Two suppressors of sel-12 encode C$_2$H$_2$ zinc-finger proteins that regulate presenilin transcription in Caenorhabditis elegans

Bernard Lakowski*, Stefan Eimer, Christine Göbel, Andreas Böttcher, Babett Wagler and Ralf Baumeister†

ABI, Department of Biochemistry, Laboratory of Molecular Neurogenetics, Ludwig-Maximilians-Universitaet, Schillerstr. 44, D-80336 Munich, Germany
*Present address: Department of Neuroscience, Pasteur Institute, Paris, France
†Author for correspondence (e-mail: ralf.baumeister@pbm.med.uni-muenchen.de)

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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$_2$H$_2$ 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 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 C2H2 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), def-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 in 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.


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


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 (itsx-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 yk247e5, kindly provided by Yuji Kohara. We found that the two cDNAs have a very similar structure, yet yk247e5 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 (Spieht et al., 1993) and gene specific reverse primers (RB1080 CATACTGAGCGC- ATCAATCG; RB1079 CATCTGCTTCTGCTGAGATCG). 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 in the 3’ UTR. The spr-3 3’UTR is shorter than the spr-4 3’UTR by 103 nucleotides. 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 the transcript we performed PCR and cloned as a C28G1.4 fragment from the cDNA (jalubowski and Kornfeld, 1999).

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. briggsae 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 TACTCTGCCACCTGTGTCGAAG and RB1629 TGTTGAACCTTTTCACCACCG 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 cloned a 2.7 kb HindIII/Xhol fragment from the cDNA yk356a2 (kindly provided by Y. Kohara) into L4440 (pPD129.36, kindly provided by A. Fire) cut HindIII/Xhol to generate a C28G1.4 RNAi feeding vector. A full-length hop-1 cDNA was amplified by PCR and cloned as a Small/NorI 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 the manufacturer’s instructions (Amersham, Freiburg, Germany). Blots were hybridized using a Megaprime labeling kit according to the manufacturer’s instructions. 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). They were 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). sel-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 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 exhibit 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

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Broods</th>
<th>Progeny</th>
</tr>
</thead>
<tbody>
<tr>
<td>N2</td>
<td>20</td>
<td>314±26</td>
</tr>
<tr>
<td>sel-12(ar131)</td>
<td>20</td>
<td>126±51</td>
</tr>
<tr>
<td>sel-12(ar171)</td>
<td>20</td>
<td>61±16</td>
</tr>
<tr>
<td>sel-12(ar131) spr-3(by108)</td>
<td>20</td>
<td>226±33</td>
</tr>
<tr>
<td>sel-12(ar171) spr-3(by108)</td>
<td>20</td>
<td>283±27</td>
</tr>
<tr>
<td>sel-12(ar171) spr-3(by108); byEx134</td>
<td>20</td>
<td>53±26</td>
</tr>
<tr>
<td>byDf1</td>
<td>20</td>
<td>230±25</td>
</tr>
<tr>
<td>sel-12(ar171) byDf1</td>
<td>19</td>
<td>205±63</td>
</tr>
<tr>
<td>spr-4(by130); sel-12(ar171)</td>
<td>20</td>
<td>239±33</td>
</tr>
</tbody>
</table>

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

†byEx143 is an extra-chromosomal array containing a 9.3 kb BamHI 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. 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.
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 C2H2 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_{2}H_{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_{2}H_{2} proteins related to transcriptional repressors**

*spr-4* was mapped between *unc-55* and *daf-8* on LG I, close to, but to the right of, the single nucleotide polymorphism (SNP) vi20a11.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 *PvuII* fragment that contains the entire coding sequence.

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**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_{2}H_{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 *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 PvuII fragment from F34G10 into pBSIIKS- cut with PvuII 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 C2H2 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=3x10^-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 Eg1 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 metazoa. 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.

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

As both SPR-3 and SPR-4 encode C3H2 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). sel-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.

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**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.
In spr-3 and spr-4 mutants, we found no differences in the temporal pattern of expression of sel-12 or spr-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 120a11.s1@186). The extent of the 12 kb Prxl 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), of the same range of phenotypes as seen when one induces RNAi of hop-1 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. 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)lin-12; glp-1; 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.

**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

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

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Phenotype without RNAi*</th>
<th>hop-1 RNAi phenotypes†</th>
<th>n‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>sel-12(ar171)</td>
<td>Wild type</td>
<td>Wild type</td>
<td>(30/30)</td>
</tr>
<tr>
<td>sel-12(ar171) spr-3(by108)</td>
<td>Wild type</td>
<td>Egl; Ste; Emb; Lag</td>
<td>(30/30)</td>
</tr>
<tr>
<td>sel-12(ar171); spr-4(by105)</td>
<td>Wild type</td>
<td>Egl; Ste; Emb; Lag</td>
<td>(48/50)</td>
</tr>
</tbody>
</table>

*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).

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**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-3 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.
We provide evidence that the mechanism by which mutants of developmental stages as mutations in hop-1 have shown recently that SPR-1 and SPR-5 proteins interact, transcription in those stages in involves de-repression of hop-1; sel-12 double mutants and strong lin-12 loss-of-function mutants.

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; Thinkaran 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 (Thinkaran 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.

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

<table>
<thead>
<tr>
<th>Phenotype*</th>
<th>Genotype†</th>
<th>Number‡</th>
<th>Percent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dpy</td>
<td>dpy-5; sel-12 spr-3</td>
<td>50/197</td>
<td>25.4</td>
</tr>
<tr>
<td>Pv1 Ste</td>
<td>hop-1; sel-12 spr-3</td>
<td>56/197</td>
<td>28.4</td>
</tr>
<tr>
<td>WT</td>
<td>dpy-5/hop-1; sel-12 spr-3</td>
<td>51/197</td>
<td>25.9</td>
</tr>
<tr>
<td>Egl§</td>
<td>dpy-5/hop-1; sel-12 spr-3</td>
<td>38/197</td>
<td>19.3</td>
</tr>
</tbody>
</table>

*Phenotypes: Dpy, dumpy; Pv1, protruding vulva; Ste, sterile; WT, wild type. 
†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.
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 C2H2 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 INHA T (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 transcriptional repressor. Both SPR-3 and SPR-4 resemble to components of known co-repressors (Eimer et al., 2002b; Jarriault and Greenwald, 2002), an additional component of the CoREST co-repressor complex. 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 INHA T 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 INHA T 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.

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