream of LET7SMJ2, and GFP3UTR is a primer that contains the reverse complement of the first 20 nucleotides of the sequence encoding *let-7* followed by the reverse complement of the last 29 nucleotides in the GFP coding sequence. The *let-7* 3′UTR including *let-7* itself was amplified from pSK+let-7 by using primers PSKDOWN and 3UTRGFP. In pSK+let-7, PSKDOWN is immediately downstream of the *Bgl*II site. 3UTRGFP is a primer containing the last 20 nucleotides in the GFP coding sequence followed by 33 nucleotides of *let-7* sequence starting with the mature *let-7* product. The two PCR products from these amplifications were used in a PCR reaction along with primers LET7SMJ2 and LET7SMJ5, a primer with the reverse compliment of the last 29 nucleotides of the 3′ end of the *let-7* rescue fragment excluding the *Bgl*II site. The resulting PCR product contains the entire *let-7* rescue fragment with the coding sequence for GFP inserted immediately before the first nucleotide of the mature *let-7* RNA.

PCR2, PCR6, PCR7, PCR8, and PCR9 were made by PCR, amplifying full-length and sequentially shorter sequences from pSJ11 using upstream primers LET7SMJ2, LET7SMJ6, LET7SMJ7, LET7SMJ8, and LET7SMJ9, respectively, along with the downstream primer GFP2C annealing to the 3′ end of the *unc-54* 3′UTR present in pSJ11. The subsequent constructs resulted in 5′ promoter deletions of 0, 593, 760, 1149, and 1432 bp, respectively.

PCRΔ8-9 was made by using overlap extension PCR analogous to the construction of PCR3, but using pSJ11 as the template for both initial PCR reactions using primer combinations LET7SMJ2/LET7SJ11 and LET7SJ10/GFP2C. The final amplification was performed containing the two initial PCR products with additional LET7SMJ2 and GFP2C added to the reaction. The resulting product deleted 283 bp between PCR8 and PCR9. pΔIR was made by using overlap extension PCR analogous to the construction of PCR3, but using pSJ11 as the template for both initial PCR reactions using primer combinations LET7SMJ2/LET7IRDN and LET7IRUP/GFP2C. The final amplification was performed containing the two initial PCR products with additional LET7SMJ2 and GFP2C added to the reaction. The resulting product deleted 22 bp containing the inverted repeat. The PCR product was then cloned into the pCR4-TOPO vector by using the TOPO TA Cloning Kit (Invitrogen) following the manufacturer's instructions. All constructs were sequenced.

### *Electrophoretic mobility shift assay*

TRE and TREΔIR were made by PCR with the upstream oligos TREUP and TREIRUP respectively and the common downstream primer TREDN using pSJ11 and pΔIR plasmids as template, respectively. These PCR products were gel purified on a 3% agarose gel and extracted by using a QIAquick gel extraction kit (Qiagen). IR and RAND were made by annealing together the oligo pairs IRUP/IRDN and RNADUP/RANDDN, respectively, by heating at 95°C for 5 min in 10 mM Tris–HCl, pH 8.0, 10 mM $MgCl_2$, 50 mM NaCl and allowing to slowly cool to room temperature. TRE, TREΔIR, IR, and TRE were end labeled with $^{32}$P-γ-ATP by using T4 polynucleotide kinase (NEB).

Nuclear extract was made from mixed stage N2 animals by homogenization in 50 mM Tris–HCl, pH 7.5, 10 mM potassium acetate, and 5 mM DTT using a dounce homogenizer on ice. After homogenization, the salt concentration was increased to 100 mM potassium acetate and 2 mM magnesium acetate followed by a 20-min incubation on ice and a 30-min, 4°C spin at 13,400*g*. After removing the supernatant, the pellet was resuspended in 50 mM Tris–HCl, pH 7.5, 400 mM potassium acetate, and 2 mM magnesium acetate, vortexed, and incubated on ice for an additional 20 min. A final 30-min, 4°C spin at maximum speed (16,100*g*) resulted in a supernatant which was removed from the pellet and called the nuclear extract.

The electrophoretic mobility shift assay was preformed by incubating the labeled construct or oligo with or without the appropriate unlabeled competitor oligo and the indicated amount of nuclear extract for 20 min in 1× Gel Shift Binding Buffer (Promega). The samples were then run on a 5% polyacrylamide TBE Criterion gel (BioRad) in 0.5× TBE at 100V until the bromophenol blue band was at the bottom in the case of the TRE or three-fourths of the way down in the case of the IR. The gel was dried and exposed to film for between 30 min to 2 h.

## Results

### *A transcriptional fusion of the let-7 promoter to GFP results in temporally regulated GFP expression*

*let-7* is a member of a large class of miRNAs. Little is known about miRNA regulation or function. *let-7,* one of the best understood miRNAs, is temporally regulated in many animals, including *C. elegans,* but the mechanism is unknown. Mature *let-7* is first observed weakly on a Northern blot at the L3 stage in *C. elegans* (Reinhart et al., 2000). Mature *let-7* is processed by DCR-1 from a larger pre-*let-7* transcript, which is also weakly detected at the L3 stage (Grishok et al., 2001). This suggests that *let-7* is likely to be transcriptionally regulated. However, in some animals, such as sea urchins, the processing of *let-7* is temporally regulated (Pasquinelli et al., 2000). To ascertain whether *let-7* is temporally regulated by transcription or by RNA processing and/or stability of pre-*let-7* RNA, we constructed a transcriptional fusion of the *let-7* promoter to the green fluorescent protein (GFP) reporter gene. If *let-7* is temporally regulated by transcription, then we predicted that GFP would be expressed in a temporal pattern recapitulating that of *let-7* RNA. If, instead, RNA processing and/or stability is responsible for the temporal expression pattern of *let-7,* then the GFP would be expressed at all stages.

Two distinct constructs were used to generate transgenic animals. The first construct, pSJ11, contains 1.8 kb of genomic DNA upstream of *let-7* followed by *gfp* and then the heterologous *unc-54* 3′UTR (Fig. 1). pSJ11 does not contain any of the mature *let-7* sequence or downstream sequences, while the upstream sequence includes all the upstream genomic DNA present in a genomic fragment capable of rescuing *let-7* mutants (Reinhart et al., 2000) (this work). The second construct, PCR3, was a PCR product containing the same 1.8 kb of genomic sequence, followed by *gfp*. The *unc-54* 3′UTR was replaced by the sequence for the mature *let-7* product followed by the complete 677-bp endogenous *let-7* 3′ region from the genomic fragment capable of rescuing *let-7* mutants (Fig. 1) (Reinhart et al., 2000). We obtained at least two independent transgenic lines from each construct. All lines showed temporally regulated *let-7::gfp* expression. GFP expression appeared in the seam cells at the very beginning of the L4 stage and continued on through adulthood. In the pSJ11-containing lines, 78% ($n = 32$) of L4 animals showed this expression (Fig. 2). GFP was never seen in the seam cells before the L3-to-L4 transition in a wild-type background (100%, $n = 80$ animals). Seam cell expression of *let-7::gfp* is significant as *let-7* is normally required for seam cell development. In *let-7* mutants, seam cells delay their terminal differentiation.

