Development 130, 2117-2128

doi:10.1242/dev.00429

Two suppressors of *sel-12* encode C_2H_2 zinc-finger proteins that regulate presenilin transcription in *Caenorhabditis elegans*

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Accepted 3 February 2003

SUMMARY

Mutations in presenilin genes are associated with familial Alzheimer's disease in humans and affect LIN-12/Notch signaling in all organisms tested so far. Loss of *sel-12* presenilin activity in *Caenorhabditis elegans* results in a completely penetrant egg-laying defect. In screens for extragenic suppressors of the *sel-12* egg-laying defect, we have isolated mutations in at least five genes. We report the cloning and characterization of *spr-3* and *spr-4*, which encode large basic C_2H_2 zinc-finger proteins. Suppression of *sel-12* by *spr-3* and *spr-4* requires the activity of the second presenilin gene, *hop-1*. Mutations in both *spr-3* and *spr-4* de-repress *hop-1* transcription in the early larval stages when *hop-1* expression is normally nearly undetectable. As *sel-12* and *hop-1* are functionally redundant, this suggests that mutations in *spr-3* and *spr-4* bypass the need for one presenilin by stage-specifically de-repressing the transcription of the other. Both *spr-3* and *spr-4* code for proteins similar to the human REST/NRSF (Re1 silencing transcription factor/neural-restrictive silencing factor) transcriptional repressors. As other Spr genes encode proteins homologous to components of the CoREST co-repressor complex that interacts with REST, and the INHAT (inhibitor of acetyltransferase) co-repressor complex, our data suggest that all Spr genes may function through the same mechanism that involves transcriptional repression of the *hop-1* locus.

Supplemental data available online

Key words: Presenilin, Alzheimer's disease, Genetic suppression, Transcription regulation

INTRODUCTION

Presenilins are a class of polytopic proteins found throughout the plant and animal kingdoms. They are part of high molecular weight complexes containing additional components, including APH-2/Nicastrin, APH-1 and PEN-2 (Capell et al., 1998; Francis et al., 2002; Li et al., 2000; Thinakaran et al., 1998; Yu et al., 1998). This complex assembles and maturates in the ER and Golgi and is subsequently transported to the cell membrane where it is required for the intra-membranous proteolytic cleavage of certain type I transmembrane proteins. These include amyloid precursor protein (APP) and Notch-type receptors (De Strooper et al., 1999; De Strooper et al., 1998; Fortini, 2001). It has been proposed that presenilins themselves provide aspartyl protease activity and are responsible for the γ-secretase cleavage involved in generating β-amyloid fragments from APP (Steiner et al., 2000; Wolfe et al., 1999).

Mutations in either of the human presenilin genes, *PSEN1* and *PSEN2*, are dominant and cause early onset Alzheimer's disease. They result in an increase in the ratio of the 42 amino acid variant to the 40 amino acid variant of β-amyloid, but do not alter the total amount of presenilin-dependent γ-secretase cleavage (reviewed by Selkoe, 2001). The 42 amino acid variant of β-amyloid is highly insoluble and tends to aggregate, nucleating the senile plaques found in brains of individuals with Alzheimer's disease (reviewed by Sisodia and St George-Hyslop, 2002).

Presenilin activity is also required for the S3 cleavage of Notch receptors after ligand binding (Struhl and Adachi, 1998). Like the γ-secretase cleavage of APP, this cleavage occurs within the transmembrane domain and releases the Notch intracellular domain (NICD). The release of the NICD is essential for Notch signaling, because the liberated NICD fragment enters the nucleus where it interacts with the transcription factor CSL (CBP, suppressor of hairless, *lag-1*) (De Strooper et al., 1999; Song et al., 1999) and additional co-activators such as *sel-8/lag-3* or mastermind (Doyle et al., 2000; Freyer et al., 2002; Petcherski and Kimble, 2000).

The *C. elegans* genome encodes three presenilin genes, *sel-12*, *hop-1* and *spe-4* that are homologous to human *PSEN1* and *PSEN2*. *spe-4* is the most divergent member of the presenilin family and appears to have a specific role in spermatogenesis (Arduengo et al., 1998; L'Hernault and Arduengo, 1992). The two other presenilins are much more similar to the human homologs and are absolutely essential for signaling through the

two *C. elegans* Notch-type receptors LIN-12 and GLP-1 (Levitan and Greenwald, 1995; Li and Greenwald, 1997; Westlund et al., 1999). The absence of both *sel-12* and *hop-1* genes leads to a completely penetrant lethal phenotype that resembles either a complete loss of GLP-1 or a complete loss of LIN-12 signaling [the exact phenotype depends on how the double mutants are constructed as both *sel-12* and *hop-1* have partial maternal effects (Westlund et al., 1999)]. On their own, mutations in *hop-1* have no obvious phenotype, while mutations in *sel-12* lead to an egg-laying defect (Egl) (Levitan and Greenwald, 1995; Westlund et al., 1999). *sel-12* and *hop-1* seem to have largely overlapping roles, as *hop-1* can rescue the *sel-12* Egl defect when expressed from a *sel-12* promoter (Li and Greenwald, 1997; Westlund et al., 1999). Not only the sequence, but also the function of presenilins is evolutionarily conserved, as both human presenilins PSEN1 and PSEN2 can also rescue the *sel-12* Egl defect when expressed under the control of appropriate promoters (Baumeister et al., 1997; Levitan et al., 1996).

In order to understand more about the biological role of presenilins, we have been studying the *sel-12* gene in *C. elegans*. Mutations in *sel-12* were first identified for their ability to suppress a *lin-12* gain-of-function mutation (Levitan and Greenwald, 1995). This suggests that *sel-12* mutations reduce *lin-12* signaling and that the SEL-12 protein normally facilitates *lin-12* signaling (Levitan and Greenwald, 1995). However, mutations in *sel-12* do not completely eliminate *lin-12* signaling, presumably owing to residual presenilin activity supplied by *hop-1* (Li and Greenwald, 1997; Westlund et al., 1999). Different levels of LIN-12 activity are required to control at least five post-embryonic signaling events (Eimer et al., 2002a). In *sel-12* null mutants, only two of these are affected to a varying degree (Eimer et al., 2002a; Cinar et al., 2001). This indicates that the presenilin activity supplied by *hop-1* is sufficient for most *lin-12* signaling events and that some *lin-12* signaling events appear to be more sensitive to presenilin dosage than others (Eimer et al., 2002a).

To elucidate the function of the *sel-12* gene further, one can study mutations that bypass the need for *sel-12*. Mutations in four genes, *sel-10*, *spr-1*, *spr-2* and *spr-5*, have already been shown to suppress the *sel-12* egg-laying defect. Mutations in *sel-10* were first found in a screen for genes that suppress a weak *lin-12* loss-of-function mutant (Hubbard et al., 1997). *sel-10* is similar to the yeast gene CDC4, and acts as an E3 ubiquitin ligase that targets the intracellular domains of LIN-12 and GLP-1 proteins for degradation (Gupta-Rossi et al., 2001; Hubbard et al., 1997). *sel-10* mutations also weakly suppress mutations in *sel-12*, but do completely bypass the need for *sel-12*. In a screen similar to the one reported here, Wen et al. have identified four genes that strongly suppress the Egl defect of *sel-12* (suppressors of presenilin) and have described the cloning and characterization of one of them, *spr-2* (Wen et al., 2000). Mutations in *spr-2* almost completely bypass the need for *sel-12*. The biochemical role of SPR-2 is presently unclear, but it may affect chromatin structure and/or transcription (Wen et al., 2000).

In this paper, we report the results of several screens for strong suppressors of *sel-12* and the isolation of 25 independent mutations. These mutations lie in several of the same complementation groups identified by Wen et al. as well as in some additional genes, indicating that neither screen has reached saturation. We also report the cloning and characterization of two suppressor genes, *spr-3* and *spr-4*, that code for C_2H_2 zinc-finger proteins similar to the transcriptional repressors REST/NRSF. *spr-3* and *spr-4* mutants bypass the need for *sel-12* by upregulating the transcription of the other presenilin, *hop-1*. As two other presenilin suppressors that were also identified in this screen, *spr-1* and *spr-5*, encode proteins of the CoREST/HDAC complex (Eimer et al., 2002b; Jarriault and Greenwald, 2002) that interacts with REST, we propose that the Spr proteins assemble into one or more repressor complexes that normally repress the *hop-1* locus in the early larval stages. Mutations in components of these complexes remove a repressor activity leading to a higher basal level of *hop-1* presenilin activity.

MATERIALS AND METHODS

General handling and mutations used

Worms were handled according to standard procedures (Sulston and Hodgkin, 1988) and grown at 20°C unless otherwise stated. The following mutations were used.

LG I: *hop-1(lg1501), dpy-5(e61), ego-1(om71), unc-55(e1170), spr-4(ar208), daf-8(e1393), unc-75(e950), unc-101(m1), unc-59(e261)*.

