Conservation of *glp-1* Regulation and Function in Nematodes

David Rudel* and Judith Kimble*,†,‡,§

**Department of Biochemistry,* †*Laboratory of Molecular Biology,* ‡*Department of Medical Genetics and* §*Howard Hughes Medical Institute, University of Wisconsin, Madison, Wisconsin 53706*

Manuscript received August 31, 2000
Accepted for publication October 26, 2000

ABSTRACT

The *Caenorhabditis elegans* (*Ce*) *glp-1* gene encodes a Notch-like receptor. We have cloned *glp-1* from *C. briggsae* (*Cb*) and *C. remanei* (*Cr*), two Caenorhabditis species that have diverged from *C. elegans* by roughly 20–40 million years. By sequence analysis, we find that the Cb-GLP-1 and Cr-GLP-1 proteins have retained the same motif architecture as Ce-GLP-1, including number of domains. In addition, two regions (CC-linker and regions flanking the ANK repeats) are as highly conserved as regions previously recognized as essential for signaling (*e.g.*, ANK repeats). Phylogenetic analysis of *glp-1* sequences suggests a *C. briggsae*/*C. remanei* clade with *C. elegans* as a sister taxon. Using RNAi to test biological functions, we find that *Ce-glp-1*, *Cb-glp-1*, and *Cr-glp-1* are all required for proliferation of germline stem cells and for specifying blastomere fates in the embryo. In addition, certain biological roles of *Cb-glp-1*, *e.g.*, in the vulva, have diverged from those of *Ce-glp-1* and *Cr-glp-1*, suggesting a change in either regulation or function of the *Cb-glp-1* gene during evolution. Finally, the regulation of *glp-1* mRNA, previously analyzed for *Ce-glp-1*, is conserved in *Cb-glp-1*, and we identify conserved 3′ UTR sequences that may serve as regulatory elements.

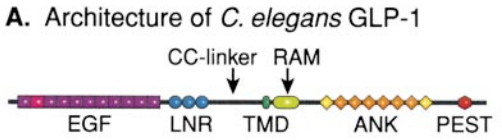
THROUGHOUT evolution, the regulators and regulatory pathways that build organisms have been amazingly well conserved (Akam 1998). While the building blocks are well conserved, their uses are varied. Comparisons of regulators from distantly related phyla that diverged more than 600 million years ago (*e.g.*, nematodes *vs.* humans, Blaxter 1998) highlight essential core molecular features of regulators and pathways. By contrast, comparisons of regulators in closely related species that diverged, for example, only 40 million years ago (*e.g.*, spider monkeys *vs.* humans, Goodman 1999), explore features particular to the function of genes in specific processes. The analysis of closely related species is the first step to understanding how changes in the regulation of ubiquitous factors can result in new roles to generate distinct species.

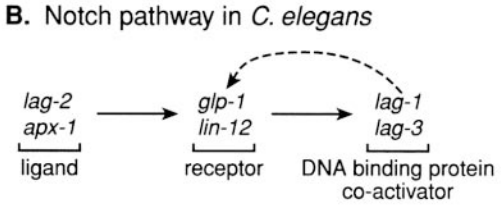
The Notch pathway controls multiple aspects of growth, fate, and patterning in virtually all metazoans (Artavanis-Tsakonas *et al.* 1995; Kimble and Simpson 1997). Many species possess multiple Notch-related receptors, which have acquired specialized functions. Vertebrates, for example, have four (del Amo *et al.* 1993; Lardelli and Lendahl 1993; Lardelli *et al.* 1994; Uyttendaele *et al.* 1996), and the genome of the nematode *Caenorhabditis elegans* encodes two Notch-related receptors, GLP-1 and LIN-12 (*C. elegans* Sequencing Consortium 1998). We have begun to explore the evolution of Notch-related receptors in three closely related nematodes, *C. elegans*, *C. briggsae*, and *C. remanei*. These three species diverged from each other roughly 40 million years ago (Kennedy *et al.* 1993). This study explores conserved features and differences between GLP-1 receptors.

As background on *C. elegans* GLP-1, we introduce its architecture (Figure 1A), its signaling pathway (Figure 1B), and its biological functions (Figure 1C). The GLP-1 receptor is composed of a series of motifs that are conserved among all Notch-related receptors (Yochem and Greenwald 1989; Figure 1A). Near the center of the receptor is a single transmembrane domain (TMD). N-terminal to the TMD is the extracellular domain containing 10 epidermal growth factor (EGF)-like repeats, 3 LNR repeats, and a region between the transmembrane domain and the LNRs that possesses two conserved cysteines, which we call the CC-linker. C-terminal to the TMD is the intracellular domain containing the RAM motif, six ANK repeats, and a PEST sequence. The EGF and LNR repeats of the extracellular domain are thought to mediate ligand binding (Kelley *et al.* 1987; Rebay *et al.* 1991; Heitzler and Simpson 1993), while the RAM domain and the ANK repeats of the intracellular domain effect the signaling response (Roehl and Kimble 1993; Roehl *et al.* 1996; Artavanis-Tsakonas *et al.* 1999 for review).

The GLP-1 receptor, like other Notch-related receptors (Blaumueller *et al.* 1997), is likely to be proteolytically cleaved and exist as a heterodimer composed of one polypeptide bearing the EGF and LNR repeats and a second polypeptide carrying the rest of the protein (Crittenden *et al.* 1994). Ligand binding is thought to dissociate this heterodimer (Rand *et al.* 2000) and per-

Corresponding author: Judith Kimble, HHMI/Department of Biochemistry, 433 Babcock Dr., Madison, WI 53706-1544.
E-mail: jekimble@facstaff.wisc.edu





C. Biological functions in *C. elegans*

	early[1] embryo	late[2] embryo	larval soma	larval/adult germline
GLP-1	+	+	–	promotes distal proliferation
LIN-12	–	+	+	prevents proximal proliferation

[1]Maternal contribution
[2]Zygotic contribution

FIGURE 1.—(A) GLP-1 is a Notch-like receptor. The functional domains of the GLP-1 receptor are shown: 10 EGF-like repeats, purple; 1 pseudo-EGF-like repeat, pink; 3 LNR repeats, blue; the CC-linker (arrow); a single-pass transmembrane domain (TMD), dark green; a RAM domain, light green; 6 ANK repeats, orange; conserved flanking regions, which may be degenerate ANK repeats (see DISCUSSION), yellow; and a PEST domain, red. (B) The core Notch pathway in *C. elegans* includes genes that encode two members of a conserved family of Notch ligands, *lag-2* and *apx-1*; two Notch-like receptors, *glp-1* and *lin-12*; a member of a conserved family of DNA-binding proteins, *lag-1*; and a novel putative activator, *lag-3*. Arrows indicate activation. The dashed arrow indicates potential transcriptional feedback regulation of the receptors by the downstream activators. (C) Functions of GLP-1 and LIN-12 in *C. elegans*. GLP-1 and LIN-12 have overlapping functions in late embryos and separate functions in early embryos, larvae, and adults.

mit further cleavage of the Notch receptor, which ultimately frees the intracellular domain for transport into the nucleus and participation in transcriptional control (SCHROETER *et al.* 1998).

Three LAG proteins (for *L*IN-12 *a*nd *G*LP-1) act together with both LIN-12 and GLP-1 receptors to achieve signaling (Figure 1B). LAG-2 serves as a ligand (HENDERSON *et al.* 1994; TAX *et al.* 1994), LAG-1 as a DNA-binding protein (CHRISTENSEN *et al.* 1996), and LAG-3 as a transcriptional activator (PETCHERSKI and KIMBLE 2000a). Two of these pathway components are conserved in other Notch-related pathways: LAG-2 is a Delta/Serrate homologue and LAG-1 is a Su(H)/CBF1 homologue (see KIMBLE and SIMPSON 1997 for review). In addition to LAG-2, APX-1 is another Delta/Serrate homologue that activates GLP-1 in the early embryo (MELLO *et al.* 1994). The function of the third component, LAG-3, may be accomplished by Mastermind in flies and vertebrates (PETCHERSKI and KIMBLE 2000b). Signaling through the worm Notch pathway has been proposed to be autoregulated by a transcriptional feedback mechanism via LAG-1 binding sites (WILKINSON *et al.* 1994; CHRISTENSEN *et al.* 1996).

The two *C. elegans* receptors, GLP-1 and LIN-12, are architecturally similar and functionally interchangeable (YOCHEM and GREENWALD 1989; LAMBIE and KIMBLE 1991; MANGO *et al.* 1991; FITZGERALD *et al.* 1993). These two receptors appear to have been generated by a gene duplication event and have both unique and common biological functions (Figure 1C). GLP-1, but not LIN-12, is required maternally during early embryogenesis for proper determination of blastomere fates (PRIESS and THOMSON 1987) and zygotically in adults for germline proliferation and maintenance of germline stem cells (AUSTIN and KIMBLE 1987). By contrast LIN-12, but not GLP-1, is required for lateral signaling between somatic cells (GREENWALD *et al.* 1983; NEWMAN *et al.* 1995). In addition, LIN-12 has a role in preventing proximal proliferation of the germline (SEYDOUX *et al.* 1990). GLP-1 and LIN-12 have overlapping roles during embryogenesis (LAMBIE and KIMBLE 1991; MOSKOWITZ and ROTHMAN 1996).

In *C. elegans*, *glp-1* mRNA is controlled at the translational level to achieve the correct time and spatial pattern of expression (EVANS *et al.* 1994). In the germline and embryo, *glp-1* mRNA is present ubiquitously. However, GLP-1 protein has a more limited distribution. In the germline, GLP-1 protein is expressed only in the distal mitotic region (CRITTENDEN *et al.* 1994), where it is required for continued mitoses, and in the embryo GLP-1 is first detectable at the two-cell stage asymmetrically localized to the anterior blastomere, AB, where it is required for blastomere fates (EVANS *et al.* 1994).

In this article, we report the isolation and characterization of *glp-1* orthologues from *C. briggsae* and *C. remanei*. Our analysis identifies potentially critical regulatory and functional regions of the gene and its protein product. In addition, while we find that the biological functions of GLP-1 receptors have remained similar, they have not been totally static.

MATERIALS AND METHODS

Strains: *C. elegans* strain Bristol N2, *C. briggsae* strain AF16, and *C. remanei* strain SB146 were used for this study. Animals were maintained at 20° and manipulated using standard *C. elegans* techniques (BRENNER 1974).

Cloning *Cb-glp-1*: Degenerate primers were designed based upon an alignment of *Ce-glp-1* and *lin-12*. Forward primer DR1 (5′-TGYAARAAYGGIGGIGTITG-3′) anneals within the 10th EGF repeat of *glp-1* and reverse primer DR3 (5′-GTISWYTC CATIGGIGGCATCCA-3′) anneals in the opposite orientation within the RAM domain of *glp-1* (degenerate bases are shown using the IUPAC code; I represents inosine). Primers DR1 and DR3 were used in a 100-μl PCR reaction [500 nM DR1, 500 nM DR3, 250 nM dNTPs, 1× Taq extender buffer, 2 μg

C. briggsae genomic DNA, 2.5 units Boehringer Mannheim (Indianapolis) Taq, and 2.5 units Stratagene (La Jolla, CA) Taq extender]. The reaction was cycled using a step down program: 10 times (94°, 1 min; 47°, 1 min, annealing temperature was lowered 1° each cycle; 72°, 2 min); 20 times (94°, 1 min; 37°, 1 min; 72°, 2 min); 10 times (94°, 1 min; 39°, 1 min; 72°, 2 min). PCR products were separated on an agarose gel, blotted, and analyzed by low-stringency hybridization. A single fragment hybridized to a *Ce-glp-1* probe. The fragment was cloned and its similarity to *Ce-glp-1* was confirmed by sequencing. This fragment was further used to screen a *C. briggsae* genomic library and cDNA library.