There was no difference in GFP expression between the endogenous and heterologous 3′UTRs that were used in the two different constructs, indicating that the endogenous

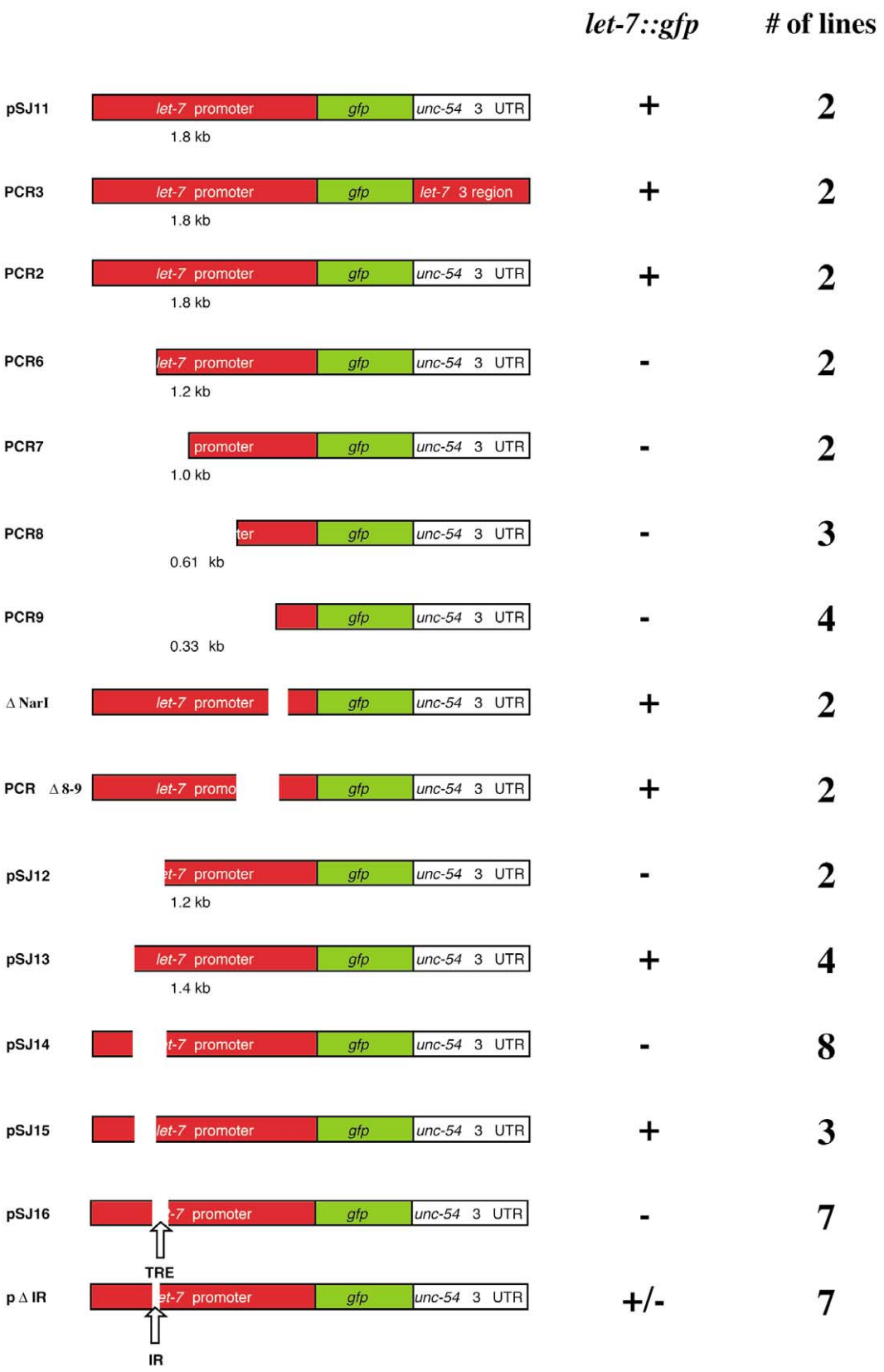


Fig. 1. *let-7::gfp* constructs and expression in transgenic animals. Plasmid and PCR products (denoted PCRX) that were used to generate transgenic animals. The first column shows the name of the construct and the portion of the *let-7* promoter driving GFP expression with either the *unc-54* 3′UTR or the endogenous *let-7* 3′region. The second column reports the expression of transgenic animals carrying the respective constructs: (+) indicates that temporally regulated GFP (*let-7::gfp* expression) is seen in the seam cell in all lines, (−) indicates that no *let-7::gfp* expression is seen in the seam cells at any stage, and (+/−) indicates weak *let-7::gfp* expression seen in some of the lines. The last column indicates the number of lines analyzed for each construct. Both the TRE and the inverted repeat within the TRE are marked by arrows.

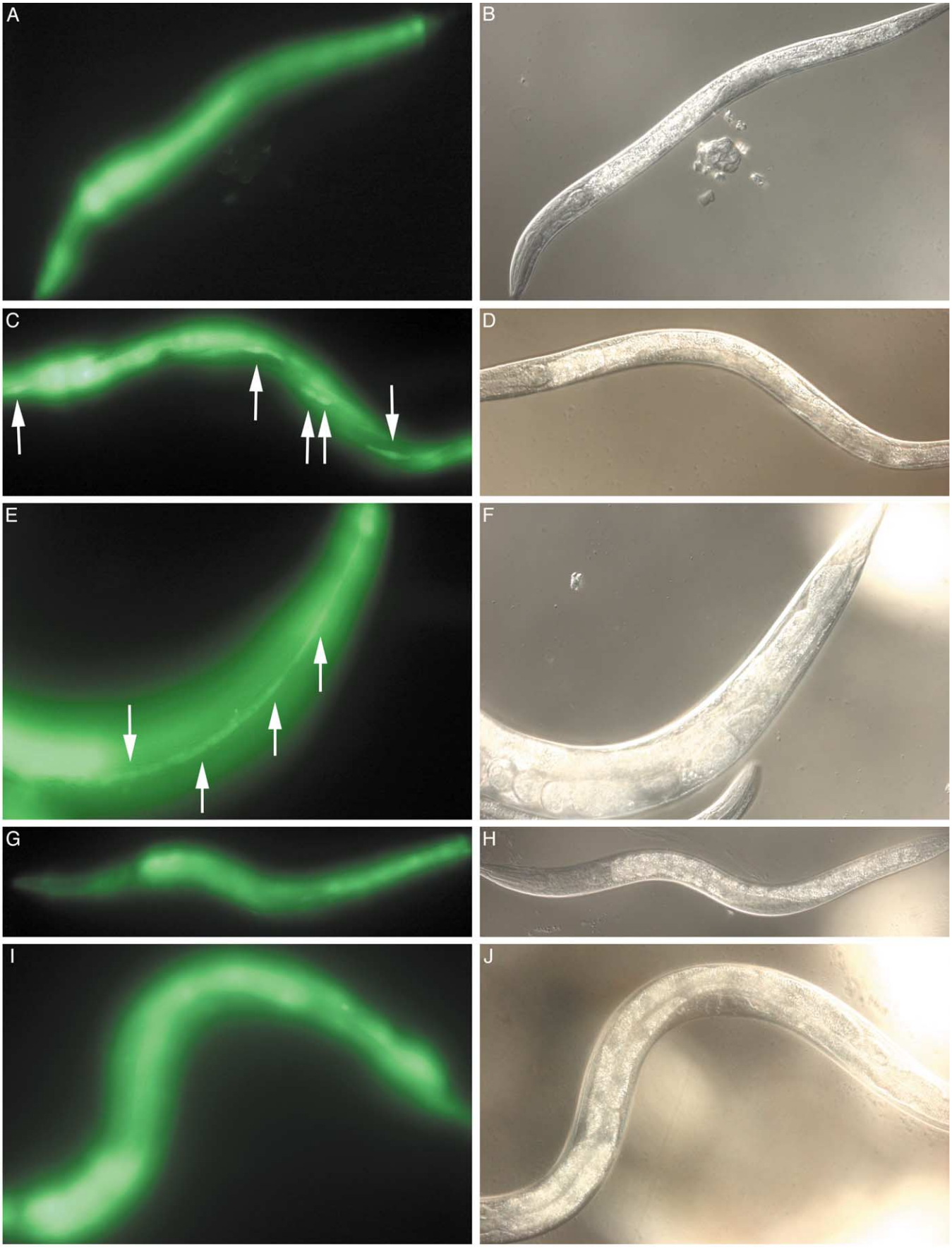


Fig. 2. An example of *let-7::gfp* expression with the full-length promoter and a loss of expression with deletion of the TRE. *zaEx5*, a transgenic line carrying the pSJ11 construct, shows temporally regulated GFP expression in the seam cells with: (A) no expression in the L3 stage, (C) expression beginning at the L4 stage and (E) expression continuing through adulthood. The white arrows mark seam cells in which *let-7::gfp* is being expressed. In the adult, the seam