LG V: *dpy-11(e224), unc-76(e911)*.

LG X: *sel-12(ar171, ar131, by125, lg1401), dpy-23(e830), spr-3(ar209), lon-2(e678), mnDp31, mnDp32*.

All mutations were obtained from the Caenorhabditis Genetics Center, except *sel-12(ar131) sel-12(ar171), spr-3(ar209)* and *spr-4(ar208)* (kindly provided by Iva Greenwald), and *hop-1(lg1501)* [described by Wittenburg et al. (Wittenburg et al., 2000)], *sel-12(by125)* and *sel-12(lg1401)* [described in Eimer et al. (Eimer et al., 2002a)].

Isolation of mutants

Ethylmethanesulfonate (EMS) and ultra violet light/tetramethylpsoralen (UV/TMP) mutagenesis were carried out according to published procedures (Anderson, 1995; Sulston and Hodgkin, 1988). The mutator screen is presented in another paper (Eimer et al., 2002b). We looked in one EMS (16,000 haploid genomes) and one UV/TMP screen (8000 haploid genomes) for dominant suppressor mutations, but did not identify any. We screened for recessive suppressor mutations in a similar manner to Wen et al. (Wen et al., 2000). Mutants were retained when the *spr; sel-12(ar171)* double mutants displayed essentially wild-type egg-laying behavior and the vast majority of their progeny (>90%) did not become Egl. All mutations were outcrossed five times before further phenotypic analysis. For each type of screen, the mutagens used, the number of haploid genomes screened and the mutations identified are as follows.

EMS: 24,300; *by105, by107, by108, by109, by112, by113, by114, by116, by117, by119*.

UV/TMP: 41,600; *by118, by128, by129, by130, by131, by132, by133, by134, by135, by136, by137, by139, by140*.

Mutator generated mutations: 9600; *by101, by110*.

Complementation tests

Complementation tests were done according to standard procedures (Sulston and Hodgkin, 1988). Assignment of complementation groups was as follows.

spr-1: *by133*.

spr-3: *ar209, by108, by109, by110, by131*(*byDf1*), *by135, by136, by137*.

spr-4: *ar208, by105, by107, by112, by114, by129, by130, by132*.

spr-5: *by101, by113, by119, by128, by134, by139*.

Uncharacterized: *by116, by117, by118, by140*.

Genetic mapping

Suppressor mutations were genetically mapped using standard techniques (Sulston and Hodgkin, 1988) maintaining, where possible, the *spr* mutation in a homozygous *sel-12(ar171)* background. The position of *spr-4* was refined further by single nucleotide polymorphism (SNP) mapping carried out essentially as described (Jakubowski and Kornfeld, 1999).

Transgenic rescue of *spr-3* and *spr-4*

We injected into the strain *sel-12(ar171) spr- 3(by108)* to rescue *spr-3* and into the strain *spr-4(by105); sel-12(ar171)* to rescue *spr-4*. We then looked for anti-suppressor activity of injection mixes (i.e. restoration of a *sel-12* phenotype). All test clones and PCR products were injected at 20 ng/μl with 100 ng/μl pRF4 (*rol-6*) and 20 ng/μl pBY218 [*ttx-3::*GFP (Hobert et al., 1997)] as co-injection markers.

Gene structures of *spr-3* and *spr-4*

We sequenced two *spr-3* cDNAs, yk64e9 and yk247c5, kindly provided by Yuji Kohara. We found that the two cDNAs have a very similar structure, yet yk247c5 has an additional intron in the largest exon of the gene. However, on staged northern blots (see Fig. 2, Fig. 4A) and by RT-PCR on each developmental stage, only a single transcript, similar in length to yk64e9, could be detected (data not shown). In the process of sequencing the cDNAs, we also discovered a sequencing error in the genomic sequence from the cosmids F46H6 and C07A12 near the 5′ end of *spr-3*, which put the first ATG out of frame (data not shown). This error was communicated to the *C. elegans* sequencing consortium and the genomic sequence has been updated. To determine the 5′ end of the *spr-3* transcript we performed PCR on a random primed cDNA library, kindly provided by Bob Barstead, using SL1 and SL2 forward primers (Spieth et al., 1993) and gene specific reverse primers (RB1080 CATACTTGACGGC-ATCATCGG; RB1079 CATCTGCTTCTCGCTCGAGAATCG). We found that *spr-3* is trans-spliced to SL1 but not to SL2. The SL1 specific product was sequenced and was found to start just 5′ to the 5′ends of the two sequenced cDNAs at a predicted splice acceptor site.

To determine the structure of *spr-4*, we sequenced the nearly full length cDNA, yk646c12, in its entirety and found that it matched the predicted gene C09H6.1 (Z81466) except that yk646c12 lacks the first five nucleotides of the ORF and the last exon starts six nucleotides more 3′ than in the annotated C09H6.1. As the cDNA, yk1178d11, uses a weak acceptor for this last exon, as annotated in WormBase (http://www.wormbase.org/), *spr-4* appears to be alternatively spliced. The two transcripts encode identical proteins except for a difference of two amino acids in the region between the 17th and 18th zinc fingers (Fig. 5C).

Comparisons with predicted *Caenorhabditis briggsae* genes

Access to the unpublished draft genomic sequence of *Caenorhabditis briggsae* is available from the Welcome Trust Sanger Institute (http://www.sanger.ac.uk/Projects/C_briggsae/) or from the Genome Sequencing Center at Washington University, St Louis (http://genome.wustl.edu/projects/cbriggsae/). The local synteny between *C. elegans* and *C. briggsae* and a preliminary prediction for *C. briggsae* genes can be viewed in Wormbase (http://www.wormbase.org).

The *C. briggase spr-3* gene

We identified a possible *spr-3* homolog in *C. briggsae* on the contig c010301474. We purified total *C. briggsae* mixed stage RNA with a Qiagen RNAeasy kit according to the manufacturer's instructions (Qiagen, Hilden). To determine its gene structure, we performed RT-PCR with various combinations of primers. We amplified a PCR product using the primers RB1627 TACTTGCCACTTGTGTCCAAG and RB1629 TGGTGAACTTTTCACCAGCG from reverse-transcribed first-strand cDNAs generated with the primer RB1629. This PCR product was sequenced and found to contain the central and 3′ regions of the *C. briggsae spr-3* gene.

Expression constructs

An *spr-3*::EGFP *promoter* fusion was made by cloning EGFP at the *spr-3* ATG behind 4 kb of *spr-3* promoter sequence. This construct also contained the *spr-3* 3′UTR. Translational fusion constructs were made by inserting EGFP into a rescuing genomic construct either at the ATG (N-terminal fusion) or before the TAA stop codon (C-terminal fusion), but transgenic lines generated with these constructs did not rescue *spr-3* and did not have detectable GFP fluorescence. The Baculovirus expression construct was made by inserting the *spr-3* cDNA into the transfer vector pBY1296 (Eimer et al., 2002b) fusing a GST-Myc-tag N-terminally to *spr-3*. The resulting construct was co-transformed along with linearized BaculoGold DNA (Becton-Dickinson/Pharmingen) into Sf9 cells to generate recombinant viruses.

RNAi by feeding

We subcloned a 2.7 kb *Hin*dIII/*Xho*I fragment from the cDNA yk356a2 (kindly provided by Y. Kohara) into L4440 (pPD129.36, kindly provided by A. Fire) cut *Hin*dIII/*Xho*I to generate a C28G1.4 RNAi feeding vector. A full-length *hop-1* cDNA was amplified by PCR and cloned as a *Sma*I/*Not*I fragment into L4440 creating pBY1575. Genes were transiently inactivated by RNAi through feeding of the *E. coli* strain HT115(DE3) expressing double stranded RNA of the gene of interest (Timmons et al., 2001; Timmons and Fire, 1998). The dsRNA expression was induced as described (Kamath et al., 2001) and the worms were transferred as L4 larvae onto seeded plates containing 50 μg/ml ampicillin and 1 mM IPTG. After 24 hours the parental worm was transferred to a new plate also containing ampicillin and IPTG. The progeny on the second RNAi plate were then scored for the relevant phenotypes. In the case of *sel-12(ar171)* animals, the parental worms were kept on the first RNAi plate until they died with a bag of worm phenotype and only the last progeny were scored for the RNAi phenotype.

