RNA Interference Can Target Pre-mRNA: Consequences for Gene Expression in a *Caenorhabditis elegans* Operon

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Manuscript received June 1, 1999
Accepted for publication July 12, 1999

ABSTRACT

In nematodes, flies, trypanosomes, and planarians, introduction of double-stranded RNA results in sequence-specific inactivation of gene function, a process termed RNA interference (RNAi). We demonstrate that RNAi against the *Caenorhabditis elegans* gene *lir-1*, which is part of the *lir-1*/*lin-26* operon, induced phenotypes very different from a newly isolated *lir-1* null mutation. Specifically, *lir-1(RNAi)* induced embryonic lethality reminiscent of moderately strong *lin-26* alleles, whereas the *lir-1* null mutant was viable. We show that the *lir-1(RNAi)* phenotypes resulted from a severe loss of *lin-26* gene expression. In addition, we found that RNAi directed against *lir-1* or *lin-26* introns induced similar phenotypes, so we conclude that *lir-1(RNAi)* targets the *lir-1*/*lin-26* pre-mRNA. This provides direct evidence that RNA interference can prevent gene expression by targeting nuclear transcripts. Our results highlight that caution may be necessary when interpreting RNA interference without the benefit of mutant alleles.

IN the last year wide acceptance of the technique called double-stranded-mediated RNA interference (RNAi) has made the investigation of gene function much more accessible (Fire *et al.* 1998). However, the mechanism underlying RNAi remains largely unknown. The potential relevance of RNAi, which was first described in *Caenorhabditis elegans*, has recently expanded with the discovery that RNAi can specifically inactivate gene function in insects (Kennerdell and Carthew 1998; Misquitta and Paterson 1999), trypanosomes (Ngo *et al.* 1998), and planarians (Sánchez Alvarado and Newmark 1999). RNAi has also been compared to post-transcriptional or homology-dependent gene silencing (PTGS) in plants (Voinnet *et al.* 1998; Waterhouse *et al.* 1998), raising the possibility that RNAi is a mechanism present in all eukaryotes (for recent reviews see Montgomery and Fire 1998; Sharp 1999).

It was initially proposed that RNAi could target four different stages of gene expression: (i) the gene itself could be targeted by direct mutagenesis; (ii) transcription could be prevented; (iii) the transcript could be targeted for degradation; (iv) translation could be prevented. Mutagenesis of the target gene was excluded because no changes in DNA sequence were found in animals affected by RNAi (Montgomery *et al.* 1998). Several different types of experiments demonstrated that initiation of transcription is not a target for RNAi (Fire *et al.* 1998; Korf *et al.* 1998; Montgomery *et al.* 1998). In particular, Montgomery *et al.* (1998) have shown that in embryonic blastomeres the accumulation of transcripts from reporter constructs is completely prevented in the cytoplasm and partially prevented in the nucleus. This reinforces the possibility that RNA is the target for RNAi and that it is degraded.

C. elegans is unusual among eukaryotes for several reasons. Most transcripts are *trans*-spliced at their 5′ ends to a small sequence called a spliced leader (SL; Blumenthal 1998) and ~25% of genes are organized in transcriptional operons (Zorio *et al.* 1994; Blumenthal 1998). Most operons conform to three criteria: (i) the genes are only 100–400 bp apart; (ii) generation of the downstream transcript is achieved by coupling polyadenylation of the upstream transcript with *trans*-splicing of the downstream transcript; (iii) if the upstream transcript is *trans*-spliced it is *trans*-spliced to SL1 whereas downstream transcripts are *trans*-spliced to SL2 or its variants.

Recently, we have been investigating the complex genomic organization of the genes *lin-26* (*lin*, lineage abnormal), *lir-1*, and *lir-2* (*lir*, ***l****in-26* **r**elated), which encode homologous putative transcription factors and define a new C2H2 motif related to TFIIIA zinc fingers (Labouesse *et al.* 1994; Dufourcq *et al.* 1999). These genes are organized in two overlapping operons, both of which conform to the classic operon described above (Dufourcq *et al.* 1999). *Trans*- and alternative splicing of *lir-1* results in at least six isoforms that can be categorized into two groups, long and short. Long *lir-1* isoforms are *trans*-spliced to SL2 at exon 1 and are organized in a transcriptional operon with *lir-2*. Short *lir-1* isoforms are *trans*-spliced to SL1 at exons 2 or 3 and are organized in a transcriptional operon with *lin-26* (Figure 1). The

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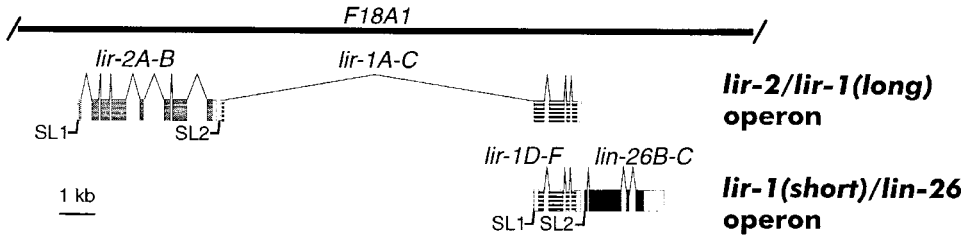

Figure 1.—The *lir-2*, *lir-1*, and *lin-26* operons. The genes *lir-2*, *lir-1*, and *lin-26* form two overlapping operons. The first operon [*lir-2*/*lir-1(long)* operon] includes *lir-2* and long *lir-1* isoforms starting at the first *lir-1* exon (*lir-1A-C*); the second operon [*lir-1(short)*/*lin-26* operon] includes short *lir-1* isoforms starting at or after *lir-1* exon 2 (*lir-1D-F*) and *lin-26*. Unshaded boxes correspond to untranslated sequences, open triangles represent introns, and variously shaded boxes represent exons of *lir-2*, *lir-1*, or *lin-26*.

first and second *lir-1* exons are separated by an unusually long intron, which contains promoter sequences for the second operon (den Boer *et al.* 1998). The *lir-2*/*lir-1(long)* operon is expressed in all cells, whereas the *lir-1(short)*/*lin-26* operon is expressed in nonneuronal ectodermal cells (Dufourcq *et al.* 1999).

lin-26, the best characterized of these three genes, is required for nonneuronal ectodermal cells to maintain their normal fates (Labouesse *et al.* 1994, 1996). Strong and null *lin-26* alleles lead to embryonic lethality due to degeneration of most hypodermal (epidermal-like) cells and glial-like cells (Labouesse *et al.* 1994, 1996). A weak loss-of-function mutation, *lin-26(n156)*, causes a set of hypodermal precursors to adopt a neural fate resulting in a vulvaless phenotype (Ferguson *et al.* 1987; Labouesse *et al.* 1994). This viable mutation leads to early larval lethality when *in trans* to a *lin-26* null allele, with defects in tissues and organs made of hypodermal cells (Labouesse *et al.* 1994). These include an abnormal tail, excretory system, and rectum, as well as a dumpy body shape (Dpy). Furthermore, loss of *lin-26* expression in the somatic gonad epithelium and its precursors leads to sterility (den Boer *et al.* 1998).

Prior to this work, no specific *lir-2* or *lir-1* mutations had been identified and efforts to ascertain their function form the background to this study. We report the identification of a *lir-1* null mutant and compare its phenotype to that of *lir-1(RNAi)* animals. We show that *lir-1(RNAi)* induces hypodermal defects reminiscent of *lin-26* phenotypes although the *lir-1* null mutation is viable. We test possible hypotheses that could explain this apparent discrepancy and conclude that *lir-1(RNAi)* targets the pre-mRNA of the *lir-1(short)*/*lin-26* operon, thereby interfering with both *lir-1* and *lin-26* gene function.

MATERIALS AND METHODS

Strains and general methods: Methods for genetic analysis and the reference wild-type strain were as described in Brenner (1974). Other strains were: CB187, *rol-6(e187)* (Brenner 1974); ML581, *lin-26(mc15) unc-4(e120)*/*mnC1 [dpy-10(e128) unc-52(e444)]* (den Boer *et al.* 1998); ML335, *dpy-2(e489) mcDf1 unc-4(e120)*/*mnC1 [dpy-10(e128) unc-52(e444)]* (Chanal and Labouesse 1997).