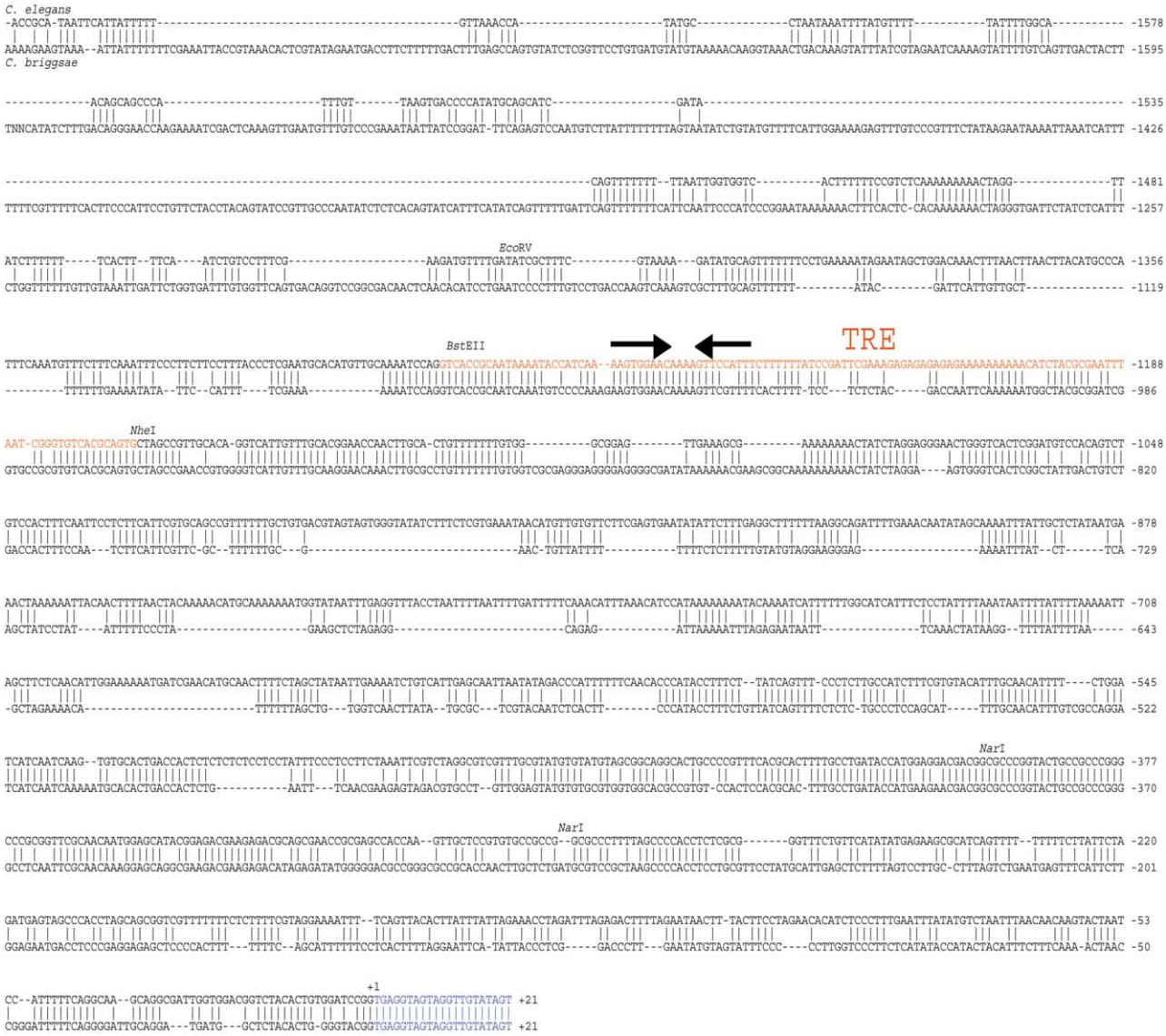


Fig. 3. Comparison of the *let-7* promoters from *C. elegans* and *C. briggsae*. The mature *let-7*-coding sequence is shown in blue, the TRE sequence is shown in red, and the black arrows mark an inverted repeat in the TRE. Positive and negative numbers represent positions in base pairs downstream and upstream of the start of the mature *let-7* product coding sequence, respectively.

mature *let-7* sequence and the *let-7* 3′ region are not required for the temporal regulation of *let-7*. Nontemporally regulated GFP expression was observed in other parts of the worm, including the pharynx, intestinal cells, muscle cells, and neurons. Because both mature product and precursor *let-7* RNA are temporally regulated and are never detected on developmental Northern blots until the late L3/ early L4 stage, and because the heterochronic phene associated with the *let-7* loss-of-function mutants is in the seam cells, we suspect that this unregulated GFP is nonspecific background. This background GFP was not the result of the *rol-6* marker, since this background was not observed with all of our DNA constructs, but we did not test whether this background was due to the high copy number in extrachromosomal arrays. Some promoter deletion lines (see below) did not exhibit this background expression, suggesting that an element(s) in the promoter inappropriately directs this expression. In the remaining studies, we concentrated on the seam cell-specific, temporally regulated *let-7::gfp* expression.

fuses to make one syncitial cell per side. *zaEx11*, a transgenic line carrying the pSJ16 construct, shows no temporally regulated GFP expression in the seam cells at any stage as seen in (G) a L3 animal and (I) a late L4 animal. (B), (D), (F), (H), and (J) are the same worms in (A), (C), (E), (G), and (I), respectively, viewed with DIC optics. All GFP pictures are taken in the focal plane of the seam cells. Animals are shown at 400× magnification with anterior to the left.

*A temporal regulatory element (TRE) in the let-7 promoter directs temporal upregulation of let-7*

To elucidate the *cis*-acting elements responsible for the temporal up-regulation of *let-7*, we compared the *let-7* promoter sequence with that of the closely related nematode, *C. briggsae* (Fig. 3). Numerous regions of the *let-7* promoter are conserved in the *C. briggsae* sequence. To identify the functionally important temporal regions, we made truncations of the 1.8−kb promoter in the pSJ11 plasmid. The full length of 1.8 kb as well as truncations resulting in 1.2, 1.0, 0.61, and 0.33 kb of *let-7* promoter DNA driving the expression of *gfp* and the *unc-54* 3′UTR were made by PCR using the pSJ11 plasmid as template and were named PCR2, PCR6, PCR7, PCR8, and PCR9, respectively (Fig. 1). A minimum of two independent lines was obtained following injection of each PCR construct. The full-length lines displayed seam cell GFP expression identical to that of lines containing the pSJ11 plasmid (Fig. 1, data not shown). However, none of the truncation lines displayed seam cell GFP expression, but the nonspecific background GFP was still observed. These experiments thus identify a *cis*-acting element necessary for temporally regulated seam cell GFP expression that is contained in the 594 bp found between −1.8 and −1.2 kb of the mature *let-7* sequence.

Further analysis of this 594-bp region of the *let-7* promoter was performed by making smaller truncations and internal deletions within this region of the pSJ11 plasmid. Plasmid pSJ13 contained a deletion of the first 327 bp (between −1762 and −1436 bp upstream of the mature *let-7* sequence) of the 594-bp region in the otherwise wild-type promoter *gfp* construct and resulted in temporally regulated seam cell GFP expression (Fig. 1, and data not shown). Plasmid pSJ14 contained an internal deletion removing the last 266 bp of this 594-bp region (between −1436 and −1169 bp upstream of the mature *let-7* sequence), but retaining the genomic sequence upstream and downstream of this internal deletion. This construct failed to express GFP in the seam cells (Fig. 1, and data not shown). Plasmid pSJ15 deletes the first 145 bp of this 266-bp region (between −1436 and −1290 bp upstream of the mature *let-7* sequence) and results in seam cell GFP expression (36%, $n = 50$ animals) (Fig. 1, and data not shown). Plasmid pSJ16 deletes the last 116 bp of this 266-bp region (between −1285 and −1169 bp upstream of the mature *let-7* sequence) and results in loss of seam cell GFP expression (0%, $n = 113$ animals) (Figs. 1 and 2). The seam cell GFP expression patterns produced by these deletions indicate that there is a *cis*-acting element, which we call the temporal regulatory element or TRE, within the 116-bp present in pSJ15 but deleted in pSJ16. Since the TRE is necessary for the temporally regulated expression of *let-7* (Figs. 1 and 3), we speculate that this element is the binding site for one or more transcription factors that are responsible for *let-7* temporal expression.