Cloning *Cr-glp-1*: We used synteny to clone *Cr-glp-1*. *C. elegans* primers CoA1 (5′-ATGTTCCGTCACGTGGCTCAA-3′) and CoA4 (5′-GTTCCTCCCTTGACAGTCGCAT-3′) were used in a PCR reaction with an annealing temperature of 45° to amplify a portion of the *C. remanei* propionyl-CoA carboxylase β-chain-like gene. In *C. elegans* this gene (F02A9.4a) is 5′ and in the opposite orientation of the *Ce-glp-1* gene. The *C. remanei* gene fragment was subsequently used to screen a *C. remanei* genomic library. Positive phage were end sequenced. Sequence analysis revealed a single phage terminated in the intercellular portion of the *Cr-glp-1* gene.

λ Libraries and library screening: A *C. briggsae* cDNA library obtained from Alex Puoti, a *C. briggsae* genomic library obtained from David Baillie, a *C. remanei* genomic library (Haag and Kimble 2000), and a *C. remanei* cDNA library (see below) were screened using standard lift and hybridization techniques (Sambrook *et al.* 1989).

A *C. remanei* directional cDNA library, λDR1, was constructed using poly(A)$^+$ selected RNA and the SuperScript Lambda System for cDNA Synthesis and λZipLox Cloning (GIBCO BRL, Gaithersburg, MD). The library was size selected for cDNAs >1 kb. Each insert is flanked by a *Sal*I site on the 5′ end and a *Not*I site on the 3′ end. The library contains an estimated 1,200,000 independent clones.

Construction of the proposed *Cb-glp-1* and *Cr-glp-1* cDNAs: The longest cDNA obtained for *Cb-glp-1* was 3.36 kb and started at the beginning of the seventh EGF repeat and terminated in a poly(A) tail. Seven putative *Cb-glp-1* cDNA clones were plaque purified. cDNA clones were screened by PCR and two clones were excised and analyzed by restriction digestion. The longest cDNA obtained for *Cr-glp-1* was 1.8 kb and started in the RAM domain and terminated in a poly(A) tail. Two *Cr-glp-1* cDNA clones were excised and analyzed by restriction digestion. All excised *Cb-glp-1* and *Cr-glp-1* cDNAs were end sequenced. Sequence data and restriction analysis concurred with the hypothesis of a single endogenous transcript for both *Cb-glp-1* and *Cr-glp-1*. Only the largest cDNA was fully sequenced for *Cb-glp-1* and *Cr-glp-1*.

The 5′ sequences were obtained from *Cb-glp-1* genomic sequence produced by the *C. elegans* Sequencing Consortium and purified *Cr-glp-1* genomic λ-clones. Subsequently, the 5′ gene structures were hypothesized based upon sequence identity. All proposed splice sites occurred at conserved exon/intron boundaries and show strong agreement with the splice site consensus sequences of *C. elegans*. We independently sequenced a purified *Cb-glp-1* genomic clone and confirmed the reported sequence.

RNA analysis: RNA extractions, poly(A)$^+$ selection, and Northern analysis were performed as described previously (Puoti and Kimble 1999). The cDNA probes used for *Cb-glp-1* and *Cr-glp-1* Northern analysis spanned the sequence encoding the LNG repeats and the transmembrane domain.

RNA interference: A *Cb-glp-1* cDNA PCR product was amplified using primers DR29 (5′-TAATACGACTCACTATAGGGC CATCGGAGGAGGCATCCATA-3′) and DR30 (5′-TAATAC GACTCACTATAGGGAACCAGGTGTCAGGAGAAGGT-3′). A *Cr-glp-1* cDNA PCR product was amplified using primers CR41 (5′-TAATACGACTCACTATAGGGATTCTGTAATTGC CCATT-3′) and CR42 (5′-TAATACGACTCACTATAGGGTG TTTCCATTGGAGGCGT-3′). All primers contained a T7 promoter sequence. *Cb-glp-1* and *Cr-glp-1* double-stranded RNA (dsRNA) was produced using a MEGAscript T7 kit from Ambion (Austin, TX) and diluted to 1 mg/ml. Young *C. briggsae* hermaphrodites were injected with *Cb-glp-1* dsRNA. Mated *C. remanei* females were injected with *Cr-glp-1* dsRNA. Injected animals were singled to plates to recover for 6 hr. Animals were subsequently transferred every 12 hr. Progeny that hatched from injected *C. remanei* animals were picked to individual plates to prevent mating.

During the 6-hr recovery window, injected animals laid mostly dead eggs. However, from 30 injected *C. briggsae* animals and *C. remanei* animals, 173 and 303 progeny hatched in this recovery window, respectively; 149/173 and 275/303 developed into adults, whereas 24/173 and 28/303 died as L1s. For adults scored, 113/149 *C. briggsae* progeny and 72/139 *C. remanei* progeny were sterile by differential interference contrast microscopy. Following the recovery window, both *C. briggsae* animals injected with *Cb-glp-1* dsRNA and *C. remanei* animals injected with *Cr-glp-1* dsRNA laid only dead embryos that lacked an anterior pharynx.

Immunostaining and 4′,6-diamidino-2-phenylindole staining: *C. briggsae* and *C. remanei* embryos and dissected germlines were fixed as described previously (Crittenden *et al.* 1997). Embryos and germlines were stained with 4′,6-diamidino-2-phenylindole (DAPI) and, where appropriate, costained with rat α-Cb-GLP-1 antibodies (see below) or mouse monoclonal antibody 3NB12. The mouse monoclonal 3NB12 antibody recognizes a subset of pharyngeal muscle cells, the two intestinal muscle cells, and two neuronal-like cells near the intestinal muscle cells (Okamoto and Thomson 1985; Priess and Thomson 1987). Cy3 donkey anti-mouse and Cy3 donkey anti-rat antibodies (Jackson Labs, West Grove, PA) were used to detect costained markers. Whole worms were DAPI stained as previously described (Kadyk and Kimble 1998).

Cb-GLP-1 antibody production: A fragment of *Cb-glp-1* cDNA encoding LNR 1-3 [amino acids (aa) 489–614] was cloned into pGEX-2T and pET28a vectors and fusion proteins were overexpressed in *Escherichia coli*. Rats were injected initially with 125 μg of affinity purified Cb-LNR/GST fusion protein suspended in Freund's complete adjuvant and boosted with 260–375 μg of fusion protein suspended in incomplete Freund's adjuvant at monthly intervals. Test bleeds were taken ~2 wk after each boost. Antibodies were purified on a column of Cb-LNR/HIS coupled to affi-gel 10 (Bio-Rad, Hercules, CA).

***Cb-glp-1* RNA *in situ* hybridization:** RNA *in situ* hybridization of *Cb-glp-1* was done on *C. briggsae* embryos and germlines as previously described (Seydoux and Fire 1994). Two digoxygenin-labeled antisense single-stranded (ss) DNA probes and their corresponding sense ssDNA control probes were used. Both probes gave similar results. The first test/control probe pair was synthesized from a *Cb-glp-1* cDNA fragment generated with the primers DR15 (5′-CGACGGTGGAGACTGTTCCG GAGG-3′) and DR20 (5′-CGACTCCAGCGACGACAGTTC CTA-3′). Probe one is complementary to the last two LNR repeats and the remaining extracellular sequence is complementary to the TMD. The second test/control probe pair was synthesized from a *Cb-glp-1* fragment generated with primers DR22 (5′-CCGAGCCGATCACCGCCGAGTC-3′) and DR27 (5′-CCGTCTTCTCCAAACCATCCAC-3′). Probe two is complementary to the ANKs. To remove primers and nucleotides before synthesis of the ssDNA probes, PCR products were purified with a QIAGEN (Valencia, CA) PCR purification kit.

DNA sequencing: DNA sequences were determined by thermal cycle sequencing with fluorescently labeled dideoxy termi-

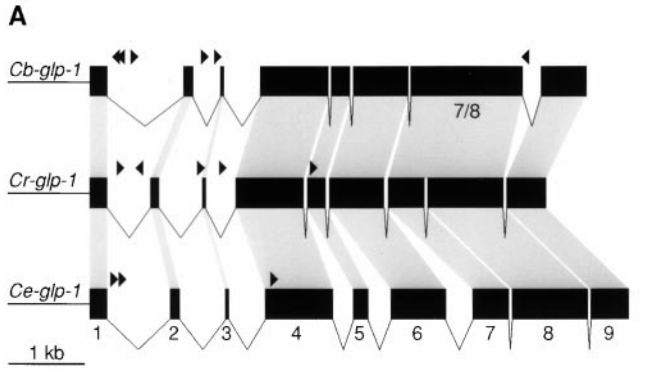


FIGURE 2.—(A) Genomic organization of *glp-1* homologues. Exons are represented by solid boxes and introns with lines. Arrowheads represent a consensus LAG-1 binding site. Direction of the arrowhead indicates the orientation of the site, with right pointing arrows forward, or sense. Shading represents conserved exons. (B) Autoradiograms of Northern blots for *Cb-glp-1* and *Cr-glp-1* show a single transcript.

nators. Automated sequencing was performed on an ABI (Foster City, CA) 377XL sequencer. All sequence analysis was performed using EDITSEQ, SEQMAN, MAPDRAW, and MEGALIGN (DNASTAR, Inc., Madison, WI). Sequence was obtained at least once from each strand. The genomic sequence for the 3′ end of *Cb-glp-1* and *Cr-glp-1* came from direct sequencing of PCR products generated using primers designed from cDNA sequence.

Phylogenetic analysis: Amino acid alignments, nucleotide alignments, and uncorrected pairwise distances were computed using SEQED, PILEUP, LINEUP, and DISTANCES from Wisconsin Package Version 10, Genetics Computer Group (Madison, WI). Phylogenetic analysis of the alignments was done using PAUP Version 4.0 for the PPC (SWOFFORD 1998).

RESULTS

***glp-1* homologues in *C. briggsae* and *C. remanei*:** We have isolated *glp-1* homologues from *C. briggsae* and *C. remanei* (see MATERIALS AND METHODS). The homologues were identified as *glp-1*, rather than *lin-12*, on the basis of amino acid identity, number of EGF motifs, conservation of gene structure, position with respect to flanking genes in the genome, and conservation of biological function (see below). Additionally, we have isolated fragments of *lin-12* homologues to confirm the presence of both *glp-1* and *lin-12* in *C. briggsae* and *C. remanei*; our analysis of *lin-12* homologues will be presented elsewhere.

***Cb-glp-1* and *Cr-glp-1* gene structure and transcripts:** We sequenced the complete genomic regions of *Cb-glp-1* and *Cr-glp-1*, as well as partial cDNAs for each gene (see MATERIALS AND METHODS). Using in part a comparison of these genomic and cDNA sequences and in part a comparison of the *C. elegans glp-1* gene sequence (YOCHEM and GREENWALD 1989) with those of *Cb-glp-1* and *Cr-glp-1*, we deduced the coding regions for both *C. briggsae* and *C. remanei* genes. The exon/intron structures of the three homologues are well conserved, though not identical (Figure 2A). Including all predicted exons, introns, 5′ untranslated region (UTR), 3′ UTR, and 1.2 kb of 5′-flanking sequence, the sizes of the *Ce-glp-1*, *Cb-glp-1*, and *Cr-glp-1* genes are 8658, 7881, and 7413 bp, respectively. These sequences have been deposited in GenBank, accession nos. AF315554 and AF315555, and AF315556 and AF315557 for the genomic and partial cDNA sequences of *Cb-glp-1* and *Cr-glp-1*, respectively. The first three introns in *Cb-glp-1* and *Cr-glp-1* are relatively large, as they are in *Ce-glp-1* (YOCHEM and GREENWALD 1989). With a few noted exceptions, splice sites are also conserved. The predicted sizes of the *Ce-glp-1*, *Cb-glp-1*, and *Cr-glp-1* transcripts are 4348, 4355, and 4435 bp, respectively, including the SL1 transpliced leader for *Ce-glp-1* and hypothesized SL1 transpliced leaders for *Cb-glp-1* and *Cr-glp-1*. Northern analyses revealed single transcripts of ~4.4 kb for *Cb-glp-1* and ~4.5 kb for *Cr-glp-1* (Figure 2B). These sizes correlate well with the proposed *glp-1* mRNAs.