Northern blots

RNA was isolated from mixed stage plates or staged plates and prepared with an RNAeasy kit according to the manufacturer's instructions (Qiagen, Hilden). For most Northern blots, 5 μg of total RNA per lane was denatured at 65°C for 5 minutes and then loaded onto a 0.8% agarose RNA gel. The gel was run overnight to separate fragments and blotted onto Hybond N+ membranes according to Sambrook (Sambrook, 1989). For the L1 northern blots, 20 μg of total RNA was used per lane. Probes were labeled with α^{32}P dCTP using a Megaprime labeling kit according to the manufacturer's instructions (Amersham, Freiburg, Germany). Blots were hybridized and washed according to the procedure of (Church and Gilbert, 1984) at 65°C. All northern blots were probed with an *ama-1* specific probe (Johnstone and Barry, 1996) as a loading control. For each blot we first made a blot with 5 μg per lane of total RNA and probed it with *ama-1*. We then used the results of this probing to adjust the amount of RNA loaded to obtain equal amounts of mRNA per lane. For the quantification of relative transcript levels, blots were placed on a storage phosphor screen (Molecular Dynamics) for several days and were read with a Storm 860 scanner (Molecular Dynamics). The intensity of bands was determined using ImageQuant version 4.2 (Molecular Dynamics) using the User Method of Volume Quantitation. Volumes were adjusted for background intensity.

For staged northern blots, worms were synchronized at the L1 stage by alkaline hypochlorite treatment (Sulston and Hodgkin, 1988). Then synchronized L1 larvae were spotted onto 9 cm plates seeded with OP50 and allowed to grow for 6 hours, 18 hours, 30 hours, 42 hours and 54 hours for L1, L2, L3, L4 and young adult stages, respectively. Worms were inspected visually before harvesting to confirm that the worms were at the correct stage.

RESULTS

The *sel-12* suppressor screen

To isolate mutations that bypass the need for *sel-12*, we performed several screens for suppressors of the egg-laying defect of *sel-12(ar171)* mutants, using chemical (EMS, UV/TMP) and genetic (Eimer et al., 2002b) mutagenesis protocols. The different screens were chosen to induce a range of types of mutations and to get some alleles with restriction fragment length polymorphisms (RFLPs). We recovered no dominant suppressor but did isolate 25 strong recessive suppressor mutations. Twenty out of the 25 mutations fall into only three complementation groups, defined by the alleles *by108*, *by105* and *by101*. *by101* was mapped to the right arm of chromosome I between *unc-101* and *unc-59* very near to *unc-59*, *by105* was mapped genetically to the cluster on LGI between *daf-8* and *unc-55*, while *by108* was mapped to LGX between *dpy-23* and *lon-2* (see Table S1 at http://dev.biologists.org/supplemental/).

Subsequently, Wen et al. identified four suppressors of presenilin (Spr genes) in a similar screen and described the cloning and characterization of one of them, *spr-2* (Wen et al., 2000). Wen et al. mapped *spr-1*, *spr-2*, *spr-3* and *spr-4* to chromosomes V, IV, X and I, respectively. By complementation analysis with *spr-3(ar209)* and *spr-4(ar208)* (kindly provided by I. Greenwald, New York), we determined that *by108* and *by105* were alleles of *spr-3 and spr-4*, respectively. Consequently, we defined a new gene, *spr-5*, with the reference allele *by101* (Eimer et al., 2002b). We examined the remaining five suppressor mutations found in our screens to see if they could be *spr-1* or *spr-2* alleles. None of the remaining five alleles had a mutation in the coding region of *spr-2*. However, *by133* showed close linkage to *dpy-11* on chromosome V and mapped to a similar region as *spr-1* (see Table S1 at http://dev.biologists.org/supplemental/). *spr-1* has recently been cloned (Jarriault and Greenwald, 2002) and we have found that *by133* contains a mutation in this gene (Eimer et al., 2002b). The remaining four suppressor mutations have not been pursued in detail, but by complementation tests define three additional genes (data not shown). The fact that we found no *spr-2* alleles and that we have found mutations in genes not identified by (Wen et al., 2000) indicates that saturation was not reached in either screen. The rest of this paper will report the cloning and characterization of two of the major complementation groups, *spr-3* and *spr-4*.

spr-3 and *spr-4* potently and specifically suppress *sel-12*

Roughly 75% of all *sel-12(ar171)* adult animals display a protruding vulva (Pvl; Fig. 1), a defect that is strongly correlated with, and presumably caused by, the mis-specification of the π lineage (Eimer et al., 2002a). Almost all *sel-12* animals retain too many eggs in the uterus (an egg-laying defective or Egl phenotype) and these eggs hatch and develop within the mother, leading to a terminal 'bag of worms' (Bag) phenotype (Fig. 1). The Egl defect severely limits the number of progeny generated (Table 1). Mutations in *spr-3* completely suppress all aspects of the *sel-12* egg-laying defect (Fig. 1). s*el-12(ar171) spr-3* double mutants also display a nearly wild-type brood size (Table 1), indicating that *spr-3* mutations restore normal egg-laying behavior and

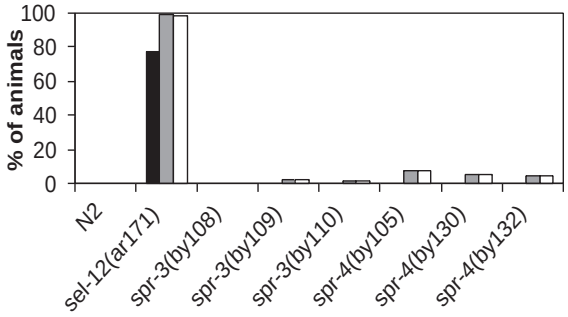


Fig. 1. Mutations in *spr-3* and *spr-4* potently suppress the egg-laying defect of *sel-12* mutants. The percentage of animals that exhibit a protruding vulva (Pvl, black bar), an egg-laying defect (Egl, gray bar) and that die of internal hatching (Bag, white bar) are shown for the wild-type (N2), *sel-12* and three separate alleles each of *spr-3* and *spr-4* in a *sel-12(ar171)* background. Number of animals examined: N2, 100; *ar171*, 95; *by108*, 97; *by109*, 99; *by110*, 99; *by105*, 92; *by130*, 95; *by132*, 87.

normal fertility. They also respond to neurotransmitters that stimulate egg laying (data not shown). *spr-4* mutations, however, lead to a less completely penetrant suppression of the *sel-12* phenotype and in all alleles, ~5% of *spr-4*; *sel-12(ar171)* animals still become Egl (Fig. 1). This Egl phenotype is similar to *sel-12(ar171)*, except that no Pvl animals are seen. This suggests that in these remaining Egl animals at least part of the *sel-12* phenotype was rescued. However, those *spr-4; sel-12(ar171)* double mutant animals that do lay eggs display a nearly wild-type brood size (Table 1). Mutations in *spr-3* and *spr-4* also suppress all other *sel-12* alleles tested (*ar131*, *by125*, *lg1401* for *spr-3*, and *ar131* for *spr-4*; Table 1 and data not shown). On their own, mutations in *spr-3* and *spr-4* have no obvious phenotype, except perhaps a slightly reduced brood size (Table 1; data not shown). For *spr-3*, we examined a clear null mutation (*by131* also known as *byDf1*) in more detail. Surprisingly even this mutation, a 31 kb deletion that deletes five genes including *spr-3* and both its upstream and downstream neighbor (Fig. 2B), has no obvious phenotype (Table 1; data not shown), indicating that none of the deleted genes is essential. Taken together these results show that *spr-3* and *spr-4* are potent and specific suppressors

Table 1. The brood size of *spr-3* and *spr-4* mutants

Genotype	Broods	Progeny
N2	20	314±26
sel-12(ar131)	20	126±61
sel-12(ar171)	20	61±16
*sel-12(ar131) spr-3(by108)**	20	226±33
*sel-12(ar171) spr-3(by108)**	20	283±27
sel-12(ar171) spr-3(by108); byEx134†	20	53±26
byDf1‡	20	230±25
sel-12(ar171) byDf1‡	19	205±63
spr-4(by130); sel-12(ar171)§	20	239±33

*Similar results were obtained with the alleles *by109* and *by110* (data not shown).

†*byEx143* is an extra-chromosomal array containing a 9.3 kb *Bam*HI fragment from F46H6 (see Fig. 2B), pBY218 and pRF4. Twenty rollers were picked as L4s and their brood size was determined.

‡Similar results were obtained with the *spr-3* allele *by135* (data not shown).

§Similar results were obtained with the *spr-4* alleles *by105* and *by132* (data not shown).