Within the TRE is 9-bp inverted repeat with one mismatch, the two halves being separated by 4 bp. This inverted repeat is well conserved in *C. briggsae* (arrows, Fig. 3). Since inverted repeats are often sites for transcription factor binding, we examined whether this inverted repeat is required for temporally regulated expression of *let-7*. pΔIR is a constant that contains all 1.8 kb of upstream *let-7* sequence except for a 22-bp internal deletion of the inverted repeat within the TRE (Fig. 1). In six out of seven independent lines generated with pΔIR, very weak seam cell GFP expression was detected (data not shown). This *let-7::gfp* expression showed reduced penetrance when compared with the *let-7::gfp* expression seen in animals containing pSJ11, with expression in only 19% ($n = 107$) of pΔIR L4 and adult animals compared with 78% ($n = 32$) of pSJ11 L4 and adult animals. Also one of the seven pΔIR lines failed to express *let-7::gfp* at all (0%, $n = 15$ L4 and adult animals), whereas all transgenic lines containing constructs with the TRE intact expressed *let-7::gfp*. These data suggest that the loss of the inverted repeat in the TRE results in a decrease in *let-7* expression.

ΔNarI and PCRΔ8-9 constructs contain internal deletions of other regions of the *let-7* promoter that are highly conserved between *C. elegans* and *C. briggsae* (e.g., from bp −399 to bp −295 and from bp −613 to bp −330 in the *C. elegans* sequence, respectively; Figs. 1 and 3). ΔNarI and Δ8-9 transgenic animals showed temporally regulated seam cell GFP expression (Fig. 1, and data not shown). The observations that further truncations down to within 330 bp of the *let-7* mature sequence show no seam cell GFP expression at any time, and that internal deletions close to the *let-7* mature sequence that leave the region from −1.8 to −1.2 kb of the promoter intact do not affect *let-7::gfp* expression, suggest that there is no seam cell specific positive or negative regulatory elements residing within these regions of the promoter, in spite of areas of high sequence conservation between the *C. elegans* and *C. briggsae let-7* sequences. In addition, none of the deletions resulted in merely a delay of *let-7::gfp* upregulation.

*The TRE is sufficient to upregulate temporal expression of let-7*

We determined what portion of the promoter is minimally required or sufficient for tissue specific, temporal upregulation of *let-7::gfp* in the seam cells. We amplified various regions of the *let-7* promoter using PCR and inserted these regions upstream of the *pes-10* minimal promoter driving *gfp* (pPD97.78; Fire Lab). The insert in pI16 contained only the TRE, 116 bp of *let-7* promoter sequence from bp −1284 (the 5′ end of the TRE) to bp −1169 (the 3′ end of the TRE), while pI1616 had two copies of the TRE inserted in tandem (Fig. 4). The insert in p2S1 contained 282 bp of *let-7* promoter sequence from bp −1284 (the 5′ end of the TRE) to bp −1003 (the 5′ end of PCR7) (Fig. 4). The insert in p1S1 contained 671 bp of *let-7* promoter sequence from bp −1284 (the 5′ end of the TRE) to bp

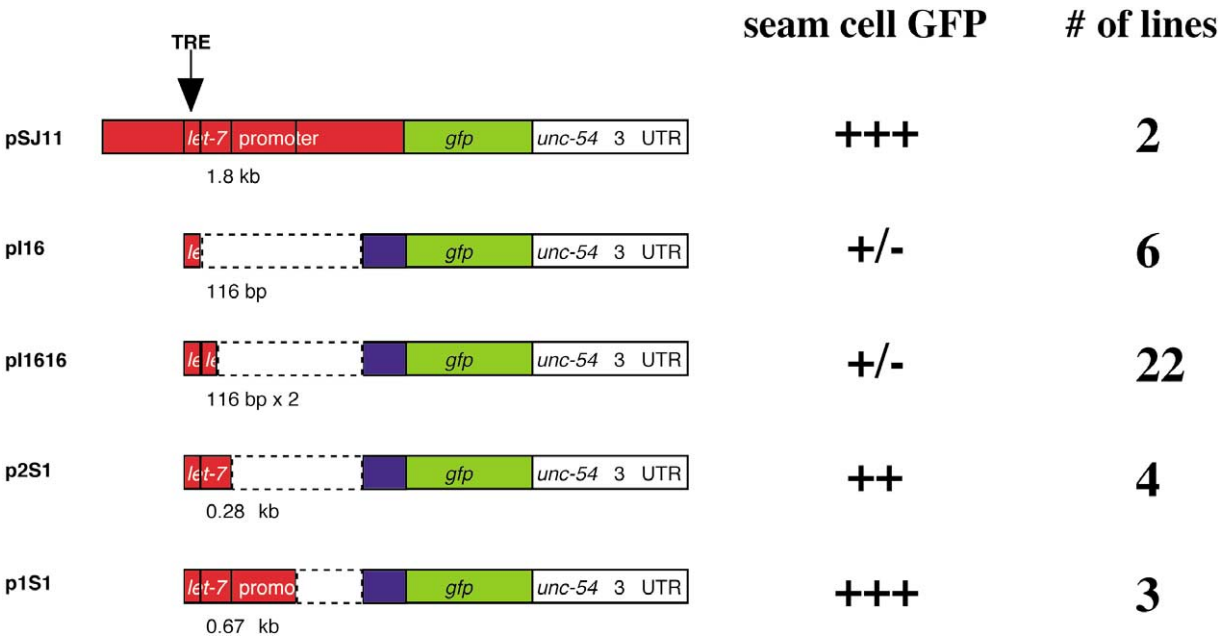


Fig. 4. Sufficiency constructs and *gfp* expression in transgenic animals. The first column shows the name of the construct and the portion of the *let-7* promoter inserted upstream of the *pes-10* minimal promoter (represented in blue) driving *gfp*. pI1616 contains two copies of the TRE. The second column reports the expression of transgenic animals carrying the respective constructs: (+++) indicates strong temporally regulated GFP is seen in the seam cell in all lines, (++) indicates moderate temporally regulated GFP is seen in the seam cell in all lines, and (+/−) indicates weak GFP is seen in the seam cells in some of the lines (experimental scale is different from Fig. 1). The last column indicates the number of lines analyzed for each construct. pSJ11 is shown as a reference for the *let-7* promoter regions used in the sufficiency constructs.

−614 (the 5′ end of PCR8) (Fig. 4). At least three independent lines were established for each construct. We found that all four of these constructs resulted in characteristic seam cell-specific, temporally regulated GFP expression (Figs. 4 and 5). Transgenic worms containing p1S1 displayed very bright, temporally regulated, seam cell GFP expression with high penetrance (93% of animals, $n = 43$) and observed in three out of three lines (Figs. 4 and 5). Transgenic worms containing p2S1 showed highly penetrant, though weaker seam cell-GFP expression that was easily observable above background and temporally regulated so as to be detectable only in the L4 and adult animals [87% of the animals ($n = 85$) and detectable in four out of four lines] (Figs. 4 and 5). Transgenic animals containing pI1616 displayed temporally regulated seam cell GFP expression that was less bright than that seen with p2S1 and with low penetrance (5–10% of animals), but detectable in 17 of 22 lines analyzed (Figs. 4 and 5). Transgenic animals containing pI16 expressed GFP predominately in the seam cells, and this expression was only in L4 and adult animals. The GFP expression was faint and difficult to detect being just above background. In addition, GFP was observed at a very low penetrance (5–10% of animals) and was detected in only three of six lines analyzed (Fig. 4, and data not shown).