The *Cb-glp-1* and *Cr-glp-1* genes contain a higher number of putative LAG-1 binding sites (rtgggaa) than would be predicted randomly, as was originally found for *Ce-glp-1* (CHRISTENSEN *et al.* 1996; Figure 2A). In all three homologues, potential LAG-1 binding sites cluster in the large introns found in the 5′ part of the gene (CHRISTENSEN *et al.* 1996; this work). Over the entire genome, LAG-1 binding sites are predicted to occur randomly every 4096 bp. The *Cb-glp-1* genomic sequence contains five binding sites clustered within the 1392 bp of the first two introns, and that of *Cr-glp-1* has four binding sites clustered within the 1556 bp of the first three introns. The conservation of a greater number of LAG-1 binding sites than expected in all three Caenorhabditis species supports the idea that the *glp-1* gene may be subject to feedback regulation at the transcriptional level.

Three additional features are worth noting. First, in both *C. briggsae* and *C. remanei* synteny is conserved for *glp-1* and its 5′-flanking gene, a propionyl CoA carboxylase β-chain-like gene. Second, exons 7 and 8 are fused in *Cb-glp-1*. This is likely to represent intron loss, based on phylogenetic relationships predicted for these three Caenorhabditis species (see below). Third, the first three amino acids are missing from exon 9 in both *C. briggsae* and *C. remanei*. This difference could have resulted from a deletion in exon 9 or a different 3′ splice site for intron 9 in both *Cb-glp-1* and *Cr-glp-1*.

Comparison of GLP-1 sequences: The overall identities between pairs of *glp-1* homologues are similar

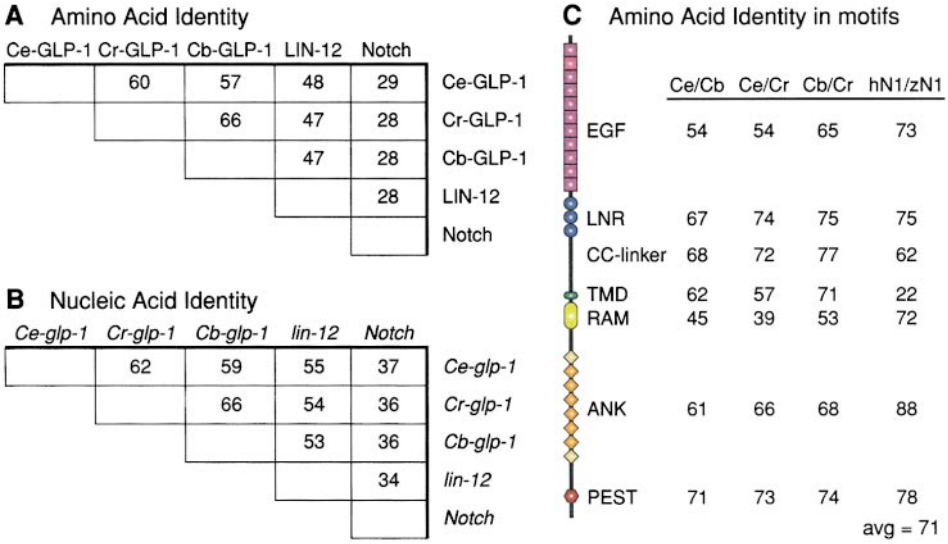


FIGURE 3.—Sequence analysis of GLP-1 homologues. The complete amino acid sequences for Ce-GLP-1, Cb-GLP-1, and Cr-GLP-1 were used in the analysis. The 10 EGF repeats of GLP-1 are, in a contiguous manner, most similar to the last 10 EGF repeats of LIN-12. The LIN-12 sequence used removed the first 3 EGF repeats and the pseudo-EGF repeat at the N terminus, as well as a small portion of the C-terminal tail where GLP-1 and LIN-12 do not align. A 5′ and 3′ truncated version of *Drosophila melanogaster* Notch was used. Notch contains 36 EGF repeats and the 3′ end of Notch is considerably longer than GLP-1 or LIN-12 and contains a glutamine-rich region not shared with GLP-1 or LIN-12. The Notch sequence was edited to remove the excess EGF repeats at the N terminus and the additional sequence at the C terminus. The alignment generated was further edited by eye to ensure the alignment accounted for functional data known for individual domains, *i.e.*, conserved amino acids and positioning within repeats and sites of post-translational processing. The corresponding nucleotide sequences were used in the cDNA analysis. Identity numbers are based upon uncorrected pairwise distances and were rounded to the nearest percentage point. (A) Percentage amino acid identity among aligned protein sequences. (B) Percentage nucleotide identity among aligned coding sequences. (C) Percentage identity within functional domains of GLP-1. Percentage identity between human Notch1/TAN1, hN1, and zebrafish Notch1, zN1, are also given for the analogous regions. The RAM domain as originally defined for mouse Notch1 (mN1; TAMURA *et al.* 1995) includes ANK flanking sequence that may harbor a cryptic ANK repeat (see DISCUSSION). For this reason and for consistency with our definition of the *C. elegans* RAM domain we defined the vertebrate RAM domain as the amino acids that correspond to mN1 amino acids 1751–1806, the minimal region that gave a strong interaction with RBP-Jκ, a vertebrate LAG-2 homologue (TAMURA *et al.* 1995). Overall identity for hN1 and zN1 is given at the bottom of the column.

whether analyzed at the amino acid (Figure 3A) or nucleotide (Figure 3B) level. Intriguingly, the Ce-GLP-1 paralogue, Ce-LIN-12, is more similar to Ce-GLP-1 than it is to Cb-GLP-1 or Cr-GLP-1 (Figure 3B). This may reflect constraints on the GLP-1 and LIN-12 receptors within *C. elegans* to conserve amino acids required to transmit a signal using the same upstream and downstream proteins (see Figure 1). Other explanations include a common codon bias for *C. elegans* genes or a slower rate of change for *glp-1* and *lin-12* in *C. elegans* in comparison to *C. briggsae* or *C. remanei*.

Cb-GLP-1 and Cr-GLP-1 have retained all functional domains of Ce-GLP-1 (Figure 3C). Both contain 10 EGF-like repeats, which is the same as Ce-GLP-1, and contrasts with LIN-12, which has 13 EGF-like repeats. Identity within the EGF repeats is essentially the same as overall identity (Figure 3, A and C). However, the LNR repeats, the CC-linker, and the ANK repeats have a greater degree of identity than the overall identity for the entire protein. The conservation among these particular motifs is likely to reflect constraints on the sequence due to intramolecular and intermolecular interactions (see DISCUSSION).

A number of molecular changes associated with a variety of mutations have been identified in *Ce-glp-1* (KODOYIANNI *et al.* 1992; LISSEMORE *et al.* 1993; BERRY *et al.* 1997). All missense mutations occur in codons for invariant amino acids, whereas nonsense mutations occur in codons for nonconserved amino acids (Figure 4). Therefore, the missense mutations are likely to identify amino acids critical for all three homologues. Complementarily, amino acid conservation identifies additional candidate residues that are likely to be critical for GLP-1 function.

A comparison of amino acids within the GLP-1 ANK repeat region is best considered in light of structural studies of ANK repeats in other proteins (see SEDGWICK and SMERDON 1999 for review). The structure of each ANK repeat consists of a β-turn followed by a pair of α-helices (Figure 5A, see legend for more detailed description). Our predictions for these structural features in the GLP-1 ANK repeats are shown in Figure 5B; it seems likely that many of these predictions will be correct at a gross level, but that details may vary (*e.g.*, positioning of ends of turns and helices). For most of the predicted GLP-1 ANK repeats, the more N-terminal α-helix (Figure 5B, α-helix 1) shows a stronger consensus than its C-terminal partner (Figure 5B, α-helix 2). Furthermore, the amino acids just N-terminal to the predicted β-turn can show a striking degree of conservation (Figure 5B, red), which may indicate critical residues for protein-protein interactions (see DISCUSSION). Flanking the classical six-ANK repeat region, both N-terminally and C-terminally, reside clusters of conserved amino acids that are reminiscent of the structural features of an ANK repeat (Figure 5B). We suggest that

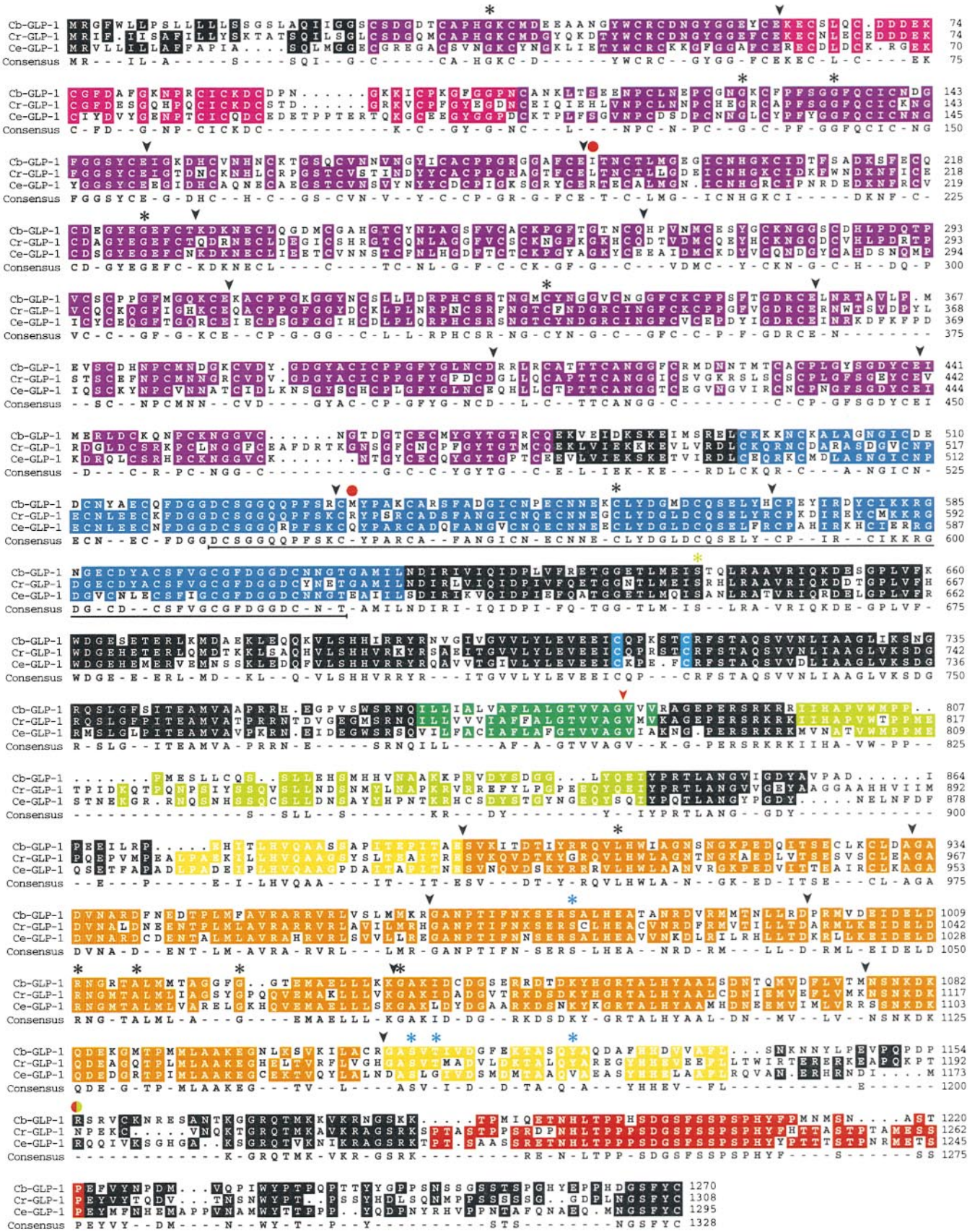


FIGURE 4.—Alignment of Ce-GLP-1, Cb-GLP-1, and Cr-GLP-1. Solid boxed residues represent identity between homologues at that position and shaded boxes represent similarity. Within functional domains solid boxes are coded by color: EGF repeats, violet; pseudo-EGF repeat, pink; LNRs, blue; the conserved cysteines in the CC-linker, light blue; TMD, green; RAM domain, light green; putative cryptic ANK repeats, yellow; ANK repeats, orange; and the PEST domain, red. Divisions between individual

these conserved regions may represent two cryptic ANK repeats that stabilize the six more central ANK repeats (see DISCUSSION).