RNA interference: The templates used for RNA synthesis were amplified by PCR with primers that have a T3 (ATTAAC CCTCACTAAAGG, to generate the sense strand) or T7 (AA TACGACTCACTATAGG, to generate the antisense strand) promoter sequence at their 5′ ends. The size and purity of PCR products was checked by agarose gel electrophoresis, but they were not sequenced. The following list gives the position of the starting nucleotide for each sense and reverse primer used in PCR reactions with cDNA or cloned genomic DNA (for introns). The first set of numbers refers to the nt coordinates of the primers in cosmid *F18A1* (GenBank accession no. U41535), the second to the length of the PCR product:

lir-2(exons4-6): 14701 and 16283; 550 bp
lir-2(exons4-7): 14701 and 16805; 1020 bp
lin-26(full length): 28166 and 29857; 1300 bp
lin-26(exon3,partial): 29102 and 29653; 190 bp
lir-1(full length): 17686 and 27907; 1000 bp
lir-1(exons3-5): 26945 and 27907; 675 bp
lir-1(exons1-3): 17720 and 27388; 570 bp
lir-1(exons1-4): 17720 and 27719; 850 bp
lir-1(exons1-2): 17686 and 26940; 315 bp
lir-1(exon3): 27165 and 27569; 405 bp
lir-1(exons4-5): 27622 and 27907; 205 bp
lir-1(exon1): 17720 and 17784; 50 bp
lin-26(intron3): 29443 and 29640; 197 bp
lir-1(intron2): 27002 and 27160; 159 bp

The following list gives the position of the starting nucleotide for each sense and reverse primer used in PCR reactions with genomic DNA:

lin-15A: 6351 and 7419 in cosmid *ZK678* (GenBank accession no. Z79605)
lin-15B: 2994 and 3975 in cosmid *ZK678*
ppp-1: 6867 and 5531 in cosmid *C15F1* (GenBank accession no. AC006608)
tra-2: 4784 and 3754 in cosmid *C15F1*
rol-6: 229 and 943 in cosmid *T01B7* (GenBank accession no. Z66499)

Sense and antisense RNA strands were individually synthesized using the mMESSAGE mMACHINE kit (Ambion, Austin, TX) following the manufacturer's instructions. DNA templates were removed with a 15-min DNaseI treatment. RNAs were extracted with phenol/chloroform and chloroform, precipitated in isopropanol, resuspended in 10 mm Tris-HCl (pH 7.5), 1 mm EDTA (pH 8.0), and annealed. Double-stranded RNA (dsRNA) was microinjected into the syncytial gonad arms of *rol-6(e187)* animals together with dsRNA corresponding to the *rol-6* locus. The rationale for using this control procedure is that *rol6(e187)* animals are Rol, whereas the null phenotype of *rol-6* is wild type (Hodgkin 1997); hence only oocytes that have incorporated the injected dsRNA will develop as nonrollers.

After being injected, animals were allowed to recover for

4 hr before they were cloned and subsequently transferred to fresh plates at 8-hr intervals for 24 hr. Terminal phenotypes were identified and analyzed especially with respect to elongation, morphology, hypodermis integrity, organ morphology, sexual identity, and fertility.

***lin-26::gfp* construct:** The *lin-26::gfp* construct (pML702) is a modification of pML301 (den Boer *et al.* 1998). The GFP (green fluorescent protein) coding sequence was PCR amplified, so that a stop codon was introduced at its 3′ end, and cloned, in frame, into the *Nhe*I site of exon 2 of *lin-26B* (Dufourcq *et al.* 1999). This fusion protein was targeted to the nucleus by cloning a nuclear localization signal just upstream of the GFP. Transgenic lines were established as described by Mello and Fire (1995). pML702 was coinjected at 10 ng/μl with plasmid pRF4 [*rol-6(su1006)*] at 100 ng/μl. One transgene was integrated (den Boer *et al.* 1998) to generate the allele *mcIs17*, which was subsequently outcrossed four times with N2 animals.

Isolation of a *lir-1* null mutation: To isolate a *lir-1* mutation, we used a protocol adapted from Jansen *et al.* (1997). Briefly, wild-type N2 animals were incubated with trimethyl-psoralen at 20 μg/ml for 15 min and subsequently exposed to a UV lamp (4 W/365-nm model VL-4L from Bioblock Scientific) for 30 sec at a distance of 10 cm (Yandell *et al.* 1994; see also Chanal and Labouesse 1997). Pools of ~400 F_1 animals were placed on a single plate. F_2 animals from six different plates were collected together and divided into two equal populations, one that was frozen at −80° and another from which DNA was prepared. We collected, in this way, the progeny from 1.1×10^6 F_1 animals and assayed them for the presence of potential deletions by nested PCR using the primers 5′ ATCACGTGAAGTGTGAAGGTC (*lir-1* intron 1) and 5′ GAG TTGGAGACTCCTCTACTT (*lin-26B* exon 4) followed by 5′ GCCGAAAATGGGTGTGCGCA (*lir-1* intron 1) and 5′ GAAT GGAATATGGAACACTCCATGC (*lin-26B* intron 3).

We recovered one mutation that deletes a fragment of 3276 nucleotides from *lir-1* (position 24453 to 27728 in cosmid *F18A1*). This mutation, which was named *lir-1(mc33)*, allows for only the synthesis of less than 42-amino-acid LIR-1 proteins and is thus likely to be a *lir-1* null allele. We demonstrated that the corresponding *lir-1* sequences were indeed deleted, rather than translocated somewhere else in the genome, by carrying out PCR reactions on homozygous *lir-1(mc33)* animals using various primer pairs internal to the *mc33* deletion: in no case did we find a band that would indicate the presence of translocated *lir-1* sequences (data not shown).

Characterization of *lir-1(mc33)* animals: The mutation *lir-1(mc33)* was outcrossed six times, marked with the mutation *unc-4(e120)* and balanced with *mnC1*. Genetic characterization of *lir-1(mc33)* showed that it is tightly linked to an ≈300-kb deficiency that we could not separate from *mc33*; we named it *mcDf3* (see Figure 6A for a genetic map). We mapped the extent of *mcDf3* by PCR (Williams *et al.* 1992) starting from dead eggs laid by heterozygous *mc33 mcDf3* animals and primers derived from the *C. elegans* genome sequence (The *C. elegans* Consortium 1998; names of cosmids that have been tested are available upon request). We mapped in a similar way the right breakpoint of the deficiency *mcDf1*, which deletes ≈350 kb including *lir-1* and sequences further to its left (Chanal and Labouesse 1997). We found that *mcDf3* deletes the equivalent of 11 cosmids to the right of *lin-26* (between *B0495* and *C08B11*), starting ≈4 kb downstream of *lin-26*, and that *mcDf1* breaks in the middle of *C06A8* (see Figure 6A for a genetic map). Therefore *mcDf1* and *mcDf3* both delete *B0495*, *B0228*, and part of *C06A8*. Although both deficiencies are embryonic lethal when homozygous, in crosses between ML335 males and *lir-1(mc33) mcDf3 unc-4(e120)*/*mnC1* hermaphrodites we observed 25% Unc larvae that failed to develop beyond the L1 stage. These presumptive *mc33 mcDf3*/*mcDf1* larvae are expected to be missing the products encoded by *lir-1* and the genes located within the cosmids *B0495*, *B0228*, and the beginning of *C06A8*. Larval lethality of these animals is probably due in part to the absence of the gene *let-253*, which can be rescued by a cosmid that overlaps with *B0495* (M. Labouesse, unpublished results).

To determine the potential phenotype of a *lir-1* null mutation, we generated *mc33 mcDf3*/*mcDf1* heterozygous animals carrying the cosmids that are deleted in the region of overlap between *mcDf1* and *mcDf3*. To this end, we first introduced the cosmids *B0495*, *B0228*, and *C06A8* (each at 5 ng/μl) into *lir-1(mc33) mcDf3 unc-4(e120)*/*mnC1* animals by germline transformation using the *myo-3::gfp* plasmid pPD93.97 (at 20 ng/μl), a body wall muscle marker (Fire *et al.* 1998). Eleven stable fluorescent lines were isolated and tested by PCR for the presence of cosmids. Similarly, in control injections using the cosmids *F18A1* (to test for *lir-1* rescue), *B0495*, *B0228*, and *C06A8*, we obtained 10 stable transgenic lines. Transgenic animals carrying either the cosmids *B0495*, *B0228*, and *C06A8*, or the cosmids *F18A1*, *B0495*, *B0228*, and *C06A8* were crossed with ML335 males. After moving parents daily to a fresh plate, mating plates were inspected under the dissecting scope for the presence of Unc cross-progeny [expected genotype *mc33 mcDf3 unc-4(e120)*/*mcDf1 unc-4(e120)* heterozygous animals] and under a GFP scope for the presence of possible GFP^+ Unc larvae (as a test for the presence of the transgene). On each mating plate there were wild-type heterozygous animals (both GFP^+ and GFP^-), paralyzed sterile animals (GFP^+ and GFP^- homozygous *mnC1*), Unc adults that were exclusively GFP^+, arrested Unc larvae that were GFP^- or rarely GFP^+, and no or very few dead eggs (Table 1).