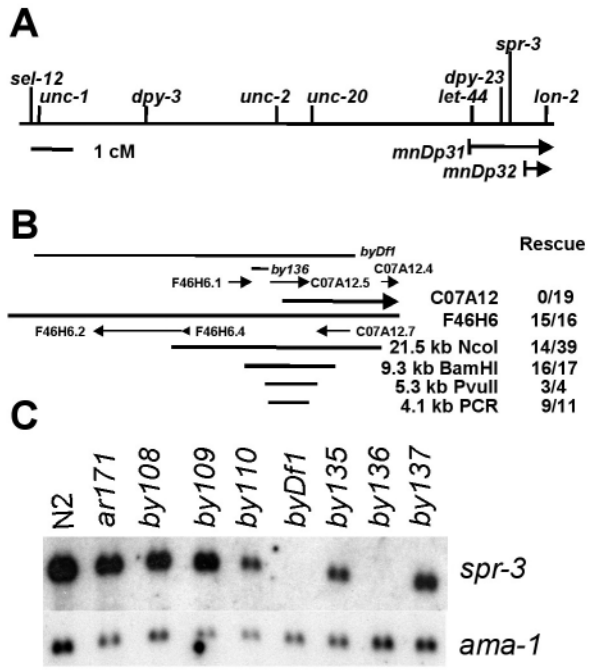


Fig. 2. The positional cloning of *spr-3*. (A) By three-factor mapping, *spr-3* was mapped to the interval between *dpy-23(e840)* and *lon-2*, outside of the duplication *mnDp32* (see Table S1 at http://dev.biologists.org/supplemental/) Note that *spr-3* was previously mapped between *dpy-3* and *unc-2*, close to *unc-2* (Wen et al., 2000). (B) Rescue of *spr-3*. *spr-3* was rescued by the cosmid F46H6 but not the partially overlapping cosmid C07A12, both shown by thick bars. The cosmid C07A12 extends further to the right. ORFs on F46H6 are named and indicated by lines with arrows. Two *spr-3* alleles generated by UV/TMP mutagenesis are large rearrangements. *byDf1* deletes 31 kb of the cosmid F46H6, while *by136* is a complex rearrangement that affects the promoter of C07A12.5. By injecting a series of restriction fragments, subclones and PCR products (see thick bars below F46H6) the minimal rescuing region was narrowed down to a 4.1 kb fragment (using RB950 CAGTATACAACTACGCTCTCC and RB951 ATCCAACACTCCTAAGTCCG), which contains only the C07A12.5 open reading frame. The number of lines that were rescued is indicated on the right for each construct. (C) Northern blot with RNA from N2, *sel-12(ar171)* and 7 *spr-3* alleles in a *sel-12(ar171)* background probed with a *spr-3* cDNA. No message is detectable in strains harboring either of the two large rearrangements, *byDf1* or *spr-3(by136)*.

of *sel-12* that are able to suppress all aspects of the *sel-12* phenotype.

SPR-3 is a C_2H_2 zinc-finger protein

Genetic mapping placed *spr-3* on chromosome X between *dpy-23(e840)* and *lon-2(e678)*, very close to *dpy-23* (Fig. 2A). *dpy-23(e840)* is a roughly 100 kb deletion with left and right breakpoints in the cosmids C15H3 and F02E8, respectively (G. Garriga, personal communication). We started sequentially injecting the sequenced cosmids from F02E8 to the right. *spr-3* was completely rescued by the cosmid F46H6 but not by the partially overlapping cosmid C07A12 (Fig. 2B). We then injected a series of purified restriction fragments, subclones and PCR products from F46H6 (Fig. 2B). All injected products containing the entirety of the predicted gene C07A12.5 produced complete rescue of *spr-3* (Fig. 2B, Table 1 and data not shown). The minimal rescuing fragment was narrowed down to 4.1 kb containing C07A12.5 as the only predicted open reading frame.

spr-3 encodes a novel, basic protein (predicted pI=9.1) of 684 amino acids. The only recognizable domains in SPR-3 are seven putative C_2H_2 zinc-finger and several regions that may act as nuclear localization signals (Fig. 3A,B). The *spr-3* alleles *ar209*, *by108*, *by110* and *by137* are all amino acid to stop codon mutations at various positions in the protein (Fig. 3B). The *by135* mutation is a single base pair deletion, which shifts frame after amino acid 183 and truncates the protein at 210 amino acids. The *by109* mutation is a C596Y transition in the second cysteine of the sixth zinc finger, indicating that this finger is essential for SPR-3 function. *by131* is a deletion of 31,069 bases from position 3052 of F46H6 to position 6698 of C07A12 with a single A base pair insertion. This mutation deletes F46H6.2/*dgk-2*, F46H6.4, F46H6.1/*rhi-1*, C07A12.5/*spr-3* and part of C07A12.7, and is clearly null for *spr-3* function (Fig. 2B). As *by131* deletes several genes, we have renamed it *byDf1*. By a combination of PCR, Southern blotting and sequencing, we determined that there are no alterations in the coding sequence of *by136*. However, *by136* has a complex promoter rearrangement in C07A12.5 (data not shown). Using northern analysis, a single transcript is detectable in *by108*, *by109*, *by110*, *by135* and *by137* lanes at nearly wild-type levels although no transcript is detectable for *byDf1* and *by136* (Fig. 2C) indicating that *by136* is also null for *spr-3* function.

SPR-3 is broadly expressed and nuclearly localized

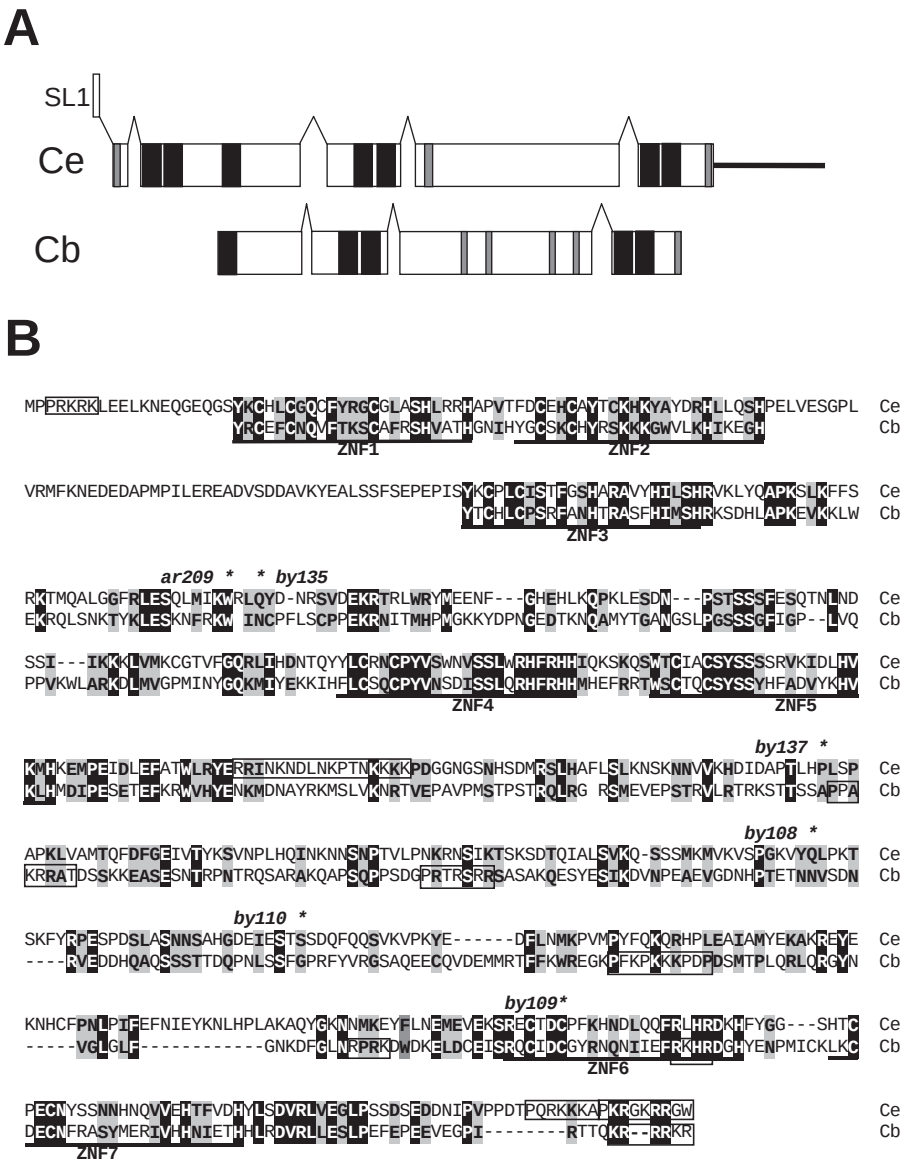
The *spr-3* transcript is expressed in all stages but at different levels. The message is present in high amounts in eggs, L2, and adult stages, but more weakly expressed in the L1, L3 and L4 stages (Fig. 4A). To determine the expression pattern of *spr-3*, we made a promoter fusion to EGFP. This construct is very broadly expressed in the embryo, larval stages and in the adult (Fig. 4B,C). When expressed in Sf9 insect cells using a baculovirus expression system, SPR-3 is localized in the nucleus (Fig. 4D,E). Attempts to purify SPR-3 from insect cells failed. SPR-3 remained in the nuclear fraction even after DNase treatment and high salt extraction (data not shown). It is therefore likely that SPR-3 is present in a nuclear subcompartment or attached to the nuclear periphery.