***Cb-glp-1* and *Cr-glp-1* form a clade with *Ce-glp-1* as a sister taxon:** A maximum parsimony analysis, using a GLP-1 amino acid alignment and Drosophila Notch as the outgroup, produced a gene tree with Cb-GLP-1 and Cr-GLP-1 more closely related to each other than either is to Ce-GLP-1 (Figure 6). The branching order of the GLP-1 homologues may be representative of a species tree. We suggest that *C. briggsae* and *C. remanei* form a clade with *C. elegans* as the sister taxon (Figure 6). The branching order of this tree is supported by high bootstrap values. Distance methods, such as neighbor joining, gave a tree with the same topology.

Conservation of *Cb-glp-1* and *Cr-glp-1* functions: To investigate the biological roles of the *Cb-glp-1* and *Cr-glp-1* homologues, we used the technique of RNA-mediated interference (RNAi). This method involves treatment of mothers with double-stranded RNA corresponding to a gene of interest and examination of progeny for defects resulting from loss of gene activity (FIRE *et al.* 1998). To test this approach for *glp-1* genes, we examined *Ce-glp-1(RNAi)* progeny and found defects typical of *glp-1* loss-of-function mutants (data not shown, Figure 1C). With the knowledge that *Ce-glp-1* activity can be inactivated using RNAi, we next injected *C. briggsae* hermaphrodites with *Cb-glp-1* dsRNA or *C. remanei* females with *Cr-glp-1* dsRNA and examined their progeny. In short, we found that both *Cb-glp-1* and *Cr-glp-1* were required for germline proliferation and embryogenesis (Figures 7 and 8). The phenotypes were grossly similar to those of *C. elegans glp-1(0)* mutants, suggesting a conservation of *glp-1* function among these three Caenorhabditis species.

Germline phenotypes of *Cr-glp-1(RNAi)* and *Cb-glp-1 (RNAi)* are not identical: The germline phenotypes of *Cr-glp-1(RNAi)* and *Cb-glp-1(RNAi)* are similar, but not identical. Whereas normal *C. remanei* adults have thousands of germ cells (Figure 7, A and B), *Cr-glp-1(RNAi)* males produced only a few sperm (Figure 7, C and D) and *Cr-glp-1(RNAi)* females produced only a few oocyte-like cells (Figure 7, F and G). DAPI staining of *Cr-glp-1 (RNAi)* males revealed an average of 32 sperm ($n = 10$ animals) and no immature germ cells; DAPI staining of *Cr-glp-1(RNAi)* L4 females revealed only a few germ cells in pachytene (Figure 7G). This germline defect of *Cr-glp-1(RNAi)* adults is similar to that of *C. elegans glp-1(0)* mutants, although the specific type of gamete made differs due to the sexual difference between these two species.

In contrast to both *Ce-glp-1(RNAi)* and *Cr-glp-1(RNAi)* animals, the *Cb-glp-1(RNAi)* germline did not produce differentiated gametes. Instead, adults possessed small gonadal arms (Figure 7H) with no sperm or obvious germline nuclei when scored by DAPI staining. *Cb-glp-1(RNAi)* L2/L3 larvae possessed two or three large granular cells (50/71) (Figure 7I). These granular cells persisted into the L4 larval stage (Figure 7J) and sometimes into adulthood. Antibodies to *C. elegans* germ cells (*e.g.*, α-PGL-1, KAWASAKI *et al.* 1998) did not cross-react with *C. briggsae*; therefore we were unable to demonstrate that these granular cells are indeed germ cells. However, the simplest explanation is that the two to three large granular cells in *Cb-glp-1(RNAi)* animals correspond to the germline progenitors Z2 and Z3 or their daughters. The *Cb-glp-1(RNAi)* defect is therefore similar to that of *Ce-glp-1(RNAi)* and *Cr-glp-1(RNAi)* with respect to the lack of germline proliferation, but different from them with respect to the final germline fate. However, ablation of the *C. briggsae* distal tip cell (DTC) at the L2 stage results in an arrest in germline proliferation and the differentiation of germ cells into sperm (data not shown). In comparison, ablation of the DTC in *C. elegans* mimics the loss of GLP-1 activity, *i.e.*, germ cells divide a few times and differentiate into sperm (KIMBLE and WHITE 1981). Therefore, the *C. briggsae* distal tip cell control of germline proliferation appears similar to that in *C. elegans*.

***Cb-glp-1* has a novel role in vulva development:** The *Cb-glp-1(RNAi)* adult hermaphrodite revealed vulval defects that are not typical of *Ce-glp-1(0)* animals. Some *Cb-glp-*

repeats are noted with a downward arrowhead above sequence. Divisions between EGF repeats are defined by the last conserved cysteine usually followed by a glutamic acid "CE." The beginning of an LNR motif is defined by its first conserved cysteine "C." Divisions between ankyrin repeats are defined by the position of a semiconserved glycine and alanine "GA" in an alignment between ANK repeats (KODOYIANNI *et al.* 1992). The RAM domain was defined as the smallest known region that could bind LAG-1 in a two-hybrid yeast screen (ROEHL *et al.* 1996). Unless otherwise noted, the positions of known mutations (MANGO *et al.* 1991; KODOYIANNI *et al.* 1992; LISSEMORE *et al.* 1993; BERRY *et al.* 1997) in the *Ce-glp-1* gene are denoted by symbols above the sequence. The allele name for each mutation is given in order of appearance (N terminus to C terminus) following its symbol's description. Alleles separated by a slash occur in the same codon although not necessarily at the same nucleotide and they do not necessarily result in the same amino acid substitution. A nonsense mutation is represented by a solid red circle (*q175*^{opal} and *q46*^{ochre}). *q35* is a nonsense allele of *glp-1* with both gain-of-function phenotypes and loss-of-function phenotypes and is represented by a half red/half light green circle. A missense mutation is represented by an asterisk (*e2142, q50, e2072, q415, oz25, q158, e2141/e2144, sy56, bn18, q224, q231*). Second site revertant mutations for missense mutations in the ANKs are represented by blue asterisks (*q278/q282/q283/q285, q335/q284, q280/q246/q277/q279, q240/q252/q333/q334/q336/q337/q286*). The *glp-1* gain-of-function (*gf*) mutation is represented by a light green asterisk (*oz112*). The line under the sequence represents a deletion allele, *q172*. The putative site of ligand-induced cleavage is represented above the sequence by a red downward pointing arrowhead (SCHROETER *et al.* 1998).

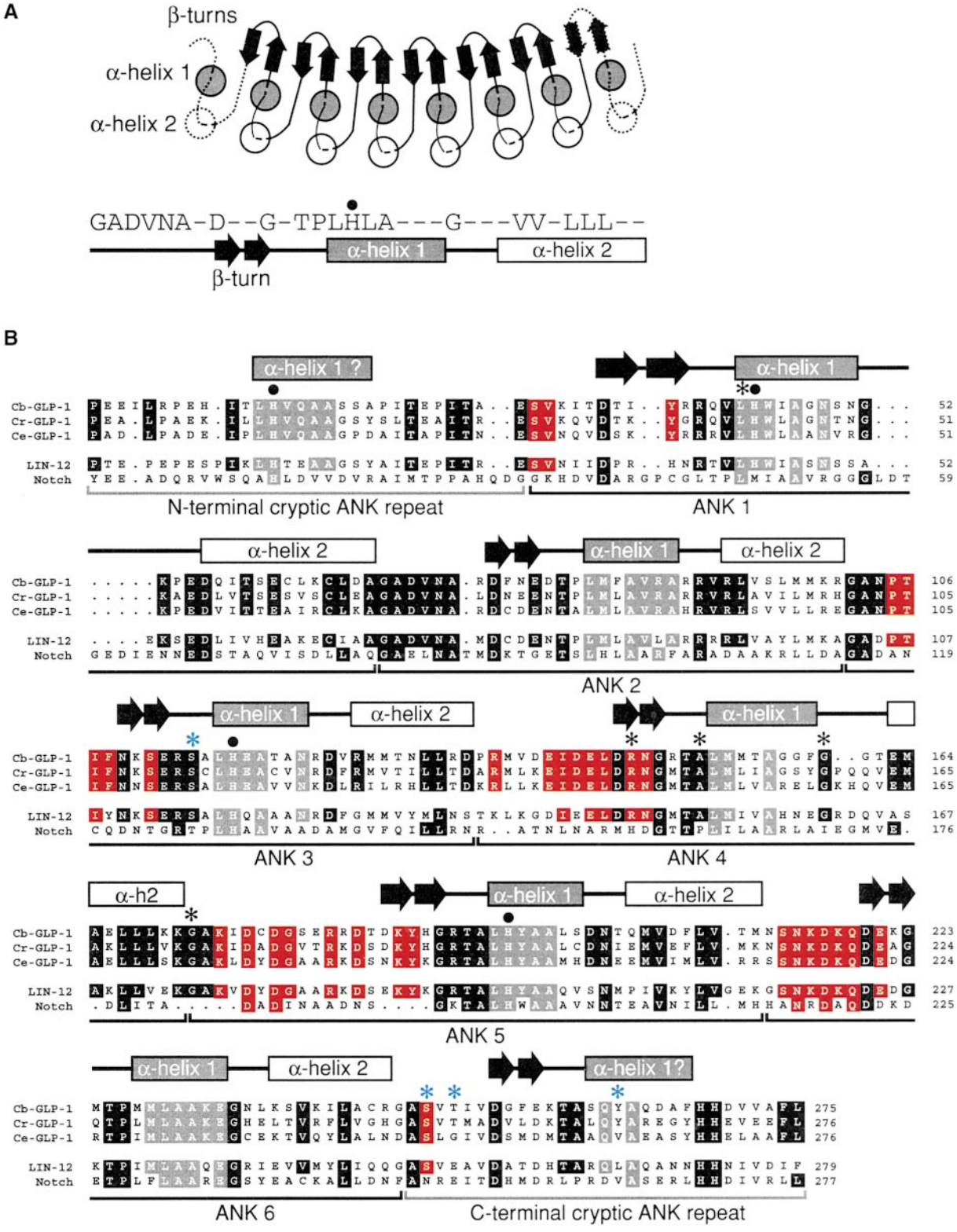


FIGURE 5.—(A) Predicted structure for GLP-1 ANK repeat region (adapted from GORINA and PAVLETICH 1996). β-Turns are represented by arrows; α-helices are viewed end on (gray and open circles); predicted cryptic ANK repeats are drawn with dotted lines. Below, sequence of a consensus ANK repeat (from SEDGWICK and SMERDON 1999). Each ANK repeat consists of a β-turn followed by a pair of α-helices forming a coiled-coil, a $\beta_2\alpha_2$ structure. This structure forms an L shape with the coiled-coil as the back and the β-turn as the base of the L. Adjacent repeats stack against each other via interrepeat and intrarepeat hydrogen bonding among β-turns and hydrophobic interactions between exposed interfaces of adjacent coiled-coils (GORINA and PAVLETICH 1996; LUH *et al.* 1997; BATCHELOR *et al.* 1998; JACOBS and HARRISON 1998). A conserved histidine, H (labeled with a solid circle above the residue), often acts to position the β-turn of the next adjacent repeat. The full ANK repeat region forms a grooved domain similar to a cupped hand with the extended β-sheet as the fingers and the α-helical bundle as the palm. (B) Amino

1(RNAi) hermaphrodites had a protruding vulva (Pvl: 17/113), some had multiple pseudovulvae (Muv: 32/113), and others were both Pvl and Muv (30/113; Figure 7H). Animals with pseudovulvae (62/113) could have one pseudovulva anterior to the main vulva (26/113), one pseudovulva posterior to the main vulva (24/113), or one on either side (12/113). The Pvl phenotype is reminiscent of a *lin-12* loss-of-function mutant, while the Muv phenotype is similar to a *lin-12* gain-of-function mutant (Greenwald *et al.* 1983). Arguments that *glp-1(RNAi)* does not affect *lin-12* activity are presented in the discussion.