These data demonstrate that the TRE is sufficient to temporally upregulate a minimal promoter in the seam cells at the L3-to-L4 transition and that this temporal regulation is confined to the seam cells. Coupled with the previous data, this suggests that the TRE is both necessary and sufficient to upregulate temporal expression of *let-7*. However, our analysis does suggest that some sequence outside of the TRE but contained within the p1S1 construct also contributes to robust expression.

### *Deletion of the TRE in the let-7 rescuing fragment caused loss of full rescuing activity*

*let-7(mn112)* is a null allele and causes all mutant animals to die by bursting through the vulva at the L4 to adult molt. Previously it has been shown that pSK+let-7, a 2.5–kb genomic fragment (from 1.8 kb of genomic sequence upstream to 677 bp downstream of the mature *let-7* sequence), will rescue *let-7* null mutant animals (Reinhart et al., 2000). In order to determine whether the TRE is important in vivo, we examined whether the TRE is required for this rescue. We deleted the 116-bp of the TRE from this rescue fragment (pΔTRE) and injected it or the wild-type rescue fragment into SP231 animals [*let-7(mn112) unc-3(e151)/mnDp1*]. Four independent transgenic lines were made for both the wild-type fragment and the pΔTRE fragment. Transgenic *unc-3* animals were picked from each line and examined for rescue. In both cases, we observed partial rescue of the *let-7(mn112)* lethal phenotype. However, we found that deletion of the TRE from the rescue fragment resulted in less effective rescuing activity. We found that, in both cases, a similar number of transgenic animals became sterile adults. We scored the viable, fertile

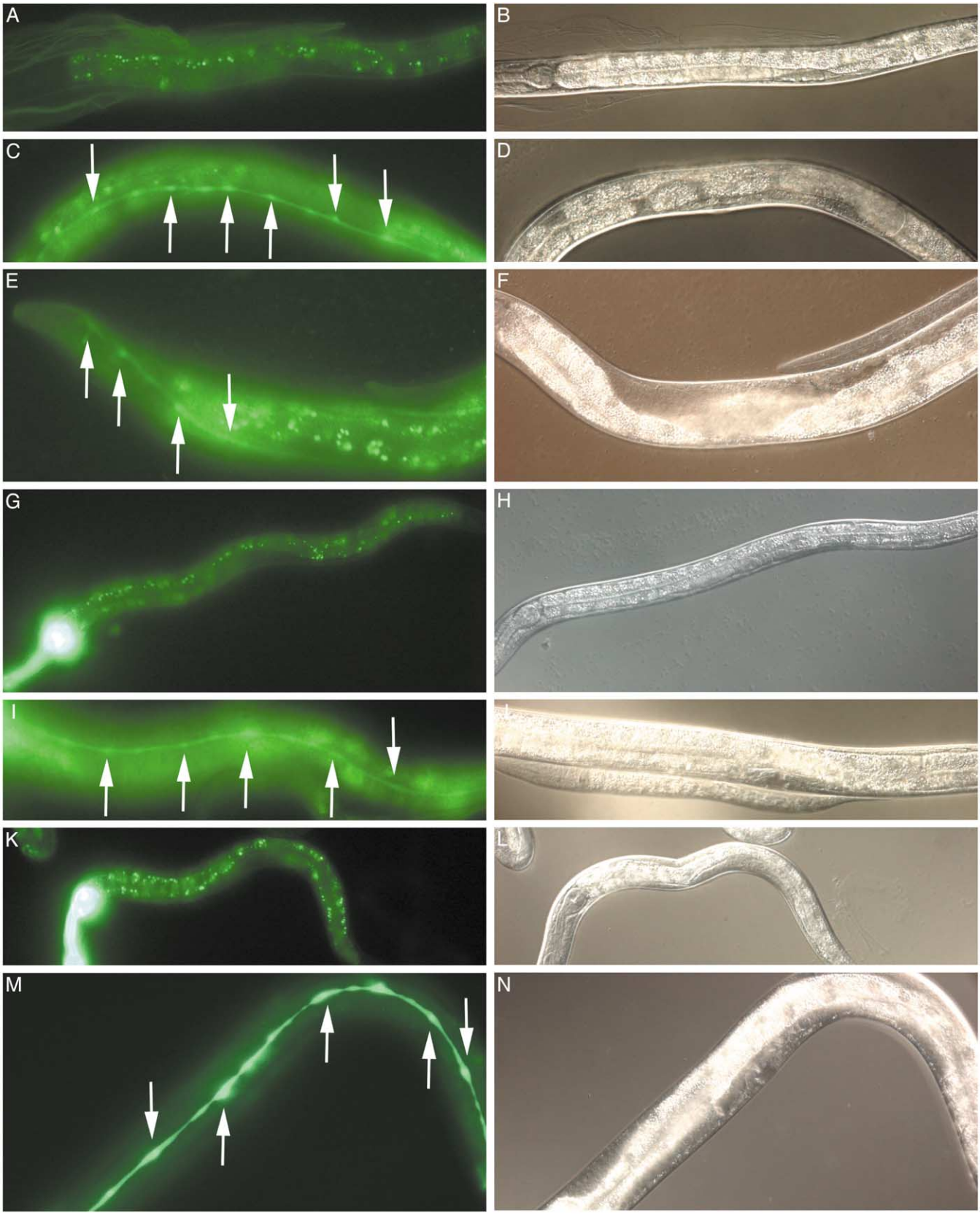


Fig. 5. Examples of TRE directed *gfp* expression from a minimal promoter. *zaEx8* (A–F), *zaEx9* (G–J) and *zaEx10* (K–N), transgenic lines containing the pI1616, p2S1, and p1S1 constructs respectively, show temporally regulated GFP expression in the seam cells with: (A, G, and K) no expression in the L3 stage, (C, I, and M) expression beginning at the L4 stage and (E) expression continuing through adulthood. The white arrows mark seam cells in which *gfp* is being expressed. (B), (D), (F), (H), (J), (L), and (N) are the same animals as (A), (C), (E), (G), (I), (K), and (M), respectively, viewed with DIC optics. Animals are shown at 400× magnification with anterior to the left.

