Genetic and Molecular Analysis of Genes Required for Axon Outgrowth in Caenorhabditis elegans

by

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B.S., Cellular and Molecular Biology University of Michigan, 1985

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Abstract

This thesis describes three approaches taken toward understanding
the molecular mechanisms that underlie axon bundle assembly in the
relatively simple nervous system of the nematode Caenorhabditis elegans:
genetic characterization of genes necessary for normal bundle assembly,
molecular analysis of one of these genes, and development of axon
labeling and cell culture techniques to expand the set of tools
available for C. elegans neurobiology.

Previous analysis of C. elegans mutants with uncoordinated
movement identified a group of genes required for axonal elongation and
bundling only in regions of the nervous system in which axons extend
along other axonal surfaces, but not where axons grow along epithelial
cells and basement membranes. Null alleles of two of these genes, unc-
34 and unc-76, were identified and shown to confer the same environment-
specific axonal outgrowth defects. Analysis of unc-34 unc-76 double
mutants suggested that the two genes function independently. Null unc-
34 alleles confer a temperature-sensitive axonal outgrowth defect,
indicating that another process functions in axonal outgrowth in the
absence of the unc-34 gene product.

The unc-76 gene was cloned and sequenced. It encodes a 385 amino
acid protein with no apparent similarity to other known proteins, and
thus defines a new class of axonal outgrowth molecules. Antibodies
raised against the protein indicated that it is present throughout the
nervous system in axons and cell bodies but not nuclei. It is not found
outside of the nervous system. The expression pattern, sequence, and
mutant phenotype of unc-76 suggest that it is likely to function in
either the formation of axonal structure or the transduction of signals
in growing axons.

To allow light and electron microscopic labeling of axons during
axon outgrowth, axonally-localized Unc-76-β-galactosidase fusion
proteins were placed under the control of promoters active in limited
numbers of cells. Initial observations suggested that these could be
generally useful markers for the study of nervous system development.
Conditions in which dissociated embryonic cells extended long axons in
vitro were developed, suggesting that analysis of mutant and wild-type
axon outgrowth in defined conditions might be possible.

Thesis Supervisor: Dr. H. Robert Horvitz
Title: Professor of Biology
To Sara,
whose love, support, and perspective
have brought joy to life
and this work to completion
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Chapter 1  Introduction
Growing axons in a developing animal face many difficult choices. They often must travel long distances, decide which cells and surfaces to grow along and which to avoid, properly enter and leave nerve bundles even when neighboring axons do not do so, and choose among many potential targets. That the $10^{12}$ neurons in the human nervous system can make these decisions accurately suggests that finely-tuned molecular mechanisms exist to ensure this accuracy; that the 302 neurons in the nervous system of a genetically-tractable nematode can make similar decisions equally accurately encourages the hope that the full complement of these molecules can be identified.

Most axons in nervous systems, large and small, are arranged in bundles. Studies of developing vertebrate and invertebrate nervous systems suggest that at least some of these bundles are formed through a process of selective fasciculation; that is, many growing axons display an ability to discriminate among axons they encounter and elongate only along specific axonal surfaces. To determine the molecular mechanisms by which these specific interactions occur, considerable research has focused on axonal surface molecules. Intracellular events in fasciculation remain poorly understood.

The studies described in this thesis address the mechanisms of bundle formation in the nematode *Caenorhabditis elegans*. This animal has a small nervous system, consisting of only 302 neurons, with only three bundles containing more than a half-dozen axons. Yet in this simple organism, the major bundles contain 50-175 axons, and they are organized with remarkable consistency from animal to animal (White et al., 1983, 1986) The relatively small size of the *C. elegans* nervous
system and the ease with which mutations affecting the nervous system can be obtained suggest that this organism could be a useful system in which to study specific neuron-neuron interactions.