Embryonic and larval phenotypes in *Cb-glp-1(RNAi)* and *Cr-glp-1(RNAi)* animals: *C. elegans* embryos lacking *glp-1* activity arrest and lack anterior pharyngeal tissue (Priess *et al.* 1987). Most *Cb-glp-1(RNAi)* and *Cr-glp-1(RNAi)* progeny also died during embryogenesis ($n > 1000$). These embryos arrested prior to elongation and morphogenesis with several hundred cells, including differentiated gut, muscle, and pharyngeal tissues. Whereas normal *C. briggsae* and *C. remanei* embryos have a pharynx resembling that of *C. elegans* (Figure 8, A and C), the *glp-1(RNAi)* animals did not possess an anterior pharynx (Figure 8, B and D). When RNAi progeny escaped, the anterior pharynx was still not made in many L1s, which subsequently died (compare Figure 8, E and F). *C. briggsae* and *C. remanei* larvae often had bumps on their heads and vacuoles adjacent to the posterior bulb of the pharynx, where the excretory cell would be in *C. elegans* L1 larvae (Figure 8F). In 2/28 *Cr-glp-1(RNAi)* dead larvae, the nose was distorted (Figure 8H). In 3/28 *Cr-glp-1(RNAi)* dead larvae, no anus was obvious and a bump was present on the tail (Figure 8I). These larval phenotypes may be analogous to Lag defects, though the bumps and twists differ in detail from those in *C. elegans lag* mutants (Lambie and Kimble 1991).

***glp-1* translational regulation is likely to be conserved in *C. briggsae*:** To ask whether Cb-GLP-1 is localized, we stained the *C. briggsae* germline and embryo with polyclonal antibodies to this protein. Cb-GLP-1 was present distally in the germline (Figure 9A), in the AB blastomere in two-cell embryos, and in AB descendants in four-cell embryos (Figure 9B). The asymmetric distribution in embryos is best seen at the four-cell stage by comparing the intense membrane staining between blastomeres ABa and ABp to the lack of staining between blastomeres EMS and P2 (Figure 9B). Thus, Cb-GLP-1 is localized in both germline and embryo in a fashion that is indistinguishable from the pattern of Ce-GLP-1 (Evans *et al.* 1994). To ask if *Cb-glp-1* mRNA was also localized, we used *in situ* hybridization. Similar to *Ce-glp-1*, *Cb-glp-1* mRNA is present uniformly in germlines, oocytes, and early embryos (Figure 9C). By contrast, staining with the antisense control was either much weaker or nonexistent (Figure 9D).

The embryonic localization of Ce-GLP-1 relies on regulatory elements in the *glp-1* 3′ UTR (Evans *et al.* 1994). Alignment of 3′ UTRs from *Ce-glp-1*, *Cb-glp-1*, and *Cr-glp-1* revealed a region of high conservation (Figure 10). This conserved region covers a portion of the *glp-1* 3′ UTR required for proper translational regulation of *glp-1* (Evans *et al.* 1994). Previously, sequences reminiscent of Nanos response elements (NREs) were noted in this region. However, critical parts of these putative NREs are either poorly conserved or absent from the *Cb-glp-1* and *Cr-glp-1* 3′ UTRs (Figure 10, asterisks).

DISCUSSION

This work on *glp-1* orthologues in *C. briggsae* and *C. remanei* has led to four main conclusions. First, *glp-1* is conserved among three Caenorhabditis species of nematodes. Second, *C. briggsae* and *C. remanei* are likely to form a clade with *C. elegans* as a sister taxon. Third, *glp-1* has similar, but not identical, roles during development in all three species. Last, *Cb-glp-1* regulation is similar to that of *Ce-glp-1*, and this regulation is likely to occur through conserved regulatory elements. The implications of these findings are discussed below.

Conservation of GLP-1 architecture: A conserved motif architecture is the signature for Notch-like receptors (*e.g.*, Artavanis-Tsakonas *et al.* 1995; Maine *et al.* 1995; Kimble and Simpson 1997). All Notch-related receptors harbor a single transmembrane domain and have a core of conserved motifs including EGF, LNR, and ANK repeats (Figure 1). In addition, all Notch receptors possess two cysteines in a region extending between the LNR repeats and the transmembrane domain; we have dubbed this region the CC-linker. Our comparison of GLP-1 homologues underscores the conservation of previously known motifs and reveals additional highly conserved features.

All vertebrate Notch-related receptors possess 36 EGF repeats, whereas GLP-1 has 10 and LIN-12 has 13 EGF repeats. Since GLP-1 and LIN-12 are functionally interchangeable (Lambie and Kimble 1991; Mango *et al.* 1991; Fitzgerald *et al.* 1993; Roehl and Kimble 1993), one might think that their specific number of EGF re-

acid alignment of the ANK repeats of Ce-GLP-1, Cr-GLP-1, and Cb-GLP-1; the LIN-12 and *D. melanogaster* Notch ANK repeats are aligned underneath. Solid boxes indicate absolute conservation between GLP-1 homologues. Gray boxes indicate the putative first α-helix, based on sequence conservation and position within the repeat. The conserved histidine, if present in a repeat, is labeled with a solid circle above the residue. Red boxes indicate conserved amino acids in the predicted β-turn and linker (from previous repeat to β-turn); these red amino acids do not agree with the consensus sequence and may participate in interactions with LAG-2, LAG-3, or other unknown factors. The mutations and the divisions of repeats shown in Figure 4 are reiterated here.

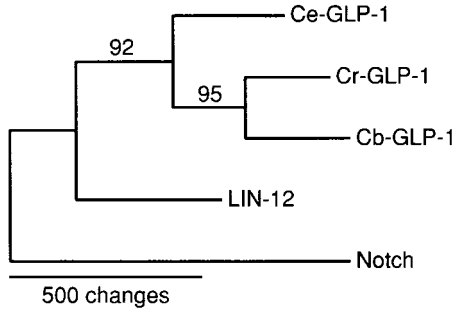


Figure 6.—A phylogram of Ce-GLP-1, Cb-GLP-1, Cr-GLP-1, LIN-12, and *D. melanogaster* Notch based on amino acid alignment. The phylogram shown is the single shortest tree resulting from a maximum parsimony exhaustive search. All characters were given equal weight and gaps were treated as characters. The alignment contained 1443 individual characters, 166 of which were informative. To generate bootstrap values, 400 characters were resampled in 1000 replicates.

peats would not be critical. However, we have found that all three GLP-1 homologues possess 10 EGF repeats. We suggest two explanations—10 repeats may be functionally significant, or changes in repeat number may be difficult to achieve. The EGF repeats have been proposed to mediate interactions with ligands (Artavanis-Tsakonas *et al.* 1995 for review). There is no evidence for any specificity of individual GLP-1 EGF-like repeats in particular interactions. Perhaps the lower degree of identity of the EGF-like repeats in comparison to the LNRs and the ANK repeats is a result of built-in redundancy. This makes the retention of all 10 EGF repeats more intriguing.

All Notch-related receptors possess three and only three LNR repeats. The LNR region has been implicated in activation of the receptor upon signaling (Greenwald and Seydoux 1990; Lyman and Young 1993; Rand *et al.* 2000), a key role that is consistent with its strong conservation. The CC-linker as well as the transmembrane region have gained prominence recently as sites of cleavage during signaling (Blaumueller *et al.* 1997; Logeat *et al.* 1998; Schroeter *et al.* 1998). Furthermore, the two cysteines have been proposed to mediate dimerization (Kidd *et al.* 1989; Greenwald and Seydoux 1990). We find that both the transmembrane domain and CC-linker are remarkably well conserved among GLP-1 receptors. Indeed, conservation of the CC-linker region is as strong or stronger than that in any part of the receptor. Examination of vertebrate Notch1 receptors also revealed extensive amino acid identity within the CC-linker region (Figure

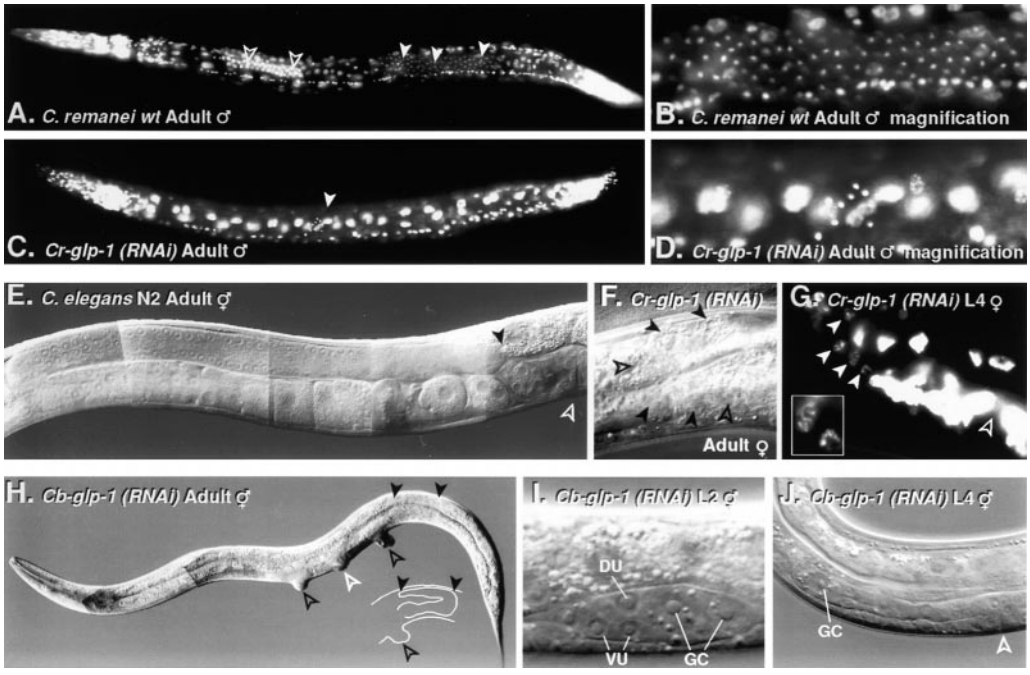


Figure 7.—Conservation of *glp-1* function in maintenance of mitosis in the germline. (A and B) Wild-type *C. remanei* male, stained with DAPI. Solid arrowheads, sperm; open arrowheads, immature germ cells. (C and D) *Cr-glp-1(RNAi)* male, stained with DAPI. Solid arrowheads, sperm in the gonad arm. (E) Wild-type *C. elegans* hermaphrodite germline. With the exception of the lack of sperm in *C. remanei* females, the germlines of adult *C. elegans* hermaphrodites, *C. briggsae* hermaphrodites, or *C. remanei* females are largely indistinguishable. Solid black arrowhead, distal tip of the germline; open white arrowhead, position of vulva. (F) *C. remanei* female germline from a *Cr-glp-1(RNAi)* experiment. Solid arrowheads, position of nuclei of oocyte-like cells; open arrowheads, position of nuclei of oocyte-like cells out of plane of focus. (G) *Cr-glp-1(RNAi)* female L4 germline, stained with DAPI. Solid arrowheads, germ-cell nuclei in pachytene; open arrowhead, position of the vulva. The inset is a magnification of two germ-cell nuclei to show chromatin characteristic of meiotic pachytene. (H) *Cb-glp-1(RNAi)* hermaphrodite: germline is outlined in a cartoon and indicated by solid black arrowheads. Open black arrowheads, pseudovulvae; open white arrowhead, main vulva. In many animals this protrudes more dramatically than that shown here. (I) *Cb-glp-1(RNAi)* late L2/early L3 hermaphrodite, cells are labeled as follows: DU, dorsal uterine precursor; VU, ventral uterine precursor; GC, germ cell. (J) *Cb-glp-1(RNAi)* late L4 hermaphrodite. The single germ cell in the gonad arm is labeled GC. White open arrowhead, vulva.