RESULTS

***lir-1(RNAi)* affects hypodermal cells:** To investigate the possible functions of *lir-1* and *lir-2*, prior to the identification of any mutations, we decided to use RNA interference. This technique was first described using single-stranded RNA (Guo and Kemphues 1995); however, it was subsequently recognized that double-stranded RNA is the active molecule (Fire *et al.* 1998); the term RNAi itself was coined by Rocheleau *et al.* (1997). As an initial control we found that RNAi using *lin-26*-specific dsRNA phenocopied *lin-26* null alleles (Figure 2C; Labouesse *et al.* 1994). Specifically, 100% of embryos failed to elongate beyond the 1.5-fold stage and contained degenerating hypodermal cells (Figures 2E and 3A; the specific stages of embryonic development are referred to by their morphology: lima bean, comma, 1.5-fold, 2-fold, 3-fold, and finally pretzel).

Full-length *lir-1(RNAi)* gave a phenotype that was reminiscent of *lin-26(mc2)*, a moderately strong mutation (Figure 2D; Labouesse *et al.* 1994). Specifically, almost all embryos arrested at or just beyond the twofold stage with cells and droplets floating within the egg shell, suggesting that defects in hypodermal cells have resulted in leakage of cells or cytoplasm (Figures 2F and 3C). This raised the possibility that *lir-1* shares a common biological function with *lin-26*, which would not be unexpected since they both code for homologous proteins and are organized in a transcriptional operon.

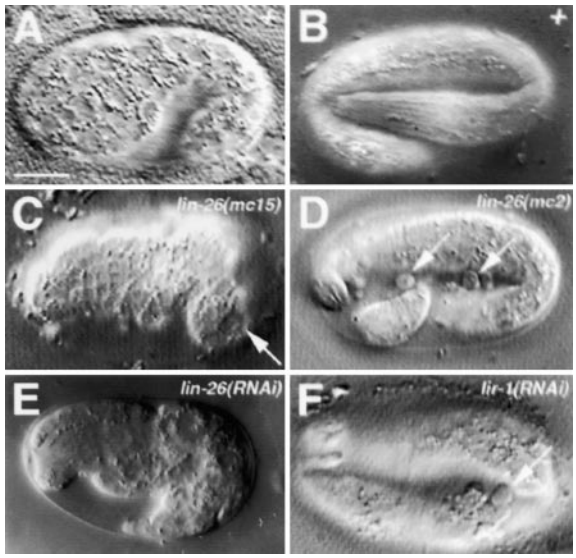

Figure 2.—*lir-1(RNAi)* and strong *lin-26* mutations induce similar embryonic phenotypes. Nomarski pictures show embryos at the 1.5-fold stage (A) or at the end of embryogenesis (B–F); anterior is to the left and dorsal is up in A, C, and E; all pictures are lateral views, except E, which is central. (A and B) Wild-type embryo. (C) *lin-26(mc15)* embryo; *mc15* is a *lin-26* null allele. Notice that it does not elongate beyond the 1.5-fold stage and shows signs of degenerating hypodermal cells (arrow). (D) *lin-26(mc2)* embryo; *mc2* is a moderately strong *lin-26* allele. This embryo could elongate slightly beyond the 2-fold stage but, due to hypodermal defects, cells or cytoplasm leaked through the hypodermis (arrows). (E) *lin-26(RNAi)* embryo. The phenotype is similar to that of *lin-26* null embryos. (F) *lir-1(RNAi)* embryo. The phenotype is similar to that of *lin-26(mc2)* embryos (see leaking cytoplasm; arrow). Bar, 10 μm.

Strikingly, *lir-1(RNAi)* using smaller stretches of *lir-1* exonic sequence resulted in a varied array of phenotypes. *lir-1(RNAi)* with sequences corresponding to exons 3–5 [*lir-1(exons3-5)*; this nomenclature will be used throughout] also resulted in a highly penetrant embryonic arrest phenotype reminiscent of *lin-26(mc2)* (Figure 3D). However, smaller stretches of sequence that included exons 1–2, 3, or 4–5 resulted in predominantly larval and/or adult phenotypes (Figure 3, G–K, and Figure 4). Most of the larvae died as L1/L2 larvae (there are four larval stages designated L1 through L4). Further analysis revealed that they had a variable range of hypodermal defects, such as a dumpy body shape, abnormal tails, excretory organs, and/or rectums (Figure 4B). Although these phenotypes have been associated with partial loss of *lin-26* function in hypodermal cells (Labouesse *et al.* 1994), they could also reflect *lir-1* function.

Animals that survived the first two larval stages sometimes displayed a molting problem resulting in a failure to shed the old cuticle. When this unshed cuticle blocked the mouth it usually led to lethality by the L3 stage; otherwise it formed a waist-like constriction that maintained its smaller diameter as the rest of the body grew (Figure 4, C and E). Since molting requires complete lysis of the matrix that attaches the cuticle to the hypodermis, this molting phenotype is consistent with defects in hypodermis function. Indeed, the only known mutation to affect the physical mechanism of molting alters a gene that acts in the hypodermis (Yochem *et al.* 1999). In addition, it has been shown that RNAi against the nuclear hormone receptor *nhr-23*, which is expressed in the epidermis among other tissues, creates a phenotype similar to that of weak *lir-1(RNAi)* animals (Kostrouchova *et al.* 1998).

Most surviving adults were Dpy with abnormal tails and had a vulval phenotype (Figure 4, E and F): they usually had a protruding vulva (Pvl), were sometimes multi-vulva (Muv), or occasionally vulvaless (Vul). In addition, these animals were often egg-laying defective (Egl) and infrequently sterile due to oocytes being absent or abnormal. These adult phenotypes are again reminiscent of known *lin-26* phenotypes: for instance, of the two viable *lin-26* alleles, *ga91* is Pvl (D. Eisenmann, personal communication) and *n156* is Vul (Ferguson and Horvitz 1985; Labouesse *et al.* 1994). Furthermore, specific loss of *lin-26* expression in the somatic gonad gives rise to Pvl and sterile animals (den Boer *et al.* 1998).

It is noticeable that variations in phenotypic severity seen in *lir-1(RNAi)* correlate with the time postinjection and the size of the dsRNA injected. As the injected dsRNA becomes smaller and as the time postinjection becomes greater, the percentage of embryonic lethality decreases (compare Figure 3, G–J). Furthermore, if two smaller dsRNAs are injected together, there is an increase in phenotypic severity and duration (for instance, compare the incidence of embryonic lethality between Figure 3, I and J, *vs.* K). Although this suggests that the overall length of sequence homology affects efficiency of interference, RNAi remains most efficient when the interfering dsRNA is present as a single molecule rather than two separate pieces (compare Figure 3, D and K).

To summarize, RNAi using dsRNA corresponding to various regions and sizes of *lir-1* sequence results in embryonic and larval phenotypes that are attributable to hypodermal cell defects and can be classified as *lin-26*-like.