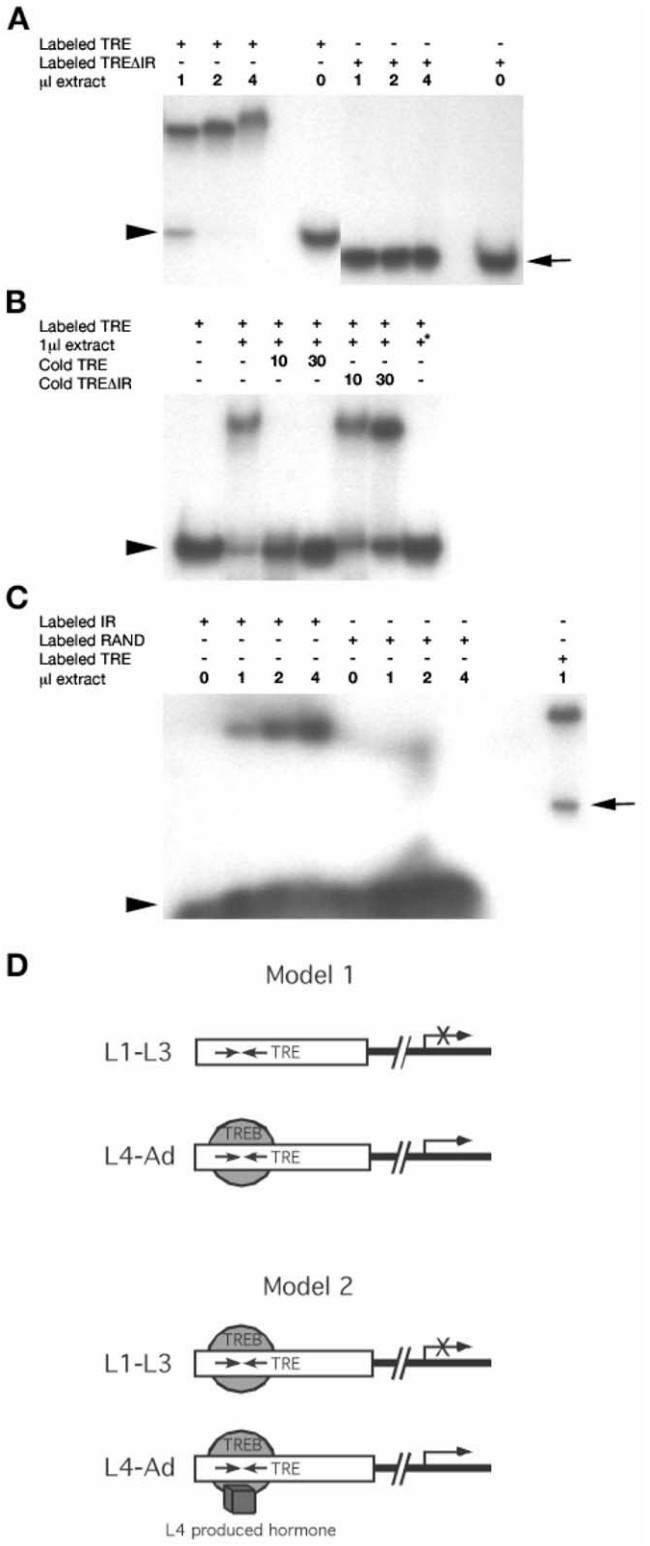


Fig. 6. TREB specifically binds the IR in the TRE. (A) The arrowhead marks free labeled TRE (116 bp) and the arrow marks free labeled TREΔIR (94 bp). Increasing amounts from 1 to 4 $\mu$l (5–20 $\mu$g) of nuclear extract protein cause retarded migration of labeled TRE with increasing amounts of extract resulting in retarded migration of all the labeled TRE. The migration of labeled TREΔIR cannot be retarded by similar amounts of the nuclear extract. (B) The arrowhead marks free labeled TRE. The retarded labeled TRE band can be specifically competed away by addition of 10- or 30-fold (1.2 pmols or 3.6 pmols) of nonlabeled TRE, but not by addition of 10- or 30-fold of nonlabeled TREΔIR. The asterisk indicates nuclear extract that has been heat inactivated for 5 min at 95°C resulting in loss of ability to retard the mobility of labeled TRE. (C) The arrowhead marks free labeled IR (22 bp), and the arrow marks free labeled TRE included for comparison. As little as 1 $\mu$l of nuclear extract (5 $\mu$g protein) is sufficient to retard the migration of labeled IR, but even four times that amount cannot retard the migration of a random 22-bp oligo from another part of the TRE (RAND). (D) Two models of TRE/TREB-induced *let-7* expression. Model 1 proposes that the TRE enhancer is not bound by transcriptional activators in the L1–L3 stages, and that the binding of TREB to the inverted repeat (IR shown by arrows) early in the L4 stage induces expression of *let-7*. In model 2, the TRE is always bound by the TREB, but the TREB is not activated until it is bound by an early L4 produced hormone or other activating factor.

adults for vulval bursting or consummation by their progeny (bag of worms). We found that 24% of *unc-3* animals transgenic for the wild-type rescue fragment ($n = 51$) burst through the vulva or died as a bag of worms. Conversely, 67% of *unc-3* animals transgenic for the pΔTRE fragment ($n = 49$) died in a similar manner. Deletion of the TRE from the rescuing fragment resulted in a greater than twofold decrease in rescuing efficiency. We therefore conclude that the TRE is necessary but not absolutely required for rescuing activity.

### *The inverted repeat in the TRE is necessary and sufficient to bind TREB*

The TRE and the inverted repeat contained therein function in temporal upregulation of *let-7*. We proposed that the TRE is the binding site for a factor (TREB) responsible for this upregulation. We used biochemical techniques to show the existence of a *trans*-acting TRE binding factor (TREB). The 116 bp of the TRE was used in electrophoretic mobility shift assays (EMSA) using nuclear extracts from N2 animals. Whereas end-labeled TRE was efficiently retarded by as little as 1 $\mu$l of nuclear extract (5 $\mu$g total protein), if the 22 bp of the inverted repeat (IR) were deleted from the TRE (TREΔIR), no shift was observed with up to four times the amount of nuclear extract (Fig. 6A). The retarded band could be competed away by the addition of increasing amounts of nonlabeled TRE, but not by nonlabeled TREΔIR, demonstrating that the shift was sequence-specific and that the inverted repeat is necessary for the shift (Fig. 6B). Additionally, heating the extract at 95°C for 5 min abolished the shift of end-labeled TRE, suggesting that TREB is most likely protein (Fig. 6B).

The IR is necessary for TREB to shift the TRE. We determined that the IR is sufficient for TREB binding by demonstrating that TREB could bind the IR by itself and cause a shift. EMSA was performed by using end-labeled IR containing just the 22 bp of the IR. Whereas end-labeled IR was shifted by as little as 1 $\mu$l of nuclear extract, four times the amount of nuclear extract could not shift an end-labeled 22-bp fragment from another part of the TRE chosen at

random (RAND) (Fig. 6C). These data indicate that the inverted repeat contained within the TRE is both necessary and sufficient to result in a shift when bound to TREB, and that TREB is protein or at least contains a necessary protein component which binds specifically to the IR in the TRE (Fig. 6D).

### *The time of appearance of let-7 is controlled by genes known to act upstream of let-7 in the heterochronic pathway*

The simplest interpretation of available genetic data allows many of the known heterochronic genes to be ordered into a linear pathway (Fig. 7A) (Banerjee and Slack, 2002; Slack and Ruvkun, 1997; Slack et al., 2000). The heterochronic genes whose loss-of-function mutations result in precocious phenotypes include *lin-14*, *lin-28*, and *lin-41*, with *lin-14* and *lin-28* acting upstream of *let-7* and *lin-41* acting downstream of *let-7*. Heterochronic genes whose loss-of-function mutations result in retarded phenotypes can be similarly ordered with *lin-4* and *daf-12*, acting upstream of *let-7*, and *lin-29*, acting downstream of *let-7* (Fig. 7A). Developmental Northern blots show that *let-7* first appears weakly in the L3 stage with subsequent strong expression in the L4 and adult stages (Fig. 7B). We hypothesized that, if the heterochronic genes which act upstream of *let-7* in the heterochronic pathway regulate the timing of *let-7* expression, then we would expect an increase in *let-7* expression in the L3 stage of the precocious mutants, and a decrease of *let-7* expression in the L3 and L4 stages of the retarded mutants. To test this hypothesis, we performed developmental Northern blots for *let-7* in each of these precocious and retarded mutant backgrounds.

Developmental Northern blots reveal that both *lin-14* and *lin-28* mutant animals display increased (precocious) expression of *let-7* in the L3 stage. We quantified this information and found an increase of *let-7* expression of more than fourfold over wild-type levels in both cases (specifically, 6.25- and 4.19-fold, respectively) (Fig. 7B). Thus, *lin-14* and *lin-28* are normally required to keep *let-7* upregulation off until the appropriate time in development. The developmental Northern blots of the *lin-4* and *daf-12* animals showed the opposite effect: *let-7* expression is decreased (retarded) in these mutants by a factor of about five in both the L3 and L4 stages. Specifically, in the *lin-4* mutant animals, *let-7* was decreased 5.56- and 7.69-fold when compared with wild-type expression in the L3 and L4 stages, respectively. In *daf-12* mutant animals, *let-7* expression was reduced 4.55- and 4.35-fold when compared with wild-type expression in the L3 and L4 stages, respectively (Fig. 7B). These molecular analyses confirm the predictions made by genetic analysis that *lin-4*, *lin-14*, *lin-28*, and *daf-12* all act upstream of *let-7*, and also show that these genes are required for the correct timing of *let-7* expression.