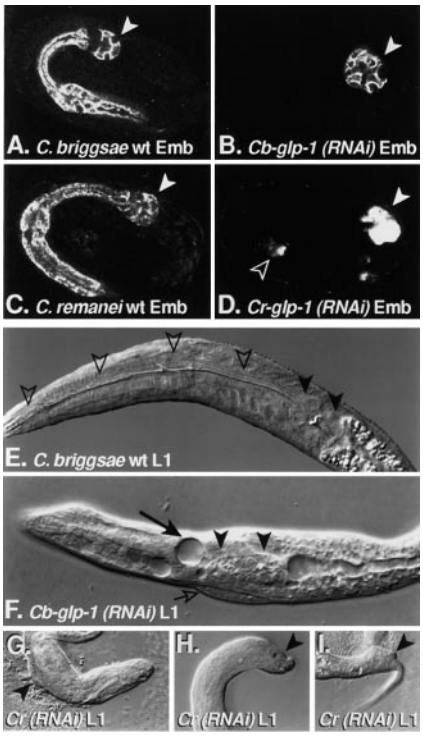


FIGURE 8.—Conservation of *glp-1* function in the embryo. (A–D) Embryos immunostained with 3NB12. Solid white arrowheads, posterior bulb of pharynx. (A) *C. briggsae* wild-type embryo. (B) *Cb-glp-1(RNAi)* embryo. (C) *C. remanei* wild-type embryo. (D) *Cr-glp-1(RNAi)* embryo. Often isolated cells not associated with the posterior pharynx stained in *Cr-glp-1(RNAi)* animals, open white arrowhead. These cells may be intestinal muscle cells. (E) Wild-type *C. briggsae* L1. Solid black arrowheads, posterior bulb of pharynx; open black arrowheads, anterior pharynx. L1 larvae of *C. elegans*, *C. briggsae*, and *C. remanei* are similar, including their pharynx. (F) *Cb-glp-1(RNAi)* L1. Solid black arrowheads, posterior bulb of pharynx; solid black arrow, vesicles adjacent to posterior bulb of the pharynx; open black arrow, bump adjacent to the posterior bulb of the pharynx. (G–I) *Cr-glp-1(RNAi)* L1s with Lag-like phenotypes. (G) Black arrowhead, bump on head. (H) Black arrowhead, twisted nose. (I) Black arrowhead, bump on tail.

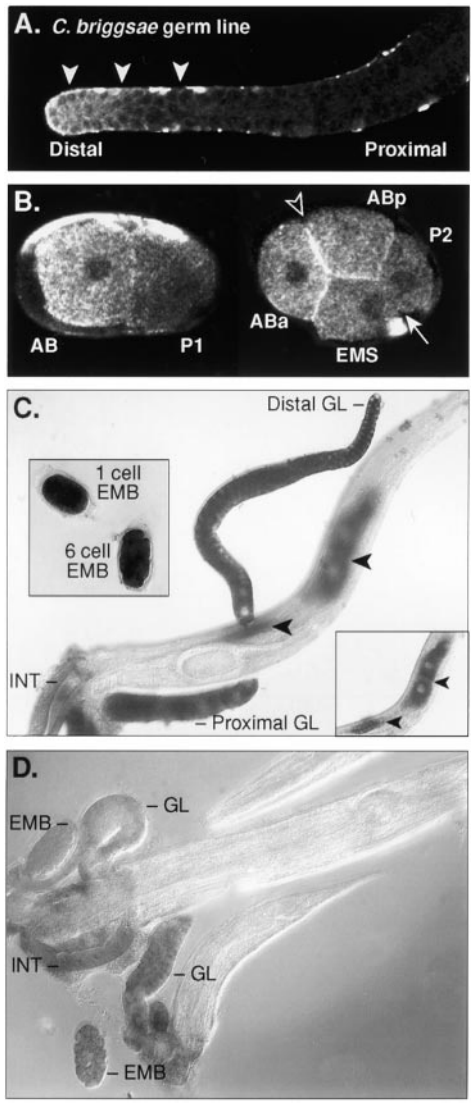


FIGURE 9.—Conservation of GLP-1 regulation in *C. briggsae*. (A) *C. briggsae* extruded germline stained with Cb-GLP-1 antibodies. Distal end is positioned to left. White arrowheads, GLP-1 staining. (B) *C. briggsae* embryos stained with Cb-GLP-1 antibodies. Left, two-cell embryo; right, four-cell embryo. Blastomeres are labeled. Open white arrowhead, boundary between ABa and ABp; white arrow, boundary between EMS and P2. (C and D) RNA *in situ* of *C. briggsae* germlines and embryos using single-stranded *Cb-glp-1* cDNA probes. GL, germline; EMB, embryos; INT, intestine. (C) Antisense probe. Distal and proximal ends of germlines are labeled. Black arrowhead, internal germline staining. Top inset: one-cell and six-cell embryos. Bottom inset: internal germline staining. (D) Sense probe.

3C), albeit not quite as strong as GLP-1. This strong conservation of the CC-linker underscores its importance in signaling.

All Notch-like receptors are predicted to possess six ANK repeats, which mediate signaling events by acting in a complex to regulate transcription of target genes (PETCHERSKI and KIMBLE 2000a,b). Sequence identity within Notch1 ANK repeats from five vertebrate taxa is higher than that within the GLP-1 ANK repeats reported here (Figure 3C). Those in fish, frogs, chickens, mice, and humans are 79% identical at the amino acid level across all five taxa, whereas those in three closely related members of the Caenorhabditis genus are only 55% identical across all three taxa. Teleost fish diverged from tetrapods over 400 million years ago (RAFF 1996), whereas *C. elegans*, *C. briggsae*, and *C. remanei* are estimated to have diverged roughly 40 million years ago (KENNEDY *et al.* 1993). Nonetheless, the nematode ANK repeats are more divergent than their vertebrate counterparts, which may be explained by either of two hypotheses. First, Caenorhabditis taxa may have an in-

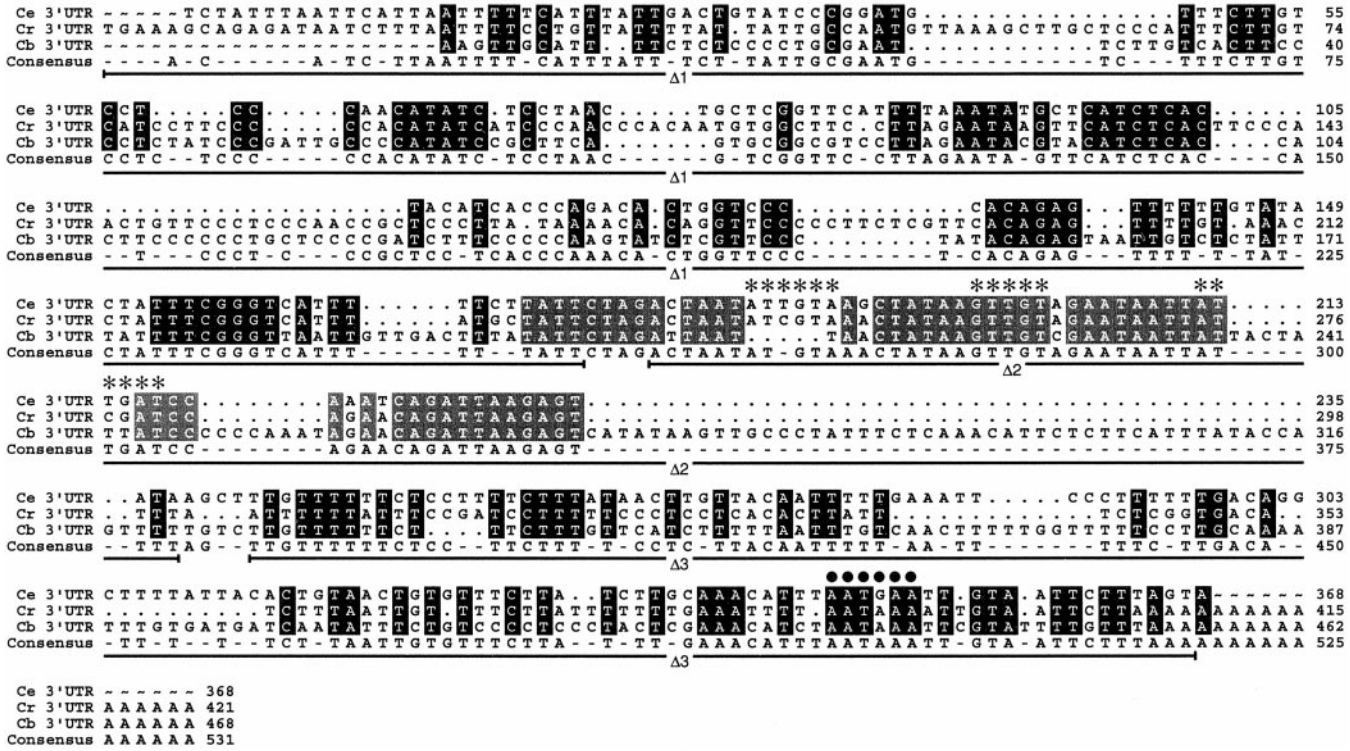


FIGURE 10.—Nucleotide alignment of *Cb-glp-1*, *Cr-glp-1*, and *Ce-glp-1* 3′ UTRs. Solid boxes indicate identity. Gray boxes note nucleotides in the SCR (see DISCUSSION). Nucleotides in the potential NREs are noted with an asterisk above sequence. Polyadenylation signal is indicated with solid circle above sequence. Brackets under the sequence represent deletions described previously (EVANS *et al.* 1994). Reporters deleted for Δ1 were expressed normally; those deleted for Δ2 were expressed at the correct time in the early embryo, but expression was uniform, suggesting the sequence deleted might harbor a spatial control element; those deleted for Δ3 were expressed prematurely in the germline, suggesting the presence of a temporal regulatory element in the region defined by Δ3.

trinsic substitution rate higher than that in vertebrate taxa, possibly due to the short life span of these nematodes. Second, more rigorous constraints may have been placed on Notch ANK repeat sequences in vertebrates than in nematodes. Consistent with this latter idea, some regions of Notch1 proteins are less well conserved among vertebrates than the analogous regions of GLP-1 among nematodes (*e.g.*, the CC-linker and the TMD, see Figure 3C).

Conservation within the ANK repeat region: ANK repeats are protein-protein interaction domains (SEDGWICK and SMERDON 1999). The GLP-1 intracellular domain participates in a ternary complex with the LAG-1 DNA-binding protein and the LAG-3 putative transcriptional activator (PETCHERSKI and KIMBLE 2000a). The GLP-1 ANK repeats are essential for formation of that complex (PETCHERSKI and KIMBLE 2000a), and they are also critical for signaling (KODOYIANNI *et al.* 1992; ROEHL and KIMBLE 1993). For other ANK repeat proteins, the β-turn tips and the exposed surface of the ANK groove (Figure 5A, legend) are critical for specific interactions. The amino acids in the GLP-1 ANK repeats that are predicted to confer specificity are strongly conserved (Figure 5B). Some of these amino acids agree with the ANK consensus and therefore are likely to play a structural role (SEDGWICK and SMERDON 1999), whereas others are unique and may be critical for specific interactions with LAG-1 and LAG-3 (Figure 5B, red). The amino acids that we propose to be critical for interactions are also conserved in Ce-LIN-12, which also must interact with LAG-1 and LAG-3 (Figure 5B).

In addition to the conservation within the ANK repeats, we note that the amino acids flanking those repeats are also conserved (Figure 4, yellow; Figure 5B). These regions may be critical for proper folding of the ANK repeats and stabilization of the full domain. Most missense GLP-1-ANK mutations change consensus amino acids that help establish ANK structural elements (KODOYIANNI *et al.* 1992; Figure 5B, black asterisk). Consistent with the idea that they disrupt structure of the domain, all of the mutations in ANK domain are temperature sensitive. A group of second site revertants of these initial ANK mutations was able to restore signaling, suggesting a restoration of structural integrity. Most of these intragenic revertants alter amino acids in the conserved C-terminal flanking region (LISSEMORE *et al.* 1993; Figure 5B, light blue asterisk). The C-terminal flanking region also contains two adjacent histidines that are

both highly conserved between nematode, insect, and vertebrate Notch-like receptors. Additionally, at least 32 amino acids on each side of the ANK repeats were required in transgenic assays for activity (Roehl and Kimble 1993; V. Kodoyianni and J. Kimble, unpublished results). These conserved flanking regions may harbor cryptic ANK repeats, a speculation made previously (Bork 1993; Lissemore *et al.* 1993). The presence of degenerate ANK repeats that cap the core ANK repeats is a common feature of ANK motif-containing proteins whose structure has been determined (Gorina and Pavletich 1996; Luh *et al.* 1997; Batchelor *et al.* 1998; Jacobs and Harrison 1998). The N-terminal degenerate repeat often lacks a β-turn, presumably because it is not stabilized by an upstream ANK repeat; the C-terminal degenerate repeat is often the least well conserved and typically lacks a conserved histidine residue that stabilizes the next β-turn. The putative GLP-1 cryptic ankyrin repeats follow this pattern (Figure 5B). Furthermore, the N-terminal flanking region in GLP-1 harbors a stretch of conserved amino acids LHVQAA that resembles the first α-helix of ANK repeats. By analogy with other ANK repeats (Figure 5, legend), the histidine in this conserved stretch may stabilize the β-turn of the first canonical ANK repeat. Inspection of the analogous region in vertebrate Notch1 receptors reveals strong conservation in the amino acids flanking the ANK repeats, and in particular a stretch bearing a histidine that may serve the same function.