LIN-26 expression is severely reduced in *lir-1(RNAi)* arrested embryos: Since *lir-1(RNAi)* results in a *lin-26*-like phenotype, we examined whether or not *lin-26* expression was normal in *lir-1(RNAi)*-arrested embryos. Using LIN-26 antiserum (Labouesse *et al.* 1996) we showed that LIN-26 is severely downregulated in *lir-1(full length)* embryos (Figure 5B). To directly confirm this observation in live animals, we examined expression of an integrated *lin-26::gfp* construct in *lir-1(exons3-5)* embryos. This construct contains genomic sequences encompassing both *lir-1* and *lin-26* (except for the very first nucleotides of *lir-1(long)* isoforms; Dufourcq *et al.* 1999). We found that *lir-1(exons3-5)*-arrested embryos

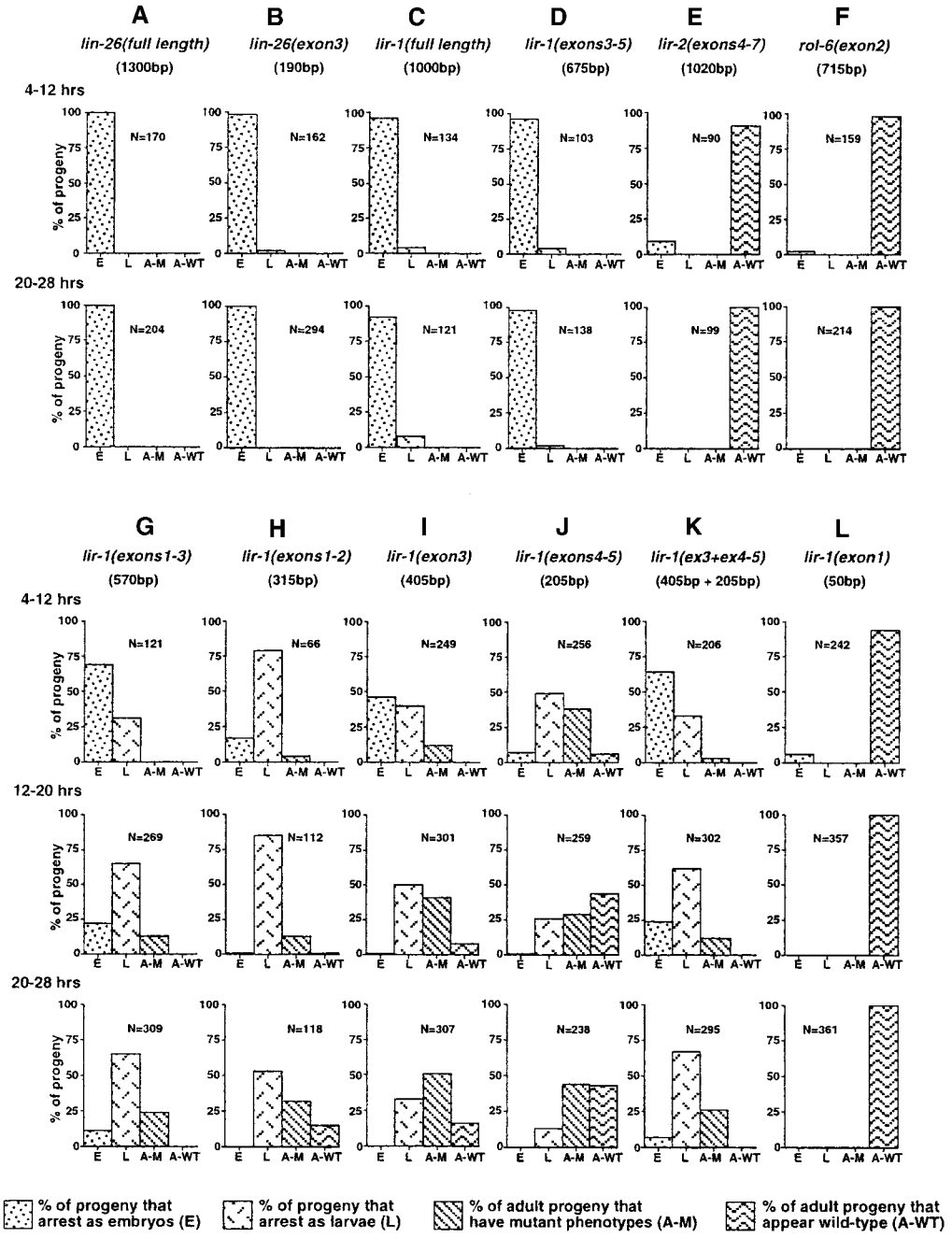

Figure 3.—Graphs illustrating the range of phenotypes obtained with RNAi. Progeny (*N*) were counted every 8 hr between 4 and 28 hr postinjection (see materials and methods). The lengths of injected dsRNA species are indicated under their names. The key for shading in the graphs is given at the bottom of the figure. (A and B) *lin-26(RNAi)* results in almost 100% arrested embryos throughout the 24 hr. (C and D) *lir-1(RNAi)* using full-length sequence or sequence corresponding to exons 3–5. These dsRNA molecules were highly potent, resulting in >92% arrested embryos throughout the time points. (E) *lir-2(RNAi)* did not induce a significant phenotype. (F) *rol-6* dsRNA injected alone results in >98% wild-type progeny. (G–J) Experiments in which dsRNAs corresponding to different regions of *lir-1* were injected. (K) Combined injection of the dsRNAs used in I and J. (L) *lir-1(exon1)* failed to induce a phenotype.

have an almost total loss of GFP expression (Figure 5F). These results demonstrate that loss of *lin-26* expression accounts for most if not all *lin-26*-like phenotypes obtained with *lir-1(RNAi)*.

It has been reported that cross-interference between homologous genes occurs when RNAi is carried out using dsRNA containing regions of high similarity (Fire *et al.* 1998). Since *lin-26* and *lir-1* define with *lir-2* and *lir-3* a new gene family (Dufourcq *et al.* 1999), there was a possibility that the *lir-1(RNAi)* phenotypes represented cross-interference with another member of this family. The nucleotide sequence similarity between these four genes is low except in the region coding for the zinc fingers, where it is ~50% identity. Yet, RNAi against full-length *lir-2* or a fragment coding for its zinc fingers (Figure 3E), or full-length *lir-3* (data not shown), failed to give a significant phenotype. This indicates that the *lir-1(RNAi)* phenotypes are not due to homologous sequences interfering with *lin-26*.

***lir-1* null animals are viable:** Two alternative explanations could explain why *lir-1(RNAi)* causes loss of *lin-26* expression: (1) LIR-1 is directly or indirectly required for the positive regulation of *lin-26*; or (2) since *lir-1(short)* and *lin-26* are organized in an operon, RNAi against the upstream gene (*lir-1*) could also interfere with expression of the downstream gene (*lin-26*).

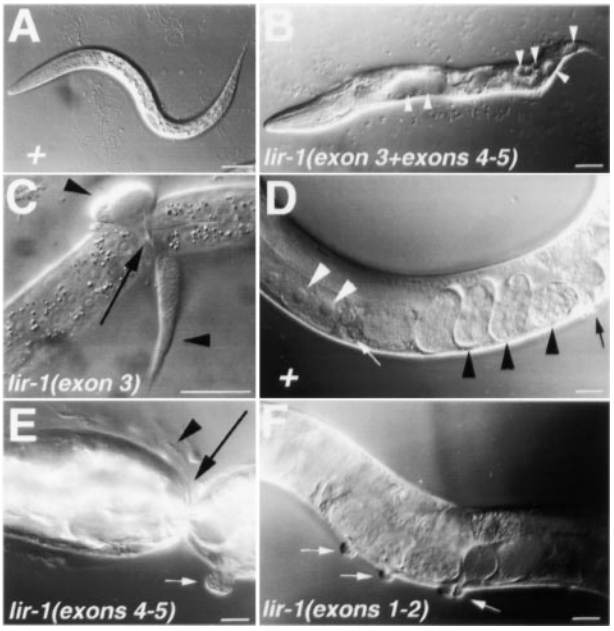

Figure 4.—Nomarski pictures showing postembryonic defects associated with *lir-1(RNAi)*. (A) Wild-type L1 larva. (B) *lir-1(exon3+exon4-5)*-arrested Dpy larva with numerous vacuoles in the hypodermis (arrowheads). (C) *lir-1(exon3)* larva showing remnants of a previous molt, which include the tail (arrowheads) still attached to the body causing a constriction (arrow). (D) Wild-type anterior gonad. Note the oocytes (white arrowheads), the sperm (white arrow), the eggs (black arrowheads), and the nonprotruding vulva (black arrow). (E) *lir-1(exons4-5)* adult with a protruding vulva or Pvl (white arrow) and a severe molting defect constriction that closed off the gut (black arrow; the arrowhead points to remnants of the cuticle). (F) *lir-1(exons1-2)* adult with a Muv phenotype (arrows). Bars, 30 μm.