The *spr-3* gene has evolved rapidly

We were unable to identify any clear homologs of SPR-3 in the sequence databases. To understand better what regions of the protein could be important for its function, we tried to identify homologs in *C. briggsae*, the closest known relative of *C. elegans* (Blaxter et al., 1998). Using RT-PCR, we have isolated a large fragment of this transcript and determined that it has a similar exon/intron structure to *spr-3* in *C. elegans* (see Fig. 3A,B). However, the *C. briggsae* gene is only 22% identical and 45% similar to *C. elegans spr-3* (see Fig. 3B), with most of the similarity confined to the zinc-finger regions. The predicted 5′ end of the *C. briggsae spr-3* gene is significantly diverged from the *C. elegans spr-3*. However, in the predicted N-terminal region of *C. briggsae* SPR-3, there is a region similar to zinc fingers 1 and 2 of *C. elegans* SPR-3 (Fig. 3B).

SPR-3 is similar to transcriptional repressors

The fact that SPR-3 is only weakly conserved in *C. briggsae*, with similarity largely confined to the zinc-finger domains, suggests that the regions between the zinc fingers are under little selective pressure and that the zinc fingers and nuclear localization signals may be the only functional domains of the protein. If this were the case, then we would expect that most mutations that result in amino acid substitutions would have no phenotypic consequences. Consistent with this, only one out of eight *spr-3* alleles is a missense mutation, and this mutation affects a conserved cysteine of one of the zinc fingers. SPR-3 contains three pairs of adjacent zinc fingers and one lone zinc finger separated by non-conserved linkers. Therefore, to analyze the zinc finger regions of the protein, we concatenated the sequences of just the zinc fingers, along with the short linkers between tandem fingers, and searched the databases for similarity. This sequence is similar to many C_2H_2 zinc-finger proteins and is most similar to members of the REST family of transcriptional repressors (Fig. 3C). This suggests that SPR-3 may also function as a transcriptional repressor.



SPR-4 belongs to a family of C_2H_2 proteins related to transcriptional repressors

spr-4 was mapped between *unc-55* and *daf-8* on LGI, close to, but to the right of, the single nucleotide polymorphism (SNP) vl20a11.s1@186 on cosmid F18C12 (Fig. 5A,B). Near this point is the gene C09H6.1, which has the greatest similarity in *C. elegans* to C07A12.5 (Fig. 5B). By probing *spr-4* alleles on a Southern blot with yk18b7, a C09H6.1 cDNA, we could identify clear polymorphisms in two UV/TMP generated *spr-4* alleles, *by130* and *by132* (data not shown). Injection of either of two cosmids that overlap C09H6, F34G10 and C48B11, gave partial rescue of *spr-4* (data not shown). Both of these cosmids contain a 12 kb *Pvu*II fragment that contains the entire coding

Fig. 3. The structure of SPR-3 from *C. elegans* and *C. briggsae*. (A) The determined structure of *C. elegans* SPR-3 and the 3′ end of the *C. briggsae* homolog. Black boxes indicate the location of the predicted C_2H_2 zinc fingers and gray boxes indicate the locations of sequences that might act as nuclear localization signals. In the region confirmed, the structure of the *C. elegans* and *C. briggsae* transcripts are very similar. (B) An alignment of the *C. elegans* and partial *C. briggsae* sequences of SPR-3. Also shown is a region 5′ of the determined sequence of the *C. briggsae* SPR-3 that is similar to the zinc fingers 1 and 2 of the *C. elegans spr-3* gene, and is predicted to be part of the *C. briggsae spr-3* gene. Identical amino acids are highlighted in black and similar amino acids are indicated in gray. Predicted zinc fingers are underlined and regions containing sequences that could act as nuclear localization signals are boxed. The positions of point mutations are shown with an asterisk. (C) Alignment of the zinc-finger regions of SPR-3 with REST (*Homo sapiens*). Identical amino acids are highlighted in black and similar in gray. Gaps in the alignment are indicated by hyphens and gaps between segments of SPR-3 are indicated by blank spaces.

region of C09H6.1, 2 kb of 3′ sequence, as well as 4.5 kb of 5′ sequence extending almost to the next gene upstream (Fig. 5B). We subcloned the *Pvu*II fragment from F34G10 into pBSIISK- cut with *Pvu*II and found that this rescues *spr-4* as well as the original cosmids (data not shown).

spr-4 codes for a large protein with 1309 amino acids (Fig. 5C) containing 18 C_2H_2 zinc-finger domains. One of the zinc fingers, labeled ZNF7, is below threshold by Pfam (http://www.cgr.ki.se/Pfam/) but this region is highly conserved in *C. briggsae* (data not shown), indicating that it may represent a functional domain. A possible splice variant has also been suggested based on the sequence of the yk1178d11 cDNA (see Materials and Methods). SPR-4 is also predicted to be nuclearly localized and contains several nuclear localization signals (NLS), including a bipartite NLS. We have identified the mutations in three *spr-4* alleles (Fig. 5C). *by130* contains a 64 bp deletion and 8 bp insertion at position 1165 of the message. This deletion shifts frame at amino acid 389 and truncates the predicted protein at position 404 (Fig. 5C). The *by112* allele is a Q97stop mutation (Fig. 5C). By Southern analysis, *by132* contains a ~500 bp deletion near the 3′ end of the gene (data not shown).

SPR-4 has clear homologs in *C. briggsae* (data not shown), and in the more distantly related nematodes *Pristionchus pacificus* (AI989188) and *Parastrongyloides tricosuri* (BM513702) (J. McCarter, personal communication). SPR-4 also has strong similarity ($e=3\times10^{-43}$) to C28G1.4 in *C. elegans* (NM 077098.1). Twenty-one of the first 34 amino acids are identical between SPR-4 and C28G1.4. This region is followed in both proteins by a highly acidic stretch suggesting that this region forms a functional domain (Fig. 5C). The central region of C28G1.4 does not resemble any other protein but C28G1.4 is 34% identical to SPR-4 in the N-terminal section from ZNF13 to the end of the protein and contains all zinc fingers in this region except ZNF15, suggesting that SPR-4 and C28G1.4 may have a related function (Fig. 5C). However, RNA interference by feeding (Kamath et al., 2001) of C28G1.4 has no obvious effects on the wild-type strain N2 nor does it influence the Egl defect of *sel-12(ar171)* and *sel-12(ar131)* (data not shown). dsRNAi against C28G1.4 also fails to produce any synthetic effect in either a *spr-4(by105); sel-12(ar171)* or a *spr-4(by105)* background (data not shown). These results may suggest that an RNAi effect was not induced by the bacterial feeding approach; however, RNAi by injection of purified double stranded RNA from yk356a2, a C28G1.4 cDNA, also induced no phenotype (Maeda et al., 2001). Interestingly, there is no C28G1.4 homolog present in the draft *C. briggsae* assembly, suggesting that C28G1.4 may have recently diverged from an ancestral *spr-4* like gene or that this gene is under very low selective pressure or can be lost without phenotypic effects.

SPR-4 also has similarity to a large number of zinc-finger proteins from other metazoans. SPR-4 is most similar to members of the REST family of transcriptional repressors (Fig. 5D) but it also has weaker similarity to other known transcriptional repressors, such as members of the CTCF/CCCTC-binding factor, suggesting that SPR-4, like SPR-3, may also function as a transcriptional repressor.

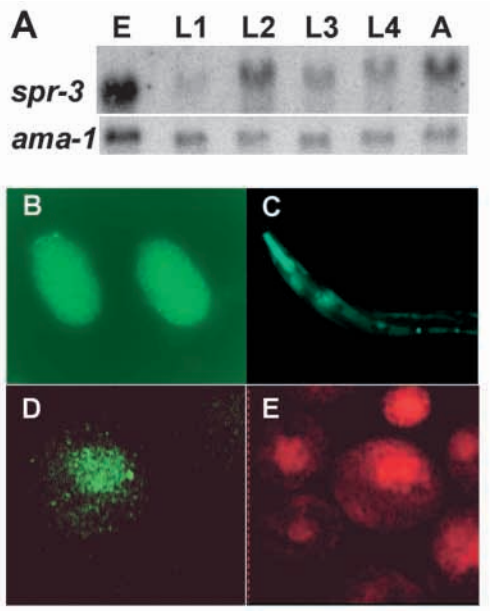


Fig. 4. The expression pattern of *spr-3*. (A) The stage-specific expression of the *spr-3* transcript with *ama-1* shown as a control; E, eggs; L1-L4, first to fourth larval stages; A, adult. (B,C) An *spr-3*::EGFP promoter fusion is expressed broadly throughout the animal. *spr-3* is expressed uniformly in eggs (B) and very broadly in the adult (C) with strong expression in the pharynx. (D,E) Expression of a GST-Myc tagged *spr-3* Baculovirus construct expressed in insect Sf9 cells and detected with a fluorescein-isothiocyanate coupled goat-anti-mouse secondary antibody. (D) Fluorescent and (E) propidium iodide staining of the same cells.

hop-1 transcription is regulated by *spr-3* and *spr-4*

As both SPR-3 and SPR-4 encode C_2H_2 zinc-finger proteins that are probably nuclearly localized and might act as transcription factors, we looked for possible targets regulated by *spr-3* and *spr-4*. Therefore, we probed northern blots prepared with mixed stage, or staged, RNA from *spr-3* and *spr-4* mutants with several genes involved in *lin-12* or *glp-1* signaling. No significant differences in transcript levels were seen between any of the strains when we probed with *lin-12*, *glp-1*, *lag-1*, *apx-1* or *sup-17* (data not shown). Although we did not probe exhaustively, this suggested that *spr-3* and *spr-4* might not have obvious effects on the transcription of genes involved in the *lin-12* and *glp-1* pathways.