The studies presented in this thesis use a genetic approach to identify molecules likely to function in fasciculation. Genes were chosen for analysis on the basis of defects in axonal morphology and bundle organization caused by loss of gene function, without presuppositions as to the biochemical nature and location of the molecules important in this process. While not without its own biases, a genetic approach provides access not only to extracellular molecules involved in cell-cell interactions, molecules also readily identified and analyzed in cell culture systems, but also has the potential for revealing molecules involved in intracellular responses to extracellular signals as well. This thesis will present the discovery of a protein required for normal axonal bundle formation, Unc-76, with a structure that has not been observed elsewhere. This introductory chapter will first describe the structure of the C. elegans nervous system and the principal events in its assembly. Specific cell-cell interactions necessary for neuronal bundle formation will then be addressed in the context of molecular mechanisms gleaned from studies in a variety of organisms. Intracellular events in axonal outgrowth and pathfinding will be reviewed. Finally, genetic screens for genes involved in axonal outgrowth in C. elegans will be reviewed, and the initial characterization of the genes under study in this thesis will be presented.

Structure and development of the C. elegans nervous system
The body of *C. elegans* is arranged as a pair of concentric tubes (for a detailed review of *C. elegans* anatomy, see Wood et al., 1988). The outer tube consists of a set of syncytial epithelial cells called the hypodermis, the outer surface of which produces the exterior cuticle of the worm and the interior surface of which is lined with a basement membrane. The nervous system lies between the hypodermis and this basement membrane, and the body muscles and gonad are found on the interior side of this membrane. The pharynx and intestine comprise the inner tube.

The nervous system of adult *C. elegans* hermaphrodites contains 302 neurons, and their complete morphology and connectivity have been determined from analysis of serial section electron micrographs (White et al., 1986; Albertson and Thomson, 1976; Hall and Russell, 1991). Most of the cell bodies of these neurons are arranged in ganglia in the head and tail of the animal or in a row along the ventral midline; a few others are located alone or in small clusters on the lateral body wall. Several bundles of neurons are present in the nervous system. The ventral nerve cord consists of a large bundle of about 50-60 axons (depending on the position along the anterior-posterior axis) on the right side of the ventral midline and a smaller bundle of 5-6 axons on the left, separated by a ridge of ventral hypodermis and neuronal cell bodies. The dorsal cord, extending along the right side of a ridge of hypodermis at the dorsal midline, contains 10-12 axons at any given point, all but 3 of which are joined to their ventrally-located cell bodies by commissures running around the body wall. These major longitudinal bundles contain processes of most motorneurons as well as a
few interneurons. Most sensory and interneurons and some motorneurons extend to the nerve ring, a dense bundle of about 175 axons that surrounds the pharynx, and make most of their synaptic contacts there. Sensory dendrites project from sensory endings in the tip of the nose to cell bodies in the head ganglia, and a few neurons send longitudinal processes along the lateral body wall.

222 of the 302 neurons are born in the embryo (Sulston et al., 1983). Most embryonic neurons are born close to where the cell bodies will lie in the larva. The basic nervous system plan is established by the embryonically-derived neurons; newly-hatched L1 larvae have well-defined nerve cords, nerve rings, sensory processes, and lateral neurons. A wave of neurogenesis adds 56 neurons to the ventral nervous system in the late L1 stage, and these axons grow into existing bundles. The remaining neurons complete their outgrowth in the L2 and L4 stages.

The outgrowth of the axons in the embryo has been described in detail from reconstructions of serial electron micrographs taken of animals at different stages of development (Durbin, 1987). In the ventral nerve cord, a single axon (AVG) first grows posteriorly from the head along the right side of the ventral cord neuronal cell bodies. Subsequently, axons of the six DD motorneurons begin to extend in contact with the AVG process. When the DD axons reach the position of the next DB motorneuron cell body in the anterior direction, they exit the ventral nerve cord and form commissures along the body wall to the dorsal cord. Commissures from the DA and DB axons, the remaining two classes of embryonic ventral cord motorneurons, extend at the same time.