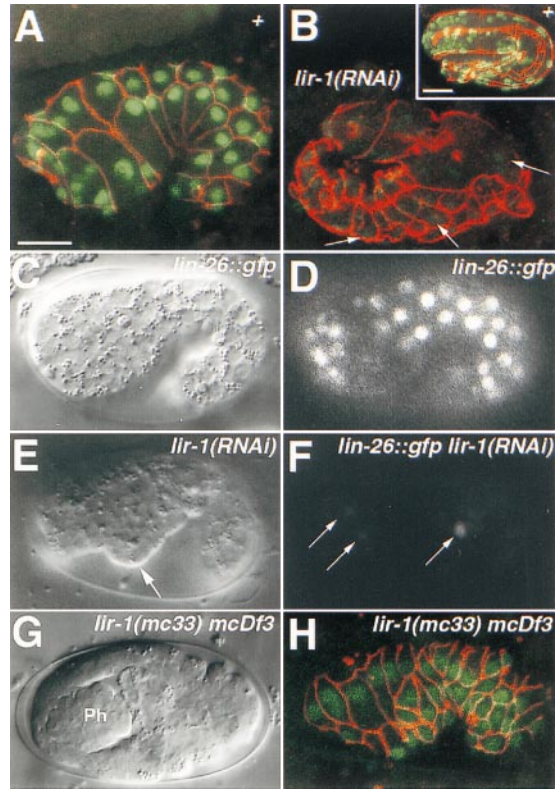

Figure 5.—*lir-1(RNAi)*, but not *lir-1(mc33)*, affects *lin-26* expression. Confocal projections (A, B, and H) show the top third of each embryo costained with a LIN-26 antiserum (green) and the monoclonal antibody MH27 (red), which recognizes an adherens junction component (Francis and Waterston 1991), Nomarski pictures (C, E, and G) or epifluorescence images (D and F) show expression of the integrated *lin-26::gfp* transgene *mcIs17*. (A) Wild-type 1.5-fold stage embryo. (B) *lir-1(RNAi)*-arrested embryo, which had elongated until the 2-fold stage (the head is above the body). LIN-26 is barely detectable (arrows); for comparison, the inset shows a wild-type pretzel embryo of the same age that had been processed on the same slide and analyzed by confocal microscopy within the same field and with the same settings. (C and D) Control *lin-26::gfp* 1.5-fold stage embryo, lateral focal plane. (E and F) *lin-26::gfp; lir-1(exon3-5)* 1.5-fold stage embryo, lateral focal plane [the ventral bulge, arrow, was often seen in *lir-1(RNAi)* and *lin-26(null)* embryos]. (F) Only three cells weakly express the *lin-26::gfp* construct (arrows). (G and H) *lir-1(mc33) mcDf3*-arrested embryo. The Nomarski phenotype (G) is much more severe than that of *lir-1(RNAi)* embryos due to the deficiency *mcDf3*, but LIN-26 (H) was still detected. *lir-1(mc33) mcDf3* embryos, like embryos homozygous for the overlapping deficiency *mnDf106* (Chanal and Labouesse 1997), had ~50% additional cells compared to wild-type embryos (data not shown). Bar, 10 μm.

Using reverse genetics (Jansen *et al.* 1997), we isolated the mutation *lir-l(mc33)*, which is probably a *lir-1* null allele since it removes 3276 nucleotides between the end of the first *lir-1(long)* intron and the end of the last *lir-1* intron. Further genetic characterization of the mutation *lir-1(mc33)* revealed that it is tightly linked to a chromosomal deficiency, which we called *mcDf3* (see materials and methods and Figure 6A). Embryos homozygous for *lir-1(mc33) mcDf3* failed to elongate and died during embryogenesis (Figure 5G). When we stained these embryos with LIN-26 antiserum, we observed that *lin-26* expression was not severely downregulated as in *lir-1(RNAi)* embryos (compare Figure 5, B and H), suggesting that *lir-1* is not essential for *lin-26* expression and that sequences deleted by *mc33* are not essential for *lin-26* expression. However, since *lir-1(mc33) mcDf3* embryonic arrest occurs prior to *lir-1(RNAi)* embryonic arrest, the presence of the deficiency *mcDf3* could potentially mask a requirement for *lir-1* function during late embryogenesis or early larval development.

To assess the phenotype of *lir-1(mc33)* independently of the deficiency *mcDf3*, we took advantage of the fact that *mcDf3* overlaps with another deficiency, *mcDf1* (Chanal and Labouesse 1997). This deficiency starts to the right of *lin-26*, extending in the opposite direction to *mcDf3*, so that they overlap by three cosmids (*B0495*, *B0228*, *C06A8*; see Figure 6A). We generated heterozygous animals carrying *mcDf1* on one chromosome, *lir-1(mc33) mcDf3* on the other, and a transgene containing the three cosmids that are deleted by *mcDf1* and *mcDf3*