***C. briggsae* and *C. remanei* form a clade with *C. elegans* as a sister taxon:** The phylogenetic relationships among *C. elegans*, *C. briggsae*, and *C. remanei* have been controversial. One group has proposed a *briggsae/remanei* clade (Fitch *et al.* 1995), whereas another has proposed a *remanei/elegans* clade (Baldwin *et al.* 1997). Our data support a *briggsae/remanei* clade. Comparison of either nucleotide or amino acid sequences indicates that the *Cr-glp-1* and *Cb-glp-1* genes are closer to each other than either is to *Ce-glp-1*. Additional distance data from comparisons of *tra-2* and *spe-11* homologues from these species agree with the *briggsae/remanei* clade (Haag and Kimble 2000; E. Polinko and S. Strome, personal communication).

Knowledge of phylogeny is essential for evaluating whether a species difference resulted from a loss or gain of character. For example, in the evolution of hermaphroditism, *C. elegans* and *C. briggsae* are male/hermaphrodite species, while *C. remanei* and most other Caenorhabditis species are gonochoristic, or male/female species. The idea that *C. briggsae* and *C. remanei* form a clade suggests that either hermaphroditism was acquired once and that *C. remanei* females lost the ability to produce sperm or that hermaphroditism evolved independently in both *C. elegans* and *C. briggsae*. The former seems more likely, suggesting that *C. remanei* is a derived male/female species and that a more distant Caenorhabditis species is more likely to represent the primitive gonochoristic state.

Conservation of GLP-1 function in the germline and embryo: GLP-1 is required for germline proliferation and early embryonic decisions in all three Caenorhabditis species (Austin and Kimble 1987; Priess *et al.* 1987; this work). We find the role of GLP-1 during embryogenesis was indistinguishable in the three species, but its role in control of germline development was divergent. In *C. elegans*, the germline of a *glp-1* null mutant undergoes one or at most two rounds of division and enters meiosis to produce sperm (Austin and Kimble 1987). Therefore, *Ce-glp-1* is required for germline mitoses, but not for spermatogenesis. Although *Cb-glp-1* mimics *Ce-glp-1* in being essential for germline mitosis, the few germ cells present in *Cb-glp-1(RNAi)* animals often fail to divide at all and arrest without differentiating. Therefore, *Cb-glp-1* appears to be necessary both for germline proliferation and for differentiation of gametes.

In *C. elegans*, the distal tip cell signals to GLP-1 to promote germline proliferation, and ablation of the distal tip cell mimics the germline defects of a *glp-1* loss-of-function mutant. Interestingly, *C. briggsae* animals with ablated DTCs not only arrest germline mitosis, but, in contrast to the *Cb-glp-1* RNAi phenotype, they also produce sperm. Therefore, spermatogenesis in *C. briggsae* does not require DTC signaling, but does require *Cb-glp-1* activity. We suggest that *Cb-glp-1* may act in early germ-cell precursor cells to permit differentiation, but that it is not required later. However, the possibility that an initial or residual signaling activity from the DTC may be sufficient to invest germ cells with the capacity to differentiate has not been ruled out. Alternatively, signaling from some other source is also possible.

***Cb-glp-1* controls vulva development:** *C. briggsae* and *C. remanei* likely form a clade with *C. elegans* as a sister taxon (Fitch *et al.* 1995; this work). On the basis of this phylogeny, we suggest that *C. briggsae* GLP-1 has acquired a new role in vulval development. In *C. elegans* and *C. remanei*, *glp-1* is not required for vulval development (Austin and Kimble 1987; this work). By contrast, *Cb-glp-1(RNAi)* animals often have vulval defects (Figure 7H). One possibility for the Pvl phenotype might have been that *Cb-glp-1* dsRNA reduces *Cb-lin-12* as well as *Cb-glp-1*. Two lines of evidence suggest this is not the case. First, other defects typical of a *lin-12* loss of function were not observed in *Cb-glp-1(RNAi)* animals (*e.g.*, proximal proliferation). Second, a stretch of nucleotide identity >75% is required to reduce gene function in *C. briggsae* (M. Montgomery, personal communication). *Ce-glp-1* and *Ce-lin-12* are ~55% identical (Yochem and Greenwald 1989). Assuming a similar percentage identity for *Cb-glp-1* and *Cb-lin-12*, RNAi experiments directed against *glp-1* would not be expected to affect *lin-12*. Furthermore, we find that *glp-1(RNAi)* in *C. elegans* does not result in *lin-12* loss-of-function defects.

We envision two alternative explanations for the *Cb-glp-1(RNAi)* protruding vulva phenotype. First, in *C. briggsae*, GLP-1 may have assumed a role played by LIN-12 in *C. elegans*, for example, the AC/VU decision and/or specification of uterine π-cell fates (Greenwald *et al.* 1983; Newman *et al.* 1995, 1996). Second, the process that results from a loss of Cb-GLP-1 activity and generates pseudovulvae (see below) may affect formation of the main vulva as well.

Why are *Cb-glp-1(RNAi)* animals Muv? In *C. elegans*, this is a phenotype associated with overactivity of either *lin-12* or *glp-1* (Greenwald *et al.* 1983; Mango *et al.* 1991; Berry *et al.* 1997). Many explanations of the *Cb-glp-1(RNAi)* Muv phenotype are possible. We offer one idea that is both simple and plausible. Perhaps, in *C. briggsae*, GLP-1 represses LIN-12 in the vulval hypodermis; by this scenario, LIN-12 would aberrantly drive vulval fates in the absence of GLP-1. If true, this role for *glp-1* may be newly acquired in *C. briggsae*, or it may be conserved. If conserved, it may be genetically invisible in *C. elegans* due to redundancy that has been lost in *C. briggsae*. Clearly further experiments are required to sort out the vulval function of *glp-1* and its regulatory relationship to *lin-12* in both species. Interestingly the pseudovulvae defect resulting in *C. briggsae* hermaphrodites from *Cb-glp-1(RNAi)* differs from those resulting from overactivation of Notch-like receptors in *C. elegans* in two ways. First, no more than two pseudovulvae are seen in *Cb-glp-1(RNAi) C. briggsae* hermaphrodites, in contrast to as many as six in *C. elegans* hermaphrodites with an overactivated Notch-like receptor (Greenwald *et al.* 1983; Mango *et al.* 1991; Berry *et al.* 1997). Second, the pseudovulvae of *Cb-glp-1(RNAi)* animals are morphologically distinct, *i.e.*, larger than those seen in *C. elegans glp-1(gf)* and *lin-12(d)* hermaphrodites.

Conservation of *glp-1* mRNA translational regulation: In *C. elegans*, *glp-1* mRNA is translationally repressed via regulatory elements within its 3′ UTR in the germline and embryo (Evans *et al.* 1994; see Introduction). In *C. briggsae*, we observe an expression pattern similar to that in *C. elegans*: *Cb-glp-1* mRNA is present throughout both the germline and embryo, but Cb-GLP-1 protein is present only in the mitotic region of the germline and only in the anterior blastomeres of the embryo. Comparison of the *Ce-glp-1* and the *Cb-glp-1* 3′ UTR sequences reveals a striking region of nucleotide identity (Figure 10, gray). This region was originally identified as critical for regulation in the embryo by a series of deletion mutants in the *Ce-glp-1* 3′ UTR (Evans *et al.* 1994). This same region is now known to be sufficient to confer regulation on a reporter mRNA and has been dubbed the SCR, for *s*patial *c*ontrol *r*egion (T. Evans, personal communication). Within the SCR are sequences reminiscent of Nanos response elements in Drosophila (Evans *et al.* 1994). However, these putative NREs are not conserved in the *Cb-glp-1* 3′ UTR, and direct mutation of NRE residues does not disrupt regulation (T. Evans and J. Kimble, unpublished data). Therefore, these sequences either are not involved in translational control or are redundant to other control elements in the region. We suggest that the sequences conserved in both *C. elegans* and *C. briggsae glp-1* 3′ UTR regulatory regions may define novel and critical elements for translational control and should be used to focus on identification of *trans*-acting regulators.

We thank Eric Haag for the *C. remanei* genomic library and insightful conversation and comments on this manuscript, David Baillie and Alex Puoti for libraries, and the *Caenorhabditis* Genetics Center for strains. The authors also thank Eileen Durkin for invaluable assistance in the production of fusion proteins and the purification of antibodies and Lisa Friedman for assistance with the 5′ portion of the *Cb-glp-1* genomic clone. We also thank Tom Evans, Eric Polinko, Susan Strome, and Mary Montgomery for sharing unpublished results. Last, we thank Kevin O'Connell, Sarah Crittenden, Christian Eckmann, and the many lab members and colleagues who provided thoughts and criticisms throughout the course of this work and the writing of this manuscript. D.R. was a National Institutes of Health Molecular Biosciences trainee; J.K. is an investigator with the Howard Hughes Medical Institute.

LITERATURE CITED

Akam, M., 1998 The yin and yang of evo/devo [meeting review]. Cell **92:** 153–155.

Artavanis-Tsakonas, S., K. Matsuno and M. E. Fortini, 1995 Notch signaling. Science **268:** 225–232.

Artavanis-Tsakonas, S., M. D. Rand and R. J. Lake, 1999 Notch signaling: cell fate control and signal integration in development. Science **284:** 770–776.

Austin, J., and J. Kimble, 1987 *glp-1* is required in the germ line for regulation of the decision between mitosis and meiosis in C. elegans. Cell **51:** 589–599.

Baldwin, J. G., R. M. Giblin-Davis, C. D. Elddleman, D. S. Williams, J. T. Vida *et al.*, 1997 The buccal capsule of *Aduncospiculum halicti* (Namata: Diplogasterina): an ultrastructural and molecular phylogenetic study. Can. J. Zool. **75:** 407–423.

Batchelor, A. H., D. E. Piper, F. C. de la Brousse, S. L. McKnight and C. Wolberger, 1998 The structure of GABPα/β: an ETS domain-ankyrin repeat heterodimer bound to DNA. Science **279:** 1037–1041.

Berry, L. W., B. Westlund and T. Schedl, 1997 Germ-line tumor formation caused by activation of *glp-1*, a *Caenorhabditis elegans* member of the *Notch* family of receptors. Development **124:** 925–936.

Blaumueller, C. M., H. Qi, P. Zagouras and S. Artavanis-Tsakonas, 1997 Intracellular cleavage of Notch leads to a heterodimeric receptor on the plasma membrane. Cell **90:** 281–291.

Blaxter, M., 1998 *Caenorhabditis elegans* is a nematode. Science **282:** 2041–2046.

Bork, P., 1993 Hundreds of ankyrin-like repeats in functionally diverse proteins: mobile modules that cross phyla horizontally? Proteins **17:** 363–374.

Brenner, S., 1974 The genetics of *Caenorhabditis elegans*. Genetics **77:** 71–94.

C. elegans Sequencing Consortium, 1998 Genome sequence of the nematode *C. elegans*: a platform for investigating biology [published errata appear in Science 1999 Jan 1;283(5398):35 and 1999 Mar 26;283(5410):2103]. Science **282:** 2012–2018.