Substantial precocious or retarded *let-7* expression was not observed in developmental Northern blots of animals mutant for heterochronic genes that genetically act downstream of *let-7*. Both *lin-41* and *lin-29* animals expressed mostly wild-type levels of *let-7* in L3, L4, and adult stages (Fig. 7B) (with the exception of a 3-fold decrease in the L3 stage of *lin-29* mutant animals). Correct timing of *let-7* expression is thus independent of (or not significantly dependent on) the heterochronic genes downstream of *let-7*.

### *daf-12 and lin-28 mutants display altered timing of let-7::gfp expression*

*lin-28* mutants display a precocious phenotype and delete L2 stage fates (Ambros and Horvitz, 1984). *daf-I2* mutants display a retarded phenotype and reiterate L2 fates at later stages (Antebi et al., 1998). These two heterochronic genes have been shown by genetics to act most proximally upstream of *let-7*. To show that the temporally regulated GFP expression in the pSJ11 transgenic animals is indeed indicative of *let-7* expression, we tested whether *lin-28* and *daf-12* mutations affect the timing of *let-7::gfp* expression. We performed crosses to establish *zaEx5* (a transgenic line containing the pSJ11 construct) in the background of *lin-28* and *daf-12* mutants. Whereas wild-type *zaEx5* animals initiated seam cell GFP expression at the L3/L4 molt, with strong expression thereafter, *lin-28(n719); zaEx5* animals showed precocious seam cell GFP expression in the early L3 stage (Fig. 8A and B) one stage earlier than normal (88%, $n = 24$). Conversely, *daf-12(rh61); zaEx5* animals displayed retarded expression of GFP in the seam cells, lacking expression in the L4 stage (100%, $n = 12$ animals) (Fig. 8C and D), with GFP appearing in these cells only at the L4 to adult molt (Fig. 8E and F), one stage later than normal (50%, $n = 10$ animals). This precocious and retarded expression of seam cell GFP corresponds to the early and late expression of *let-7* RNA in these two mutants. Thus, *let-7::gfp* expression faithfully mimics *let-7* RNA expression in another important manner.

## Discussion

*let-7* is a prototype member of a large family of miRNAs (Lagos-Quintana et al., 2001; Lau et al., 2001; Lee and Ambros, 2001). With the exception of *let-7* and *lin-4*, little is known about the function of these miRNAs, and until now, little was known about the regulation of any miRNA. *let-7* functions as a key temporal regulator of *C. elegans* development (Reinhart et al., 2000), and *let-7* RNA is temporally regulated. The mechanism by which this temporal regulation occurs (i.e., through transcription or RNA processing and/or stability, and what other factors regulate *let-7*) had been heretofore unexplored. Understanding what regulates the production of the *let-7* RNA timekeeper would be of great value in understanding the regulation of temporal cell fate transitions in *C. elegans*, and since *let-7* is

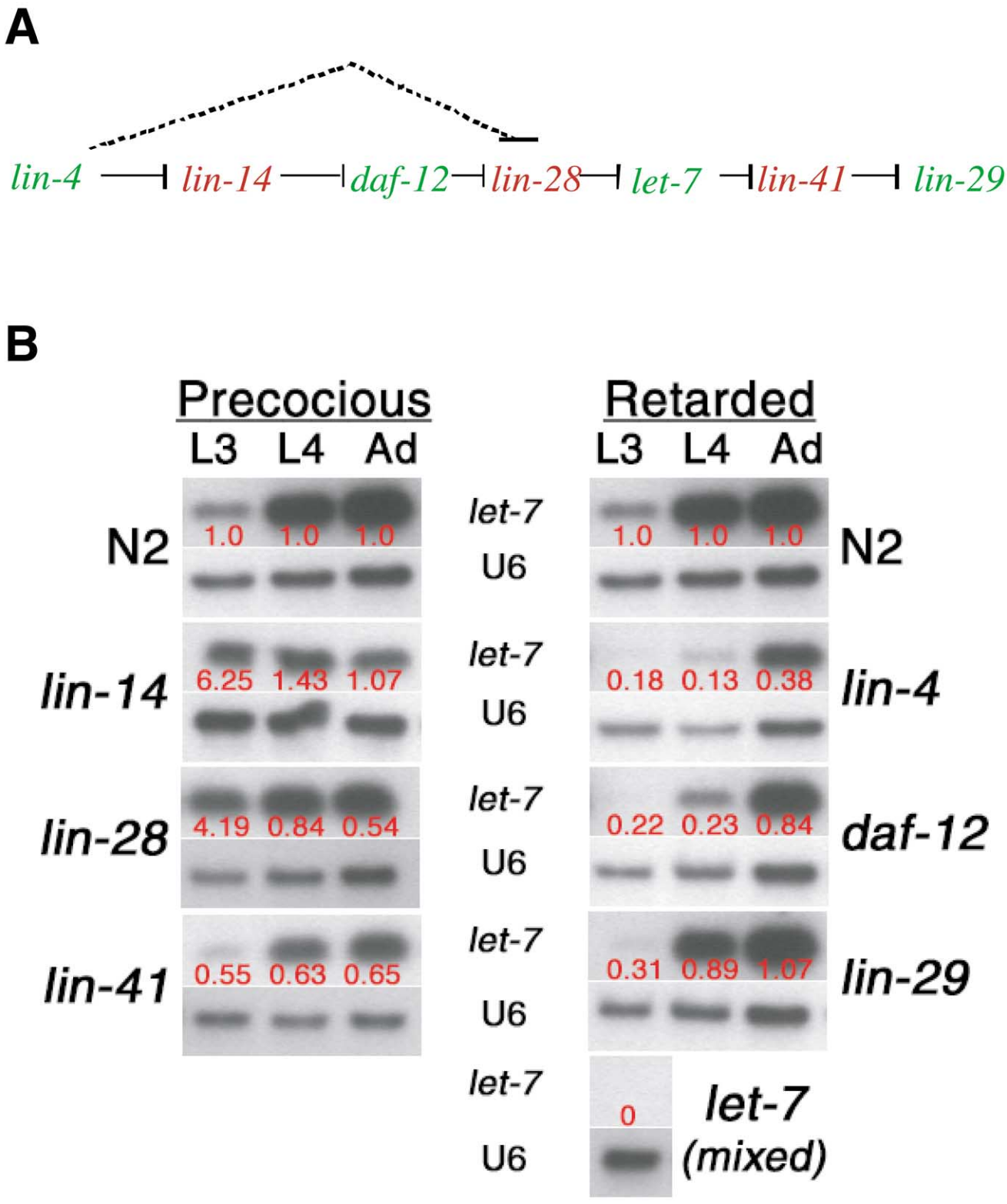


Fig. 7. Developmental Northern blots for *let-7* from heterochronic mutants. (A) A depiction of the linear heterochronic pathway in *C. elegans*. Loss-of-function of the genes in red results in precocious heterochronic phenotypes and loss-of-function of the genes in green results in retarded heterochronic phenotypes (Banerjee and Slack, 2002; Slack and Ruvkun, 1997). (B) Developmental Northern blots of the precocious mutants upstream of *let-7* show precocious *let-7* expression, developmental Northern blots of the retarded mutants upstream of *let-7* show retarded *let-7* expression, and developmental Northern blots of both precocious and retarded mutants downstream of *let-7* show near wild-type expression of *let-7*. For each mutant, the top blot is probed for *let-7* and the bottom blot is the same membrane stripped and probed for U6 snRNA to standardize loading of the RNA. Numbers in red under the *let-7* bands represent the ratio of mutant expression of *let-7* to wild-type expression of *let-7* for the same stage. The mutant alleles of the heterochronic genes used for the Northern blot are as follows: *lin-4(e912), lin-14(n179ts), daf-12(rh61), lin-28(n719), let-7(mn112); lin-28(n719), lin-41(ma104),* and *lin-29(n546)*.