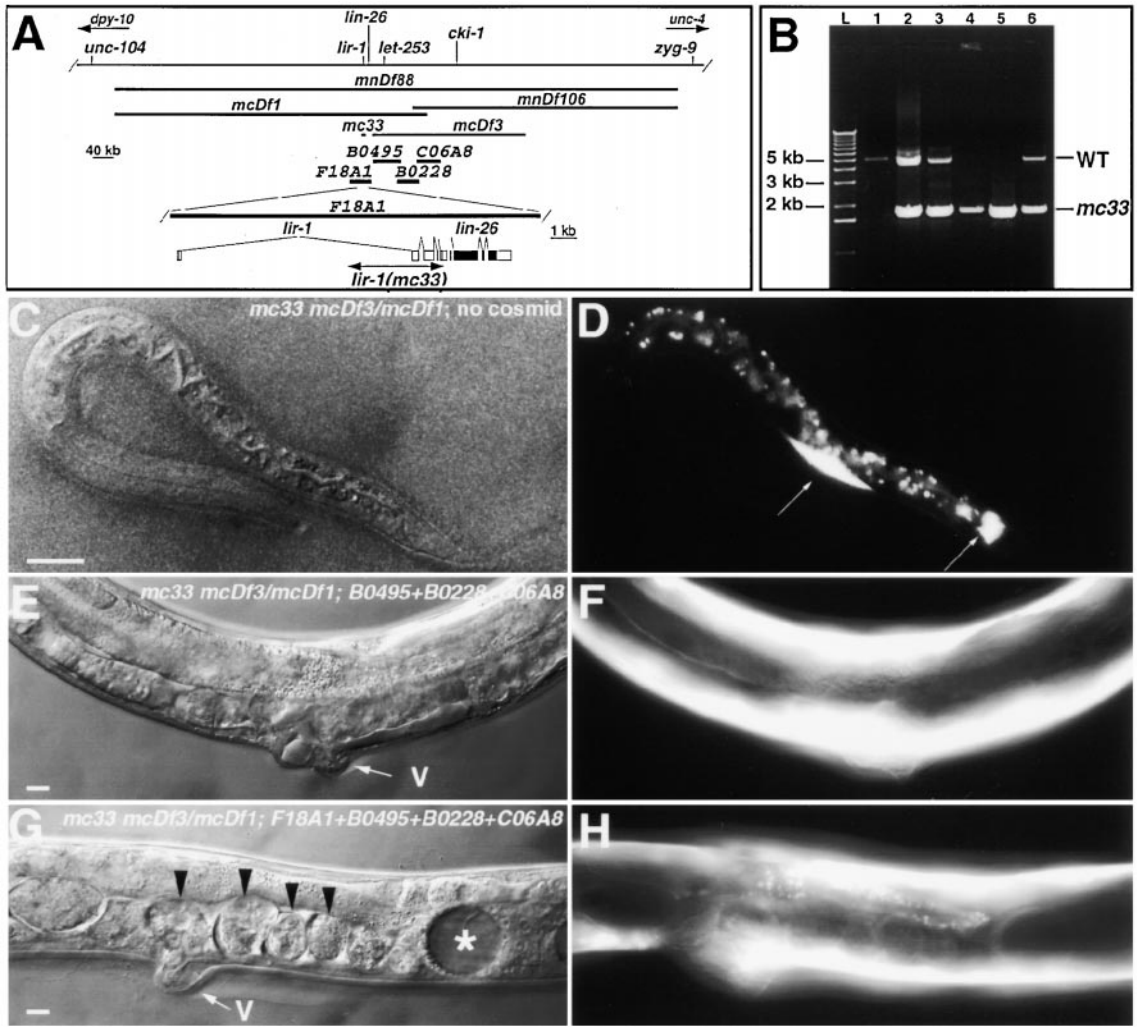

Figure 6.—Hemizygous *lir-1(mc33)* animals reach adulthood. (A) The top line shows part of linkage group II. The middle part shows the extent of four chromosomal deficiencies and four cosmids that map to this area. The deficiencies *mcDf1* and *mcDf3*, which is very tightly linked to *lir-1(mc33)*, overlap with most of *B0495*, all of *B0228*, and part of C06A8. Notice that neither *lir-1(mc33)* nor *mcDf3* deletes *lin-26*. At the bottom is an enlargement of the *lir-1*/*lin-26* region. The extent of *lir-1* that is deleted by the mutation *mc33* is symbolized by the double-headed arrow. (B) PCR was performed on single animals using primers located on both sides of *lir-1(mc33)* to test for the presence or absence of a wild-type copy of *lir-1*. Lane L, 1-kb ladder (GIBCO-BRL, Gaithersburg, MD); lane 1, wild-type animal; lane 2, *lir-1(mc33) mcDf3 unc-4(e120)*/*mnC1* animal; lane 3, DNA purified from a *lir1(mc33) mcDf3 unc-4(e120)*/*mnC1* population; lane 4, GFP$^-$-arrested *lir-1(mc33) mcDf3 unc-4(e120)*/*mcDf1 unc-4(e120)* Unc L1 larva; lane 5, GFP$^+$ viable *lir-1(mc33) mcDf3 unc-4(e120)*/*mcDf1 unc-4(e120)* Unc adult transgenic for the cosmids *B0495* + *B0228* + *C06A8*; lane 6, GFP$^+$ viable *lir-1(mc33) mcDf3 unc-4(e120)*/*mcDf1 unc-4(e120)* Unc adult transgenic for the cosmids *F18A1* + *B0495* + *B0228* + *C06A8*. (C, E, and G) Nomarski images and (D, F, and H) matching epifluorescence images (in the same focal planes) showing expression of the *myo-3::gfp* construct used as a cotransformation marker. (C and D) Four-day-old *lir-1(mc33) mcDf3 unc-4(e120)*/*mcDf1 unc-4(e120)* Unc L1 larva. Although the cause of death of these larvae was not analyzed in detail the hypodermis appeared normal, unlike hypodermis in *lir-1(RNAi)* larvae. Almost all larvae that died at the L1 stage were GFP$^-$; this exceptional larva was highly mosaic and inherited the transgene in very few cells (arrows). (E and F) Viable *lir-1(mc33) mcDf3 unc-4(e120)*/*mcDf1 unc-4(e120)* Unc adult transgenic for the cosmids *B0495* + *B0228* + *C06A8* and the *myo-3::gfp* marker (see F). This animal, like most animals of this genotype (see Table 1), was Pvl (arrow) and sterile with no visible gametes (compare to Figure 4D). (G and H) Viable *lir-1(mc33) mcDf3 unc-4(e120)*/*mcDf1 unc-4(e120)* Unc adult transgenic for the cosmids *F18A1* + *B0495* + *B0228* + *C06A8* and the *myo-3::gfp* marker (see H). This animal was Pvl (arrow) and sterile (see Table 1) with abnormally small eggs (arrowheads) and vacuoles (asterisk). Bars, 20 μm.

(see materials and methods). The resulting animals, which we refer to as "*lir-1(mc33)* hemizygous animals," have no functional *lir-1* but should have at least one copy of the remaining genes deleted by *mcDf1* and *mcDf3*. In principle, their phenotype should reflect the phenotype of *lir-1* null animals. We found that *lir-1(mc33)* hemizygous animals were completely viable and could reach adulthood, thereby ruling out that *lir-1* is zygotically necessary for normal *lin-26* expression (Figure 6; Table 1).

TABLE 1

"*mc33* hemizygous animals" develop into sterile adults

lir-1(mc33) mcDf3/mcDf1	Viable adults[a] (%)	GFP$^+$ dead (%) larvae[a]	Normal H gonad (%) arm[b]	Normal M gonad (%) arm[b]	Normal (%) vulva[c]	Normal male (%) tail[c]	*N*[d]
B0495 + B0228 + C06A8							
mcEx184[e]	90	10	2	0	32	14	51 (25 + 21 + 5)
mcEx185[f]	96	4	3	0	20	0	53 (35 + 16 + 2)
mcEx186[g]	86	14	3	0	31	0	49 (32 + 10 + 7)
F18A1 + B0495 + B0228 + C06A8							
mcEx187	NA	NS	50	44	19	9	77 (43 + 34)
mcEx188	NA	NS	4	9	32	14	58 (37 + 21)

[a] *mc33* hemizygous animals were generated as described in materials and methods using *myo-3::gfp* as a marker to follow transgenic animals. Presumptive *lir-1(mc33) mcDf3/mcDf1* were recognized on the basis of the Unc phenotype conferred by the linked *unc-4(e120)* mutation. To calculate the percentages tabulated, we did not take into consideration GFP$^-$ Unc animals. Note that GFP$^-$- and GFP$^+$-arrested larvae did not display hypodermal defects (see Figure 6). In addition to the transgenic lines listed here, we examined another transgenic line obtained with the cosmids *B0495 + B0228 + C06A8* and two with the cosmids *F18A1 + B0495 + B0228 + C06A8*; on the basis of smaller samples (10–15 adults) we found for all three lines that GFP$^+$ Unc animals were sterile and had abnormal gonads. We did not score arrested Unc larvae in the cross-progeny from the lines carrying the cosmid *F18A1*. NS, not scored; NA, not applicable.

[b] Percentage of normal gonad arms among GFP$^+$ Unc hermaphrodites (H), or of normal gonad arms among GFP$^+$ Unc males (M). Gametes were absent, in reduced number, or very abnormal, in which case they were leading to the production of small early arrested embryos; in addition the germline had a tendency to become vacuolated.

[c] Percentage of GFP$^+$ Unc hermaphrodites with a normal vulva, or of GFP$^+$ Unc males with a normal male tail. The vulva was generally protruding (Pvl) or asymmetric or, occasionally, absent; male tails had missing rays, abnormal fans, and/or protruding spicules.

[d] Total number of GFP$^+$ animals examined with the number of GFP$^+$ hermaphrodites, males, and arrested larvae, respectively, in parentheses.

[e] In addition to GFP$^+$ animals, 79 GFP$^-$-arrested larvae were examined.

[f] In addition to GFP$^+$ animals, 121 GFP$^-$-arrested larvae were examined.

[g] In addition to GFP$^+$ animals, 81 GFP$^-$-arrested larvae were examined.

Although *lir-1(mc33)* hemizygous animals were viable, they were completely sterile and generally had a protruding vulva (Figure 6C; Table 1). The most likely cause for sterility is a germline differentiation defect as gametes were either absent or abnormal (Figure 6E). We are not certain whether the sterility and Pvl phenotypes are attributable to the lack of *lir-1* function, for the following reason: In control experiments in which we introduced the cosmid *F18A1*, which should complement *lir-1*, in addition to the three cosmids that are deleted by *mcDf1* and *mcDf3*, most animals were still partially sterile and often had a protruding vulva (Figure 6G; Table 1). Possible explanations for the sterility phenotype will be discussed later.

In summary, the fact that *lir-1(mc33)* hemizygous animals are viable strongly argues that the phenotypes of *lir-1(RNAi)* animals are primarily, if not entirely, due to their organization in an operon, so that interference of the upstream gene also induces loss of downstream gene expression.

RNAi and operons: To test whether or not this is generally the case we selected two other operons (*ppp-1/tra-2* and *lin-15B/lin-15A*) for which the null phenotypes of the downstream genes had been identified. Null mutations in *tra-2* result in XX animals that have the soma and germline of males (Kuwabara *et al.* 1992). The null phenotype of *ppp-1*, which encodes a pyrophosphorylase, is unknown, but it has not been linked to sex determination (Cline and Meyer 1996). Null mutations affecting *lin-15B* or *lin-15A* do not result in a mutant phenotype, but those affecting both lead to a Muv phenotype (Clark *et al.* 1994; Huang *et al.* 1994). In both cases, we found that injection of dsRNA corresponding to the upstream gene in the operon does not interfere with expression of the downstream gene. Although XX *ppp-1(RNAi)* animals were Dpy, sterile, and barely viable, they had the body and gonad morphology of hermaphrodites whereas XX *tra-2(RNAi)* animals looked like males. In agreement with Montgomery *et al.* (1998) we also found that *lin-15A(RNAi)* and *lin-15B(RNAi)* animals had a normal vulva whereas injection of *lin-15A* dsRNA together with *lin-15B* dsRNA caused a Muv phenotype (data not shown; see also Tabara *et al.* 1998).