Christensen, S., V. Kodoyianni, M. Bosenberg, L. Friedman and J. Kimble, 1996 *lag-1*, a gene required for *lin-12* and *glp-1* signaling in *Caenorhabditis elegans*, is homologous to human CBF1 and *Drosophila* Su(H). Development **122:** 1373–1383.

Crittenden, S. L., E. R. Troemel, T. C. Evans and J. Kimble, 1994 GLP-1 is localized to the mitotic region of the *C. elegans* germ line. Development **120:** 2901–2911.

Crittenden, S. L., D. Rudel, J. Binder, T. C. Evans and J. Kimble,

1997 Genes required for GLP-1 asymmetry in the early *Caenorhabditis elegans* embryo. Dev. Biol. **181:** 36–46.

del Amo, F. F., M. Gendron-Maguire, P. J. Swiatek, N. A. Jenkins, N. G. Copeland *et al.*, 1993 Cloning, analysis, and chromosomal localization of Notch-1, a mouse homolog of Drosophila Notch. Genomics **15:** 259–264.

Evans, T. C., S. L. Crittenden, V. Kodoyianni and J. Kimble, 1994 Translational control of maternal *glp-1* mRNA establishes an asymmetry in the C. elegans embryo. Cell **77:** 183–194.

Fire, A., S. Xu, M. K. Montgomery, S. A. Kostas, S. E. Driver *et al.*, 1998 Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. Nature **391:** 806–811.

Fitch, D. H. A., B. Bugaj-Gaweda and S. W. Emmons, 1995 18S ribosomal RNA gene phylogeny for some Rhabditidae related to *Caenorhabditis*. Mol. Biol. Evol. **12:** 346–358.

Fitzgerald, K., H. A. Wilkinson and I. Greenwald, 1993 *glp-1* can substitute for *lin-12* in specifying cell fate decisions in *Caenorhabditis elegans*. Development **119:** 1019–1027.

Goodman, M., 1999 The genomic record of Humankind's evolutionary roots. Am. J. Hum. Genet. **64:** 31–39.

Gorina, S., and N. P. Pavletich, 1996 Structure of the p53 tumor suppressor bound to the ankyrin and SH3 domains of 53BP2. Science **274:** 1001–1005.

Greenwald, I., and G. Seydoux, 1990 Analysis of gain-of-function mutations of the *lin-12* gene of *Caenorhabditis elegans*. Nature **346:** 197–199.

Greenwald, I. S., P. W. Sternberg and H. R. Horvitz, 1983 The *lin-12* locus specifies cell fates in Caenorhabditis elegans. Cell **34:** 435–444.

Haag, E. S., and J. Kimble, 2000 Regulatory elements required for development of *Caenorhabditis elegans* hermaphrodites are conserved in the *tra-2* homologue of *C. remanei*, a male/female sister species. Genetics **155:** 105–116.

Heitzler, P., and P. Simpson, 1993 Altered epidermal growth factor-like sequences provide evidence for a role of *Notch* as a receptor in cell fate decisons. Development **117:** 1113–1123.

Henderson, S. T., D. Gao, E. J. Lambie and J. Kimble, 1994 *lag-2* may encode a signaling ligand for the GLP-1 and LIN-12 receptors of *C. elegans*. Development **120:** 2913–2924.

Jacobs, M. D., and S. C. Harrison, 1998 Structure of an IκBα/NF-κB complex. Cell **95:** 749–758.

Kadyk, L. C., and J. Kimble, 1998 Genetic regulation of entry into meiosis in *Caenorhabditis elegans*. Development **125:** 1803–1813.

Kawasaki, I., Y. H. Shim, J. Kirchner, J. Kaminker, W. B. Wood *et al.*, 1998 PGL-1, a predicted RNA-binding component of germ granules, is essential for fertility in C. elegans. Cell **94:** 635–645.

Kelley, M. R., S. Kidd, W. A. Deutsch and M. W. Young, 1987 Mutations altering the structure of epidermal growth factor-like coding sequences at the Drosophila *Notch* locus. Cell **51:** 539–548.

Kennedy, B. P., E. J. Aamodt, F. L. Allen, M. A. Chung, M. F. Heschl *et al.*, 1993 The gut esterase gene (*ges-1*) from the nematodes *Caenorhabditis elegans* and *Caenorhabditis briggsae*. J. Mol. Biol. **229:** 890–908.

Kidd, S., M. K. Baylies, G. P. Gasic and M. W. Young, 1989 Structure and distribution of the Notch protein in developing *Drosophila*. Genes Dev. **3:** 1113–1129.

Kimble, J., and P. Simpson, 1997 The LIN-12/Notch signaling pathway and its regulation. Annu. Rev. Cell Dev. Biol. **13:** 333–361.

Kimble, J. E., and J. G. White, 1981 On the control of germ cell development in *Caenorhabditis elegans*. Dev. Biol. **81:** 208–219.

Kodoyianni, V., E. M. Maine and J. Kimble, 1992 Molecular basis of loss-of-function mutations in the *glp-1* gene of *Caenorhabditis elegans*. Mol. Biol. Cell **3:** 1199–1213.

Lambie, E. J., and J. Kimble, 1991 Two homologous regulatory genes, *lin-12* and *glp-1*, have overlapping functions. Development **112:** 231–240.

Lardelli, M., and U. Lendahl, 1993 Motch A and Motch B—two mouse Notch homologues coexpressed in a wide variety of tissues. Exp. Cell Res. **204:** 364–372.

Lardelli, M., J. Dahlstrand and U. Lendahl, 1994 The novel Notch homologue mouse Notch 3 lacks specific EGF-repeats and is expressed in proliferating neuroepithelium. Mech. Dev. **46:** 123–126.

Lissemore, J. L., P. D. Currie, C. M. Turk and E. M. Maine, 1993 Intragenic dominant suppressors of *glp-1*, a gene essential for cell-signaling in *Caenorhabditis elegans*, support a role for cdc10/SWI6/ankyrin motifs in GLP-1 function. Genetics **135:** 1023–1034.

Logeat, F., C. Bessia, C. Brou, O. LeBail, S. Jarriault *et al.*, 1998 The Notch1 receptor is cleaved constitutively by a furin-like convertase. Proc. Natl. Acad. Sci. USA **95:** 8108–8112.

Luh, F. Y., S. J. Archer, P. J. Domaille, B. O. Smith, D. Owen *et al.*, 1997 Structure of the cyclin-dependent kinase inhibitor $p19^{Ink4d}$. Nature **389:** 999–1003.

Lyman, D., and M. W. Young, 1993 Further evidence for function of the *Drosophila* Notch protein as a transmembrane receptor. Proc. Natl. Acad. Sci. USA **90:** 10395–10399.

Maine, E. M., J. L. Lissemore and W. T. Starmer, 1995 A phylogenetic analysis of vertebrate and invertebrate Notch-related genes. Mol. Phylogenet. Evol. **4:** 139–149.

Mango, S. E., E. M. Maine and J. Kimble, 1991 Carboxy-terminal truncation activates *glp-1* protein to specify vulval fates in *Caenorhabditis elegans*. Nature **352:** 811–815.

Mello, C. C., B. W. Draper and J. R. Priess, 1994 The maternal genes *apx-1* and *glp-1* and establishment of dorsal-ventral polarity in the early C. elegans embryo. Cell **77:** 95–106.

Moskowitz, I., and J. Rothman, 1996 *lin-12* and *glp-1* are required zygotically for early embryonic cellular interactions and are regulated by maternal GLP-1 signaling in *Caenorhabditis elegans*. Development **122:** 4105–4117.

Newman, A. P., J. G. White and P. A. Sternberg, 1995 The *Caenorhabditis elegans lin-12* gene mediates induction of ventral uterine specialization by the anchor cell. Development **121:** 263–271.

Newman, A. P., J. G. White and P. W. Sternberg, 1996 Morphogenesis of the *C. elegans* hermaphrodite uterus. Development **122:** 3617–3626.

Okamoto, H., and J. N. Thomson, 1985 Monoclonal antibodies which distinguish certain classes of neuronal and supporting cells in the nervous tissue of the nematode Caenorhabditis elegans. J. Neurosci. **5:** 643–653.

Petcherski, A. G., and J. Kimble, 2000a LAG-3 is a putative transcriptional activator in the *C. elegans* Notch pathway. Nature **405:** 364–368.

Petcherski, A. G., and J. Kimble, 2000b Mastermind is a putative activator for Notch. Curr. Biol. **10:** R471–R473.

Priess, J. R., and J. N. Thomson, 1987 Cellular interactions in early C. elegans embryos. Cell **48:** 241–250.

Priess, J. R., H. Schnabel and R. Schnabel, 1987 The *glp-1* locus and cellular interactions in early C. elegans embryos. Cell **51:** 601–611.

Puoti, A., and J. Kimble, 1999 The *Caenorhabditis elegans* sex determination gene *mog-1* encodes a member of the DEAH-box protein family. Mol. Cell. Biol. **19:** 2189–2197.

Raff, R. A., 1996 *The Shape of Life: Genes, Development, and the Evolution of Animal Form.* University of Chicago Press, Chicago.

Rand, M. D., L. M. Grimm, S. Artavanis-Tsakonas, V. Patriub, S. C. Blacklow *et al.*, 2000 Calcium depletion dissociates and activates heterodimeric Notch receptors. Mol. Cell. Biol. **20:** 1825–1835.

Rebay, I., R. J. Fleming, R. G. Fehon, L. Cherbas, P. Cherbas *et al.*, 1991 Specific EGF repeats of Notch mediate interactions with Delta and Serrate: implications for Notch as a multifunctional receptor. Cell **67:** 687–699.

Roehl, H., and J. Kimble, 1993 Control of cell fate in *C. elegans* by a GLP-1 peptide consisting primarily of ankyrin repeats. Nature **364:** 632–635.

Roehl, H., M. Bosenberg, R. Blelloch and J. Kimble, 1996 Roles of the RAM and ANK domains in signaling by the *C. elegans* GLP-1 receptor. EMBO J. **15:** 7002–7012.

Sambrook, J., E. F. Fritsch and T. Maniatis, 1989 *Molecular Cloning: A Laboratory Manual.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Schroeter, E. H., J. A. Kisslinger and R. Kopan, 1998 Notch1 signaling requires ligand-induced proteolytic release of intracellular domain. Nature **393:** 382–386.

Sedgwick, S. G., and S. J. Smerdon, 1999 The ankyrin repeat: a diversity of interactions on a common structural framework. Trends Biochem. Sci. **24:** 311–316.

Seydoux, G., and A. Fire, 1994 Soma-germline asymmetry in the distributions of embryonic RNAs in *Caenorhabditis elegans*. Development **120:** 2823–2834.

Seydoux, G., T. Schedl and I. Greenwald, 1990 Cell-cell interac-

tions prevent a potential inductive interaction between soma and germline in C. elegans. Cell **61:** 939–951.

Swofford, D. L., 1998 *PAUP*. Phylogenetic Analysis Using Parsimony (*and Other Methods), Version 4*. Sinauer Associates, Sunderland, MA.

Tamura, K., Y. Taniguchi, S. Minoguchi, T. Sakai, T. Tun *et al.*, 1995 Physical interaction between a novel domain of receptor Notch and the transcription factor RBP-Jκ/Su(H). Curr. Biol. **5:** 1416–1423.

Tax, F. E., J. J. Yeargers and J. H. Thomas, 1994 Sequence of *C. elegans lag-2* reveals a cell-signalling domain shared with *Delta* and *Serrate* of *Drosophila*. Nature **368:** 150–154.

Uyttendaele, H., G. Marazzi, G. Wu, Q. Yan, D. Sassoon *et al.*, 1996 *Notch4/int-3*, a mammary proto-oncogene, is an endothelial cell-specific mammalian *Notch* gene. Development **122:** 2251–2259.

Wilkinson, H. A., K. Fitzgerald and I. Greenwald, 1994 Reciprocal changes in expression of the receptor *lin-12* and its ligand *lag-2* prior to commitment in a C. elegans cell fate decision. Cell **79:** 1187–1198.

Yochem, J., and I. Greenwald, 1989 *glp-1* and *lin-12*, genes implicated in distinct cell-cell interactions in C. elegans, encode similar transmembrane proteins. Cell **58:** 553–563.

Communicating editor: R. K. Herman