Fig. 8. *lin-28(n719); zaEx5* and *daf-12(rh61); zaEx5* animals show precocious and retarded *let-7::gfp* expression, respectively. The white arrowheads mark seam cells in which *let-7::gfp* is being expressed. A *lin-28(n719); zaEx5* L3 animal (A, B) shows precocious expression of *let-7::gfp* expression. L4 and adult *daf-12(rh61); zaEx5* animals are shown in (C, D) and (E, F), respectively, and *let-7::gfp* is not seen in the L4, but is present in a mosaic pattern in the adult. Animals are shown at 400× magnification with anterior to the left.

temporally regulated in other animals, including zebrafish, this analysis may shed light on developmental mechanisms in these animals and on regulation of miRNAs in general. In summary, we provide evidence that *let-7* is temporally regulated in seam cells by transcription and not by processing of its 70 nucleotide precursor molecule or by stability of the mature RNA product itself. We have also shown the existence of a 116-bp temporal regulatory element (TRE) that is both necessary and sufficient for the upregulation of *let-7* at the L3/L4 molt and a TRE binding factor (TREB) that specifically binds the 22-bp inverted repeat (IR) found in the TRE. This regulation is modulated either directly or indirectly by the heterochronic genes genetically upstream of *let-7*, but is unaffected by the heterochronic genes that are genetically downstream of *let-7*.

Transcriptional fusions of the *let-7* promoter to a GFP reporter gene reveal expression of *let-7*. A transcriptional GFP fusion to 1.8 kb of genomic sequence upstream of the start of the mature *let-7* sequence displayed temporal upregulation of GFP at the L4 stage in seam cells, cells known to require *let-7* for their development. We thus believe that expression of this transcriptional *let-7::gfp* fusion in seam cells recapitulates *let-7* RNA expression. In this analysis, the GFP is observed slightly later than the appearance of

*let-7* RNA, which may reflect the time required for the GFP to become expressed and active.

If *let-7* RNA were regulated at the level of RNA processing, we would predict that *let-7::gfp* would be expressed at all stages. A transcriptional *let-7::gfp* fusion was not expressed in seam cells at all stages of development, and the temporal upregulation of *let-7::gfp* did not require any of the *let-7* mature sequence or 3′ downstream sequences, including the other arm in the stem of the pre-*let-7* RNA. Thus, *let-7* temporal upregulation is not the result of temporally regulated processing of the pre-*let-7* RNA or stability of the *let-7* RNA. We conclude that *let-7* is transcriptionally regulated in seam cells. However, our experiments do not rule out additional regulation at the level of processing and do not fully address the reasons for non-temporally regulated background GFP expression observed in some tissues.

Developmental Northern blot analysis probing for *let-7* in heterochronic mutants, as well as expression of the *let-7::gfp* fusion in heterochronic backgrounds demonstrate that the heterochronic genes upstream of *let-7* are required for correct temporal expression of both *let-7* RNA and *let-7::gfp*. Specifically, we found that temporal upregulation of *let-7* requires the stRNA *lin-4* and the nuclear hormone receptor DAF-12. We also show that LIN-14 and LIN-28 are required to keep high-levels of *let-7* expression off until the L4 stage. Our unpublished data show that not all temporally regulated microRNAs are affected by both *lin-4* and *daf-12*, suggesting that these genes are not just global regulators of stage, but have some specificity on *let-7*.

Temporal upregulation of *let-7* is critical to the correct progression of seam cell fates from the L4 to adult stage, but the mechanism is unknown. We identified the *cis*-acting sequences in the *let-7* promoter that are responsible for temporal regulation of *let-7* by making truncations and deletions in the 1.8 kb of genomic sequence upstream of the mature *let-7* sequence. We found a 116-bp region that is absolutely required for temporal upregulation of *let-7::gfp* that we have named the temporal regulatory element (TRE). We also demonstrated that the TRE is sufficient to temporally upregulate a minimal promoter specifically in the seam cells; however, other elements in the promoter contribute to full expression. The TRE is required for a genomic fragment to optimally rescue the *let-7* mutant phenotype—the 2.5 kb genomic rescuing fragment, deleted for sequences, including the TRE, but retaining everything else abrogates rescuing activity. Since the TRE is important in vivo, this supports our assertion that the TRE is critical for *let-7* expression. Within the TRE is a 9-bp inverted repeat with one mismatch, the two halves separated by 4 bp (Fig. 3). Whereas deletion of this inverted repeat from the *let-7* promoter did not abolish *let-7::gfp* expression, it did reduce it to much lower levels. We have also shown that this sequence, which is highly conserved in *C. briggsae*, is the binding site for a *trans*-acting TRE binding factor(s) (TREB) that could be a transcriptional activator of *let-7*.

One model for the action of this *cis*-acting TRE is one of positive regulation, where in the early larval stages (L1–L3) the TRE is free and unbound by transcriptional activators and does not induce *let-7* expression. Upon entering the L3/L4 transition, TREB binds to the IR in the TRE and activates the transcription of *let-7* (Fig. 6D, model 1). TRE therefore acts as an enhancer element. Since *let-7* expression appears coupled to the L3 molt, we predict that TREB activity is likely to be regulated by a hormone that signals the molt. TREB could itself be a nuclear hormone receptor or another factor that is regulated by hormonal signaling. An alternative model would be that the TREB is always bound to the TRE and becomes activated by binding of an early-L4-produced hormone or other ligand (Fig. 6D, model 2). Future work will distinguish between these two models and reveal the identity of TREB. Additionally, we suspect that TREB activity is likely to be controlled by *lin-4*, *lin-14*, *daf-12*, and *lin-28*, the heterochronic genes upstream of *let-7*.

The identity of the TREB is open to speculation, but various nuclear hormone receptors in *C. elegans* play roles in molting (Asahina et al., 2000; Gissendanner and Sluder, 2000; Kostrouchova et al., 2001) and may correspond to TREB or be activators of TREB. There are approximately 270 nuclear receptor genes found in the *C. elegans* genome (Sluder and Maina, 2001), at least 10 of which have been shown to be expressed in seam cells (Miyabayashi et al., 1999). All of these genes are potential TREB candidates, but only 1, *daf-12*, plays a role in the heterochronic pathway. We have shown that *daf-12* regulates the timing of *let-7* expression: mutations in *daf-12* that result in retarded heterochronic phenotypes cause retarded expression of *let-7* and *let-7::gfp*. A DAF-12::GFP fusion revealed high expression in the hypodermal seam cells (Antebi et al., 2000), and both the DAF-12::GFP fusion protein and *daf-12* mRNA are expressed throughout development (Antebi et al., 2000; Snow and Larsen, 2000), placing DAF-12 in the right place at the right time. One possibility is that DAF-12 regulation of *let-7* could be direct with DAF-12 filling the role of the TREB and binding to the TRE to activate the transcription of *let-7*. However, our TREB binding activity is not affected in a *daf-12(rh61)* mutant (data not shown), suggesting that DAF-12 may act indirectly in promoting *let-7* expression.

We have addressed some fundamental questions about *let-7* regulation in *C. elegans*, but the answers we have found might have broader implications. *let-7* is a broadly conserved stRNA, and is also a member of the large class of recently discovered miRNAs. miRNAs are nonprotein coding genes which encode mature RNA products of about 20–24 nt in length (Ambros, 2001; Grosshans and Slack, 2002; Lagos-Quintana et al., 2001; Lau et al., 2001; Lee and Ambros, 2001). Each of these genes is hypothesized to be transcribed as a longer precursor molecule that can fold back on itself to form a hairpin loop. Three newly discovered miRNAs, *mir-48*, *mir-69*, and *mir-84*, have similar

temporal expression patterns to *let-7*, and *mir-48* and *mir-84* share sequence identity to *let-7* (Lau et al., 2001). The homologies between both the expression patterns and the sequences of *let-7*, *mir-48*, and *mir-84* suggest a possible common role for these genes and, more strongly, the possibility of common transcriptional regulation. In support of this, we have identified possible common promoter elements upstream of these genes.

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