Clearly, it is not a general feature of all operons that RNAi directed against the upstream gene will also induce loss of expression of the downstream gene as happens in the *lir-1(short)/lin-26* operon.

RNAi with intronic sequences from *lir-1* and *lin-26* induces a phenotype: It is generally accepted that, for

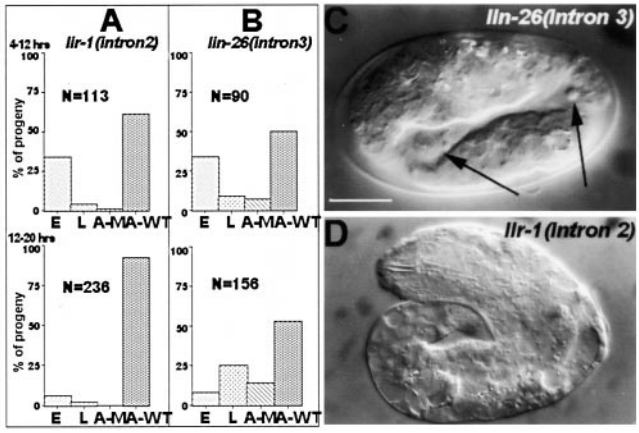

Figure 7.—RNAi against *lir-1* and *lin-26* introns resulted in specific phenotypes. (A and B) These graphs illustrate the range of phenotypes seen with *lir-1(intron2)* and *lin-26(intron3)*. The stronger and most persistent phenotypes are obtained with *lin-26(intron3)*, which is the largest intron at 197 bp. (C) A *lin-26(intron3)*-arrested embryo that had elongated to threefold but had leaking cells within the egg shell (arrows). (D) A *lir-1(intron2)* early L1 larvae that was Dpy with many vacuoles. Bar, 10 μm.

most genes, transcription is coupled to pre-mRNA processing so that as the pre-mRNA is being synthesized it is also being modified to produce the mature transcript (Neugebauer and Roth 1997). For this reason the steady-state level of pre-mRNA varies between genes. A possible explanation for our results is that in the *lir-1(short)*/*lin-26* operon transcript maturation occurs less efficiently than in the *ppp-1*/*tra-2* and *lin-15B*/*lin-15A* operons, thereby allowing the pre-mRNA to be targeted by RNAi.

It has been reported that RNAi using intronic or promoter sequences does not result in detectable interference (Fire *et al.* 1998), but if the *lir-1(short)*/*lin-26* pre-mRNA accumulates, then dsRNA specific for intronic sequences should induce a phenotype. We tested this with dsRNA corresponding to three introns: *lir-1(intron2)*, which is 158 bp; *lin-26(intron2)*, which is 147 bp; and *lin-26(intron3)*, which is 197 bp. The smaller dsRNA molecules, *lir-1(intron2)* and *lin-26(intron2)*, induced a *lin-26*-like embryonic arrest phenotype between 4 and 12 hr postinjection (34 and 21%, respectively) but no significant phenotype at later developmental stages or time points. *lin-26(intron3)* produced a stronger phenotype in both severity and endurance but this is probably because it is 35% larger than the other two (Figure 7).

To confirm that *lir-1(RNAi)* induces *lin-26*-like phenotypes by targeting the pre-mRNA, we performed an additional RNAi experiment, which capitalizes on the fact that *lir-1(mc33) mcDf3* and the *lin-26* null allele *mc15* complement each other for lethality. In *lir-1(mc33) mcDf3*/*lin-26(mc15)* animals, the functional copy of *lin-26* is linked *in cis* to the mutation *lir-1(mc33)*. If the *lin-26*-like phenotypes are the result of the pre-mRNA being targeted, then these animals should be immune to injection of dsRNA corresponding to *lir-1* sequences deleted by *mc33* (Figure 6A). *lir-1(mc33) mcDf3*/*lin-26(mc15)* animals subjected to *lir-1(exon1-4)* RNAi had the same level of lethality and fertility as uninjected animals, whereas injected control animals had nearly 100% lethality (Table 2). This result also demonstrates that the *lir-1* maternal contribution does not account for the *lir-1(RNAi)* phenotypes described previously.

These results provide direct evidence that RNAi can target the pre-mRNA. We conclude that *lir-1(RNAi)* induces loss of *lin-26* expression because the pre-mRNA for this operon is targeted by the dsRNA, thereby resulting in loss of expression of both genes.

DISCUSSION

We have found that RNA interference against *lir-1* leads to severe *lin-26*-like hypodermal defects that result in embryonic or larval lethality. We have excluded the possibility that this is due to cross-interference between homologous sequences because injection of *lir-2* dsRNA, which is as similar to *lin-26* as *lir-1* is, fails to give any phenotype. We have also demonstrated that the *lir-1(RNAi)* phenotypes are not due to a maternal *lir-1* contribution by showing that these phenotypes are entirely dependent on the presence of *lir-1* sequences *in cis* to *lin-26*. We account for the *lin-26*-like phenotypes by showing that *lin-26* expression is severely downregulated in *lir-1(RNAi)*-arrested embryos. Since a null *lir-1* mutant is viable, we exclude the possibility that the lethal phenotypes are attributable to *lir-1* zygotic function. Finally, we have shown that injection of *lir-1* and *lin-26* intron sequences leads to phenotypes similar to those resulting from injection of exon sequences. We conclude that *lir-1(RNAi)* specifically interferes with *lin-26* expression because *lir-1* and *lin-26* are organized in an operon (referred to as the *lir-1(short)*/*lin-26* operon) for which the common *lir-1*/*lin-26* pre-mRNA is available for targeting.

***lir-1* function:** This work started because we were trying to obtain information about the function of *lir-1* and *lir-2*. We presently derive our conclusions about *lir-1* function from two complex genetic backgrounds: hemizygous *lir-1(mc33)* animals (null for *lir-1*; see results) and *lir-1(mc33) mcDf3*/*lin-15(mc15)* animals in which *lir-1* dsRNA had been injected. We believe that *lir-1(mc33)* represents a true null allele because we have demonstrated absence of the sequences corresponding to wild-type *lir-1* and because *mc33* deletes *lir-1* exons 2–4, which include the putative zinc-finger domains (see Figure 6A). Since hemizygous *lir-1(mc33)* animals are viable, we can conclude that, unlike *lin-26*, *lir-1* is not essential for hypodermal development. Although sterility is observed in hemizygous *lir-1(mc33)* animals, *lir-1(RNAi)* did not affect *lir-1(mc33) mcDf3*/*lin-15(mc15)* fertility, so we suggest that *lir-1* is not essential for devel-

TABLE 2

lir-1(RNAi)* induces a phenotype only if *lir-1* is in *cis* to *lin-26

Strain	dsRNA injected	Embryonic arrest (%)	N^a
lir-1(mc33)mcDf3/lin-26(mc15)[b]	Uninjected	48[c]	193
lir-1(mc33)mcDf3/lin-26(mc15)[b]	*lir-1(exons1-4)*	50[c]	133
lir-1(mc33)mcDf3/lin-26(mc15)[b]	*lin-26(full length)*	97	62
rol-6(e187)[d]	*lir-1(exons1-4)*	99	74

[a] Total number of progeny counted between 4 and 12 hr postinjection or cloning.
[b] Full genotype of the strain is *lir-1(mc33) mcDf3 unc-4(e120)/lin-26(mc15) unc-4(e120)*.
[c] Uninjected animals laid 156 ± 55 eggs ($N = 16$); the progeny of injected animals laid 166 ± 46 eggs ($N = 6$); and in both cases egg counts were based on Egl^+ animals (50% of the animals were Egl^+).
[d] The injected control strain is CB187 (see materials and methods).

opment of the germline. There is an alternative explanation for the sterility observed in hemizygous *lir-1(mc33)* animals. In previous deletion mapping experiments, we have shown that animals heterozygous for *mcDf1* and *mnDf106*, a deletion that breaks in *B0228* (see Figure 6A), are viable but sterile (Chanal and Labouesse 1997), which suggests the existence of a locus necessary for germline development in *B0228* and/or in *C06A8*. Complete rescue of a deletion of this locus [as occurs in hemizygous *lir-1(mc33)* animals] is expected to be difficult as transgenes are generally poorly expressed in the germline (Kelly *et al.* 1997). Since we observed that *lir-1(mc33) mcDf3/lin-26(mc15)* and *lir-1(RNAi)* animals are predominantly Pvl (protruding vulva), it is possible that *lir-1* has a late function in vulval and/or uterine development (normal uterine development is necessary for proper eversion of the vulva; Seydoux *et al.* 1993). We do not think that *mc33* deletes a *cis*-acting sequence essential for normal *lin-26* expression during vulval or uterine development. Indeed if we introduce the deletion *mc33* on a *lin-26* transgene that normally completely rescues the null phenotype of *lin-26(mc15)*, we still observe full rescue (S. Quintin and M. Labouesse, unpublished results). However, reduced gene dosage of *lir-1* and *lin-26* may reveal synthetic effects or more subtle requirements for a *cis*-acting sequence. A definitive assessment of the role of *lir-1* in vulval morphogenesis awaits the isolation of another *lir-1* mutation that leads to a less complex genetic background.