We then investigated whether *spr-3* and *spr-4* might bypass the need for *sel-12* by up-regulating one of the other presenilin genes, *spe-4* or *hop-1*. Results from mixed stage blots suggested that *hop-1* and *spe-4* may be differentially expressed (data not shown). Therefore, we then probed staged Northern blots. Transcript levels of the three *C. elegans* presenilin genes in the various developmental stages have not been reported previously. We find, consistent with its only known role in spermatogenesis, that *spe-4* is only expressed in the L4 larval stage when hermaphrodites produce sperm (Fig. 6A,C). s*el-12* is expressed strongly and uniformly throughout development (Figs 6B,C), consistent with the strong and ubiquitous expression of a *sel-12*::EGFP promoter fusion (Baumeister et al., 1997). Surprisingly, *hop-1* has a very dynamic expression. It is most strongly expressed in the adult stage, more weakly in the embryo and is almost undetectable in the L1 stage (Figs 6A,C). *hop-1* expression slowly increases through the remaining larval stages.

In *spr-3* and *spr-4* mutants, we found no differences in the temporal pattern of expression of *sel-12* or *spe-4*, but we did see an increase in *hop-1* expression in the L1, L2 and L3 larval stages, those stages, in which *hop-1* expression is lowest (data not shown). As the egg-laying defect in *sel-12* animals is due to developmental defects occurring in the mid-larval stages, the suppression of *sel-12* by Spr genes could be explained by stage specific alterations in gene expression. To confirm this result, and to directly compare different strains, we prepared a northern blot with L1 RNA from N2, *sel-12(ar171)* and several Spr strains and probed it with *hop-1*. As the expression of *hop-1* is lowest in the L1 stage, we reasoned that increased expression in this stage might be easiest to detect. We increased the amount of total RNA used from 5 μg/lane to 20 μg/lane because the expression of *hop-1* in wild-type worms is near the detection level. We tested L1 RNA prepared from N2, *sel-12(ar171*), four *spr-5* alleles, one *spr-4* allele and three *spr-3* alleles (Fig. 7). In all Spr strains tested, *hop-1* is upregulated. This suggests that all Spr genes may use the same mechanism to bypass the requirement for *sel-12*. *spr-4* upregulates *hop-1* expression 11-fold, whereas *spr-3* alleles upregulate *hop-1* between four- and 12-fold (Fig. 7). These data were confirmed by separate experiments in which we looked at *hop-1* expression in the L1 stage in: 1) all seven *spr-3* alleles isolated in our screens; and 2) one *spr-5* allele and two *spr-3* alleles without *sel-12* in the background. In all *spr* alleles, *hop-1* was more strongly expressed than in the controls (see Figs S1 and S2 at http://dev.biologists.org/supplemental/).

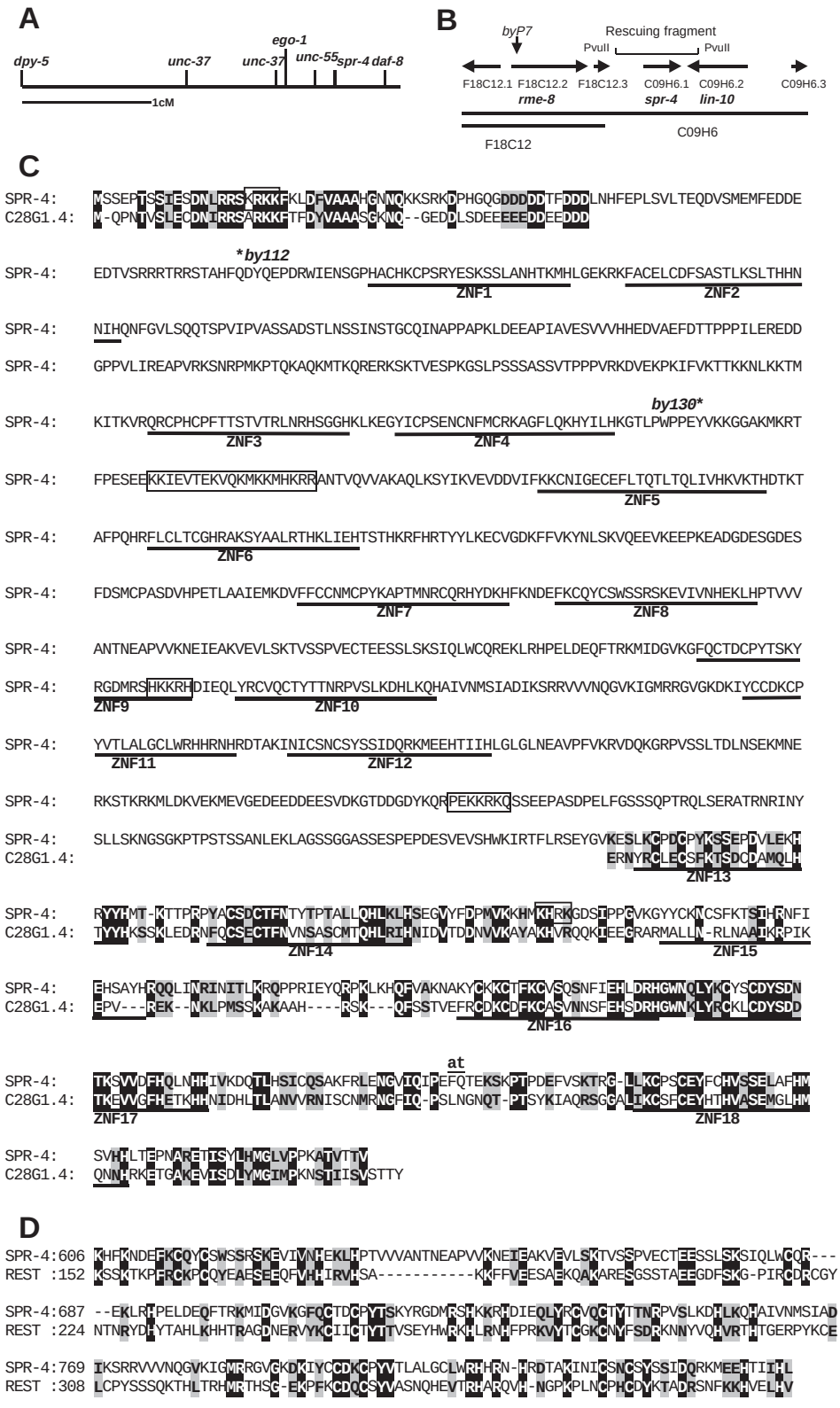


Fig. 5. The cloning of *spr-4*. (A) The genetic position of *spr-4* (see Table S1 at http://dev.biologists.org/supplemental/). (B) Physical map near C09H6.1. *spr-4* was mapped close to but to the right of byP7 (SNP v120a11.s1@186). The extent of the 12 kb *Pvu*II rescuing fragment is shown. (C) The predicted SPR-4 protein aligned with the 5′ end of C28G1.4 and the 3′ end of G28G4.4. Identical amino acids are highlighted in black and similar amino acids are highlighted in gray. The 18 putative C2H2 zinc fingers of SPR-4 are underlined, and regions that could act as nuclear localization signals are boxed. The two amino acids present only in the alternatively spliced form of SPR-4 are overlined and labeled at. Mutations are indicated with an asterisk.
(D) Alignment of SPR-4 with REST (*Homo sapiens*). Identical amino acids are highlighted in black and similar amino acids are highlighted in gray.

spr-3 and *spr-4* do not suppress the synthetic lethality of *hop-1; sel-12* double mutants

If the Spr genes bypass the need for *sel-12* by upregulating the expression of *hop-1*, then *spr-3* and *spr-4* mutations should not suppress the synthetic lethal phenotype induced by reducing both *hop-1* and *sel-12* activity. This is exactly what we find. RNAi by feeding of the *hop-1* gene in a *sel-12(ar171) spr-3(by108)* strain or a *spr-4(by105); sel-12(ar171)* strain induces the same range of phenotypes as seen when one induces RNAi of *hop-1* in *sel-12(ar171)* (Table 2) (Li and Greenwald, 1997). Most animals display a lethal phenotype (Emb, Lag, Ste), consistent with a strong reduction of *lin-12* and/or *glp-1* signaling, but those few animals that do produce progeny are Egl. This indicates that *spr-3* and *spr-4* mutations do not bypass the need for presenilins per se, but only for *sel-12* and that this effect is dependent on the activity of *hop-1*. We confirmed this result by constructing *hop-1; sel-12 spr-3* triple mutant strains (Table 3). Similar experiments with *spr-4* have not been performed because of the genetic proximity of *spr-4* and *hop-1*. We find that *spr-3* does not affect the phenotype of *hop-1; sel-12* double mutants as has also been shown for *spr-1*, *spr-2* and *spr-5* (Eimer et al., 2002b; Jarriault and Greenwald, 2002; Wen et al., 2000). Furthermore, 43% of all *hop-1(lg1501)/dpy-5(e61) I; sel-12(ar171) spr-3(by108)* animals with only one remaining copy of *hop-1* and no functional *sel-12* gene display an Egl phenotype (Table 3). As *sel-12(ar171) spr-3(by108)* animals are never Egl (see Fig. 1) this indicates that in a *sel-12 spr-3* background *hop-1* is haploinsufficient. In *sel-12 spr-3* mutants, presenilin activity is very near the threshold necessary for correct egg laying and reducing *hop-1* activity by half brings the worms below this threshold. Taken together, all of these results are consistent with *spr-3* and *spr-4* suppressing *sel-12* by upregulating *hop-1*.