RNAi and operons: As summarized before, we have determined that targeting the upstream gene with RNAi also interferes with downstream gene expression for the *lir-1(short)/lin-26* operon. However, for two other operons (*lin-15B/lin-15A* and *ppp-1/tra-2*), we found that targeting the upstream gene has no effect on the downstream gene and vice versa. This is in agreement with results reported for *lin-15B/lin-15A* (Montgomery *et al.* 1998) and *mes-6/cks-1* (Korf *et al.* 1998). We have two possible explanations, which are not mutually exclusive, to account for the RNAi response of different operons: (i) the processing of *lin-15B/lin-15A* and *ppp-1/tra-2* pre-mRNA is more efficient so that a single target for RNAi does not exist; (ii) in all operons a significant proportion of pre-mRNA is targeted by RNAi, but *lin-26* is much more sensitive to gene dosage. To clarify, in other operons there remains enough expression of the downstream gene to ensure wild-type function, whereas reduction of *lin-26* expression by *lir-1(RNAi)* is fatal.

In the case of the *lir-1(short)/lin-26* operon, Northern blots and RT-PCR experiments have confirmed that several pre-mRNA species do exist, which are at least 20-fold less abundant than the mature *lir-1* and *lin-26* transcripts. Specifically, we can detect three precursors at 3.3, 4.2, and 6.2 kb; the 3.3-kb precursor (the most abundant of them) starts, based on its size, at the beginning of *lir-1* exon 3, while the other two presumably start in *lir-1* intron 1 (data not shown; note that we have previously reported the existence of a *lir-1* RT-PCR product beginning at *lir-1* exon 3; Dufourcq *et al.* 1999). So the degree of susceptibility of *lin-26* gene function to *lir-1(RNAi)* probably reflects the competition between RNA processing and RNAi-mediated degradation of the pre-mRNA. This is supported by the observation that for a given length intronic *lin-26* dsRNA is less potent than exonic *lin-26* dsRNA and that short *lir-1* dsRNA molecules are less potent than longer ones, suggesting that a proportion of pre-mRNA escapes the RNAi effect. In addition, we believe that gene dosage is an important aspect of *lin-26* biology in that its correct function depends on specific levels of LIN-26 activity. The precise requirement for LIN-26 in certain cells might explain the very particular phenotypes seen in viable *lin-26* alleles (*n156* and *ga91*). For instance, LIN-26 activity in the hypodermal cells of homozygous *n156* animals allows them to reach adulthood but does not allow the Pn.p cells to adopt a hypodermal fate, resulting in a vulvaless phenotype (Ferguson *et al.* 1987). Furthermore, when *n156* is *in trans* to *lin-26* null alleles, LIN-26 activity is reduced to a level that results in larval lethality (Ferguson and Horvitz 1985; Labouesse *et al.* 1994).

On the basis of the relatively low abundance of the *lir-1(short)*/*lin-26* pre-mRNA, we hypothesize that when the pre-mRNA of an operon exists it provides a target for multi-gene RNAi. Although many operons have been predicted from the physical map, only the few discussed in this work have been biochemically and genetically characterized. For this reason, further testing of our prediction with other operons is not feasible. The recently dissected operon, *mes-6*/*cks-1*, was shown to accumulate pre-mRNA. However, contrary to our hypothesis, RNAi against the upstream gene (*mes-6*) does not interfere with the downstream gene (*cks-1*; Korf *et al.* 1998). How can we explain this apparent contradiction? The *mes-6*/*cks-1* operon is one of three operons recently classified as belonging to a new group of operons (Hengartner and Horvitz 1994) where the genes are only a few nucleotides apart, so that 3′-end formation of the upstream transcript and *trans*-splicing of the downstream transcript are competing processes (Williams *et al.* 1999). This competition results in a single molecule of pre-mRNA being processed to produce either the upstream transcript or the downstream transcript, but not both. We suggest that, since *trans*-splicing of the downstream transcript occurs preferentially to 3′-end formation of the upstream transcript, enough *cks-1* mature transcript is processed to carry out *cks-1* function.

Our results allow us to make several important predictions concerning the mechanism of RNA interference. First, showing that small dsRNA molecules are less potent than long molecules suggests that RNAi efficiency depends on the length of sequence homology between the dsRNA and the target RNA molecules. It is very likely that RNAi utilizes a number of cellular enzymes to ultimately degrade the transcripts that have been targeted in a sequence-specific manner by the injected dsRNA. Length dependence could reflect that as the dsRNA gets longer more cofactors (for instance, nucleases) are recruited to degrade the target RNA at multiple positions. Second, demonstrating that dsRNA is able to target pre-mRNA provides the first direct evidence that RNAi can target transcripts in the nucleus. Thus our results show that the cellular proteins involved in RNAi must be located in the nucleus, at least. This conclusion is consistent with the observations that the *smg* RNA surveillance system is not essential for RNAi in *C. elegans* (Montgomery *et al.* 1998) and that PTGS in plants, a phenomenon possibly related to RNAi, does not involve ribosomes (Holtorf *et al.* 1999). Third, showing that, for a given length, intronic *lin-26* dsRNA is less potent than exonic *lin-26* dsRNA supports the hypothesis that a certain proportion of pre-mRNA escapes intron-mediated RNAi by RNA splicing, or that pre-mRNA is partially protected against RNAi (for instance, by splicing factors). An alternative explanation for the reduced potential of intronic sequences could be that RNAi is predominantly active in the cytoplasm. In other species, there is mounting evidence that RNAi is active mostly or only in the cytoplasm. On the basis of the observation that RNAi does not take place in *Trypanosoma brucei* if pre-mRNA processing is drug inhibited, Ngo *et al.* (1998) have suggested that RNAi can occur only in the cytoplasm or that the pre-mRNA is protected. In Drosophila, the successful targeting of maternal RNA, which is localized in the cytoplasm of the syncytial blastoderm, indicates that RNAi occurs in the cytoplasm (Kennerdell and Carthew 1998; Misquitta and Paterson 1999). In *C. elegans*, the experiments performed by Montgomery *et al.* (1998) show that RNAi completely prevents the accumulation of transcripts in the cytoplasm and, partially, in the nucleus. On the basis of our study and results from different species, we suggest that the cellular cofactors involved in RNA interference must exist both in the nucleus and in the cytoplasm, where they might be more active (or more abundant).

Now that the *C. elegans* genome has been sequenced (The *C. elegans* Consortium 1998), the easiest and fastest way to ascertain a gene's function is by RNAi. Since ~25% of *C. elegans* genes are organized in operons (Zorio *et al.* 1994), it is probable that there will be other operons in which targeting of the pre-mRNA will also produce phenotypes not specific for the gene being tested. We have so far referred to the fact that *lir-1* is the upstream gene and *lin-26* is the downstream gene, but there is no evidence to suggest that the same result would not occur if the genes were in the opposite orientation. Finally, we suggest that interpretation of RNAi results should be carefully considered and, where possible, corroborated before being accepted as fact, at least for genes organized in operons.

We are grateful to Andy Fire for the gift of plasmid pPD93.97. We thank Bernard Boulay for pictures, Grégoire Michaux, Sophie Quintin, Nick Skaer, James Stévenin, and Uwe Strähle for critical reading of the manuscript. J. M. Bosher was supported by an European Molecular Biology Organization fellowship. This work was supported by funds from the CNRS, INSERM, Hôpital Universitaire de Strasbourg, the Human Frontier Science Program Organization, the Association pour la Recherche sur le Cancer, the Groupement de Recherche et d'Etudes sur le Génome, the CNRS Genome Program, and the Ministère de la Recherche.

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Communicating editor: R. K. Herman