Table 2. *hop-1* activity is required for *spr-3*- and *spr-4*-mediated suppression of the *sel-12* egg-laying defect

Genotype	Phenotype without RNAi*	*hop-1* RNAi phenotypes†	*n*‡
N2	Wild type	Wild type	(30/30)
sel-12(ar171)	Egl	Egl; Ste; Emb; Lag	(30/30)
sel-12(ar171) spr-3(by108)	Wild type	Egl; Ste; Emb; Lag	(48/50)§
sel-12(ar171); spr-4(by105)	Wild type ¶	Egl; Ste; Emb; Lag	(50/50)

*RNAi with the empty vector resulted in the same phenotypes as seen without RNAi.

†Phenotypes obtained by RNA interference against *hop-1* through bacterial feeding of dsRNA of *hop-1*. Phenotypes as described in Li and Greenwald (Li and Greenwald, 1997): Egl, egg-laying defective; Ste, sterile; Emb, embryonic lethal; Lag, *lin-12* and *glp-1*.

‡The numbers in brackets correspond to the number of parental L4 worms whose progeny show the annotated phenotypes.

§Two lines showed a Gro phenotype and did not develop.

¶Approximately 5% of the animals showed an Egl phenotype (see Fig. 1).

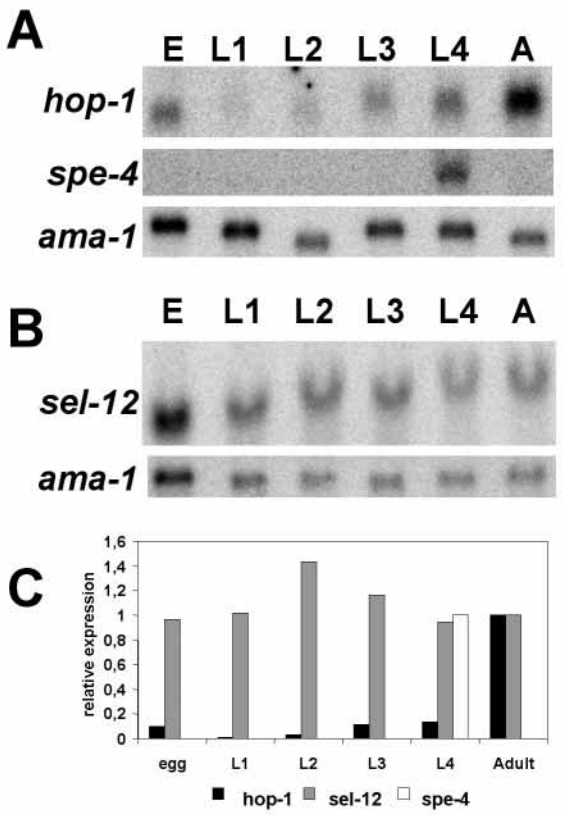


Fig. 6. The stage-specific expression of *sel-12*, *spe-4* and *hop-1* transcripts. Stages are as for Fig. 4A. (A) The stage-specific expression of *hop-1* and *spe-4* from the same blot, with *ama-1* as an equal loading control. (B) The stage-specific expression of *sel-12* with *ama-1* as a loading control. (C) The relative expression of the various presenilin genes from A and B after correction for equal loading.

DISCUSSION

The difference in expression patterns may explain the phenotypes of *sel-12* and *hop-1*

Although *sel-12* and *hop-1* have very similar biochemical functions and are interchangeable in transgenic experiments, mutations in *sel-12* and *hop-1* result in different phenotypes. This suggests that although these genes may encode functionally equivalent proteins, the genes are not redundant. The different phenotypes may be explained by their different

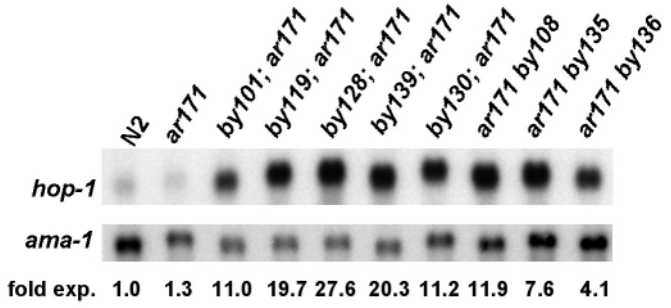


Fig. 7. Mutations in *spr-3* and *spr-4* de-repress the expression of the *hop-1* message at the L1 stage. Expression of the *hop-1* message with *ama-1* loading control in the L1 stage for N2 (wild type), *sel-12(ar171)*, four *spr-5* alleles (*by101*, *by119*, *by128*, *by139*), one *spr-4* allele (*by130*) and three *spr-3* alleles (*by108*, *by135*, *by136*). All Spr mutants are in a *sel-12(ar171)* background. Fold expression of *hop-1* compared with N2 is given, after correction for equal loading. The first six lanes are reproduced, with permission, from Eimer et al. (Eimer et al., 2002b). Preliminary experiments with 5 μg/lane RNA gave qualitatively similar results. See Fig. S1 at http://dev.biologists.org/supplemental/ for additional *spr-3* alleles.

Table 3. *hop-1* is haploinsufficient in a *sel-12 spr-3* background

Progeny of *hop-1(lg1501)/dpy-5(e61) I; sel-12(ar171) spr-3(by108)* hermaphrodites

Phenotype*	Genotype†	Number‡	Percent
Dpy	*dpy-5; sel-12 spr-3*	50/197	25.4
Pvl Ste	*hop-1; sel-12 spr-3*	56/197	28.4
WT	*dpy-5/hop-1; sel-12 spr-3*	51/197	25.9
Egl§	*dpy-5/hop-1; sel-12 spr-3*	38/197	19.3

*Phenotypes: Dpy, dumpy; Pvl, protruding vulva; Ste, sterile; WT, wild type, and Egl, egg-laying defective. The Pvl Ste animals had a phenotype very similar to *hop-1; sel-12* double mutants and strong *lin-12* loss-of-function mutants.

†All worms are the progeny of *hop-1(lg1501)/dpy-5(e61) I; sel-12(ar171) spr-3(by108)* hermaphrodites. The genotypes were inferred from the phenotypes. We verified for several animals that the Egl animals had the genotype noted. In a separate cross, 20/20 Egl progeny of a *+/hop-1(lg1501); sel-12(ar171) spr-3(by108)* strain were heterozygous for *hop-1*.

‡The broods of three Egl animals were scored.

§Forty three percent of all animals heterozygous for *hop-1* displayed an Egl phenotype.

expression patterns. *sel-12* is strongly and uniformly expressed, while *hop-1* expression is dynamic and very low throughout most of the larval stages. Thus, in the absence of *hop-1* expression, there is still enough of (*sel-12*) presenilin activity at all times of development and, consequently, *hop-1* mutants have only a very mild phenotype. However, in the absence of *sel-12* expression, there are probably insufficient copies of the *hop-1* transcript in the early larval stages to compensate completely for the loss of *sel-12* expression. Therefore, *sel-12* mutants only display postembryonic defects.

All Spr genes may function through the same mechanism

We provide evidence that the mechanism by which mutants of *spr-3* and *spr-4* suppress *sel-12* loss-of-function alleles involves de-repression of *hop-1* transcription in those stages in which *hop-1* expression alone does not suffice. Similarly, we have shown recently that SPR-1 and SPR-5 proteins interact, and that *spr-5* upregulates *hop-1* expression at the same developmental stages as mutations in *spr-3* and *spr-4* (Eimer et al., 2002b). This suggests that *spr-1*, *spr-3*, *spr-4* and *spr-5* all suppress *sel-12* by upregulating *hop-1*, replacing one presenilin with another. Similarly, mutations in *spr-2* genetically bypass the need for *sel-12*, but do not bypass the need for both *sel-12* and *hop-1* (Wen et al., 2000). Although *hop-1* activity is required for the suppression mechanism, Wen et al. did not find evidence for *hop-1* transcriptional de-repression (Wen et al., 2000). However, the stage specific de-repression of *hop-1* transcription we have seen would not be detectable on a mixed-stage northern blot. Thus, we propose that *spr-2* may also bypass the need for *sel-12* by the same mechanism as *spr-3*, *spr-4* and *spr-5*.

Upregulation of *hop-1* transcription in the early larval stages can explain the suppression of *sel-12* by *spr-3* and *spr-4*

Mutations in *spr-3* and *spr-4* clearly de-repress the transcription of *hop-1* in the early larval stages. However, even in the suppressor strains, the absolute *hop-1* transcript levels in the early larval stages are still much lower than in the adult stage. We believe that the stage-specific increase in *hop-1* expression is sufficient to explain why *spr-3* and *spr-4* suppress *sel-12*. Even in a strong, putative null *sel-12* mutant, there is sufficient HOP-1 protein in the larval stages to enable most *lin-12*-dependent developmental decisions to occur correctly. In *sel-12* mutants, the ventral uterine/anchor cell decision, lateral inhibition in the vulval precursors and the sex myoblast/coelomocyte decision are not affected (Levitan and Greenwald, 1995), indicating that there is sufficient presenilin activity, provided by HOP-1, present in many cell types. In *sel-12(ar131)* even the π cell fate is executed correctly in the vast majority of animals and in *sel-12(ar171)* it is executed correctly in some animals, while in a *sel-12 hop-1* double mutants, 100% of animals have a defective vulval uterine connection (Cinar et al., 2001; Eimer et al., 2002a). This indicates that in *sel-12* mutants the expression of *hop-1* is almost sufficient for wild-type π cell induction. Therefore, it is likely that small increases in *hop-1* expression could be sufficient to compensate completely for the loss of *sel-12* in all developmental decisions.

There are also reasons to believe that small amounts of presenilin message may be sufficient to provide adequate levels of presenilin activity. Presenilins are normally found as part of a high molecular weight complex (Capell et al., 1998; Li et al., 2000; Thinakaran et al., 1998; Yu et al., 1998). This complex is assembled in the ER and Golgi, and proteins that are not incorporated into this complex are not targeted to the cell membrane and are rapidly degraded (Ratovitski et al., 1997). Presenilins may be required in small amounts because the amount of other components of the complex are limiting for assembly (Edbauer et al., 2002). Consequently, it has been found that, in cell culture, presenilins cannot be overproduced (Thinakaran et al., 1996). Furthermore, as the presenilin complex is thought to have enzymatic activity, the levels of the complex necessary for its biochemical function may normally be in vast excess of what is required. Thus, even if the amount of the complex present at the cell membrane in *spr; sel-12* double mutants should be slightly lower than the wild-type levels, the wild-type phenotype of the double mutants indicates that it suffices to ensure sufficient levels of *lin-12* signaling.

Is the increased expression of *hop-1* in the early larval stages seen in *spr-3* and *spr-4* mutants sufficient to rescue the later larval defects seen in *sel-12* mutants? We have several reasons to believe this is the case. First, the cell signaling events that lead to the π cell induction and the correct alignment of the sex muscles, occur prior to the developmental changes (Cinar et al., 2001; Eimer et al., 2002a) and presenilin activity is presumably required at the time of signaling. Second, our initial experiments suggested that the relative expression of *hop-1* is increased in the L1, L2 and L3 stages in both *spr-3* and *spr-4* mutants. We chose to pursue this further at the L1 stage because we thought the upregulation of *hop-1* expression might be most obvious at this stage. Furthermore, it has been demonstrated in cell culture that, once assembled, the high molecular weight presenilin complex is very stable over a long time period (Edbauer et al., 2002; Ratovitski et al., 1997). Finally, we have indications that the presenilin complex is necessary in small amounts and can persist for up to 24 hours in *C. elegans* because we see rescue of sex myoblast/coelomocyte cell-fate decision in *hop-1;sel-12* double mutants with maternally provided *hop-1* (Eimer et al., 2002a). Thus, presenilin protein produced in the embryo is sufficiently stable

and produced in sufficient amounts for a cell fate decision occurring in the L2 stage.

Do *spr-3* and *spr-4* perform a conserved function?

Although SPR-3 and SPR-4 do not have clear mammalian homologs, they may be performing a similar function to known transcriptional repressors. Both SPR-3 and SPR-4 resemble known transcriptional repressors, especially REST/NRSF (Re1 silencing transcription factor/neural-restrictive silencing factor) in different vertebrates. The C_2H_2 zinc-finger factor REST mediates repression of neuronal genes in non-neuronal cells, by recruiting the co-repressor complexes Sin3 and CoREST (Humphrey et al., 2001). Both of these co-repressor complexes contain multiple proteins, including histone deacetylases, and presumably repress transcription in part by removing activating acetyl groups from histones H3 and H4 at the target locus. It is possible that SPR-3 and SPR-4 may also function by recruiting conserved co-repressor complexes to the *hop-1* locus. Three other Spr genes, *spr-1*, *spr-2* and *spr-5*, encode proteins similar to components of known co-repressors (Eimer et al., 2002b; Jarriault and Greenwald, 2002; Wen et al., 2000). SPR-2 is a member of the Nucleosome Assembly Protein (NAP) family and is most similar to the human oncogene SET (Wen et al., 2000). Human SET was purified as part of the INHAT (inhibitor of acetyltransferases) co-repressor complex, which helps to repress transcription by binding to histones and masking them from being acetyltransferase substrates for p300/CBP and PCAF (Seo et al., 2001). Recently, it has been shown that upregulation of SET also inhibits demethylation of methylated DNA and may integrate the epigenetic states of DNA and associated histones (Cervoni et al., 2002). In another paper, we have reported the identification and characterization of SPR-5 that encodes a polyamine oxidase-like protein most similar to a known component of the CoREST co-repressor complex (Eimer et al., 2002b). The core CoREST complex contains only six proteins (Hakimi et al., 2002). *spr-1* encodes a homolog of the MYB domain-containing protein CoREST (Eimer et al., 2002b; Jarriault and Greenwald, 2002), an additional component of the CoREST co-repressor complex. We have shown that SPR-1 and SPR-5 interact biochemically in vitro and in vivo (Eimer et al., 2002b). This suggests that a similar complex is present in *C. elegans* and functions to repress *hop-1* transcription. Interestingly, CoREST has been found to associate with at least two large, basic C_2H_2 zinc-finger proteins, ZNF217 and REST (You et al., 2001), and may be a general co-repressor complex that is recruited to different loci in different cell types by binding to a different C_2H_2 zinc-finger proteins. Recent work also suggests that CoREST may interact with components of the SWI-SNF complex (Battaglioli et al., 2002) and may be involved in silencing of chromosomal regions (Lunyak et al., 2002).

The proteins encoded by the Spr genes may form one or more transcriptional repressor complexes

Thus, SPR-3 and SPR-4 may recruit one or more conserved co-repressor complexes, including one similar to the CoREST co-repressor complex, to target loci. We propose the following model for how the Spr genes are functioning. The fact that loss of function mutations in either *spr-3* or *spr-4* suppress *sel-12* and de-repress *hop-1* transcription, suggest that both SPR-3 and SPR-4 are normally recruited to the *hop-1* locus. There they associate with co-repressor proteins similar to members of the INHAT and CoREST co-repressor complexes. It is unclear if the two zinc-finger proteins co-operatively bind the co-repressor proteins, or if each zinc-finger protein associates with a different complex. The assembled complex (or complexes), probably acts as a basal repressor of *hop-1* transcription that is overridden in later developmental stages.

The mammalian INHAT and CoREST complexes were purified and studied by biochemical approaches. However, as yet little is known about their biological function. The data now available on Spr gene function suggest that INHAT and CoREST complexes can be studied both genetically and biochemically in *C. elegans*. We suggest that through *C. elegans* genetics we may identify additional genes that interact with these complexes and we may help to elucidate their biological function.

We thank Gian Garriga, Sophie Jarriault, Iva Greenwald, Jim McCarter and the Sanger Institute and the Genome Sequencing Center (Washington University, St Louis) for communicating unpublished information; Jean-Claude Labbé for advice on RNA isolation and northern analysis; Jonathan Hodgkin and Iva Greenwald for strains; Yuji Kohara for cDNA clones; Bob Barstead for a cDNA library; and Alan Coulson for cosmid clones. Some strains used in this work were provided by the *Caenorhabditis* Genetics Center, which is funded by the National Center for Research Resources of the NIH. This work was supported by an EMBO fellowship to B.L. and by grants from the DFG, EC (DIADEM) and BMBF (NGFN) to R.B.

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