The posterior portion of the ventral nerve cord is pioneered on both the right and left sides by a pair of axons growing in close
association with one another, PVP and PVQ (Durbin, 1987). PVQ normally contacts the hypodermal cell surface, while PVP contacts the basement membrane. Elimination of PVPR but not PVQL (pioneers of the left side of the ventral cord) causes remaining axons on the left side of the cord to grow on the right side instead, while elimination of PVPL on the right side of the cord has no effect on later axonal growth in the right side of the cord. This probably results from the asymmetric distribution of axons in the ventral cord: growth cones entering the right side of the ventral cord when PVPL is missing can still follow interneurons growing from the head or motorneuron processes, but none of these cells is present on the left side of the cord. The outgrowth of PVP, PVQ, AVG, and the DD axons establishes a scaffold on which additional longitudinally-growing axons extend.

Reconstructions of the nervous systems of older animals from serial electron micrographs indicate that axons added to the ventral cord postembryonically are often surrounded by embryonically-derived axons, suggesting that these later axons can insinuate themselves into preexisting fascicles (White et al., 1986). Axons of the HSN motorneurons, the primary cells analyzed in this thesis, extend to the ventral nerve cord in the L2 stage but do not appear to grow anteriorly in the cord until the L4 stage (White et al., 1986; G. Garriga, personal communication). In adults, the HSN axons are found in close proximity to the PVP and PVQ axons near the dorsal surface of the ventral nerve cord on either side.

Extracellular influences on axonal outgrowth and bundling
What extracellular signals influence the establishment of nervous system structure? The neurons that pioneer the *C. elegans* ventral nerve cord encounter several different potential sources of guidance information. They contact hypodermal cells and neuronal cell bodies, and they also have access to the basement membrane that overlies the ventral cord. The posterior pioneers, PVPR and PVQL on the left and PVPL and PVQR on the right, also run in close proximity to one another and could therefore provide guidance information to one another. Laser ablation studies have shown that the PVP axon is required for guidance of the PVQ axon on the left side of the ventral nerve cord, but not on the right, where other axons are extending (Durbin, 1987). Pioneers of the commissures extending to the dorsal nerve cord are primarily in contact with the hypodermis and basement membrane. Neurons following the pioneers in the nerve cords make contact with the axons that came before them; axons that join the ventral cord early, however (such as DA, DB, DD) can also easily contact hypodermal cells or neuronal cell bodies. Axons arriving later in the nerve cords can sometimes grow into internal positions, in which the primary neighbors are other axons. (The genes under study in this thesis, *unc-34* and *unc-76*, primarily affect the outgrowth of longitudinal neurons in the nerve cords.) Thus, both the extracellular matrix (ECM) and the surfaces of other cells could be the sources of information that direct the formation of the organized nerve cord structure.

Both the ECM and cell surfaces of vertebrates and some invertebrates have a large and expanding set of characterized molecules that support axonal outgrowth *in vitro*. The function of such molecules in setting up a three-dimensional array of axons depends on the
distributions of these molecules and their receptors, the response such molecules cause in different growing axons, and the response of axons confronted with multiple possible molecules on multiple cells, as is the case in the developing C. elegans nerve cords. With a few exceptions, the roles these molecules play in shaping the directions individual axons take (as opposed to simply mediating axonal adhesion) remain largely untested.

**Extracellular matrix molecules and axonal outgrowth**

The composition of the basement membrane contacted by growing C. elegans axons is not well characterized. Mammalian ECM composition varies in different body regions at different times, but it is primarily made of large, multidomain molecules such as fibronectin, collagen, and laminin crosslinked in a meshwork (Reichardt and Tomaselli, 1991). Vertebrates have several well-characterized ECM components that stimulate axonal outgrowth in vitro, and in some assays, axons are capable of discriminating among them. Cultured axons can extend on laminin, fibronectin, collagens I and IV, thrombospondin, and in some assays, tenascin (reviewed in Sanes, 1989; Bixby and Harris, 1991; Reichardt and Tomaselli, 1991; Letourneau et al., 1992).

Laminin is the most potent stimulator of axonal elongation among vertebrate ECM molecules. Axons grown on substrates containing strips of laminin, fibronectin, and the nonspecific substratum polylysine—all of which support neurite extension when present alone—nearly always grow preferentially on laminin (Letourneau, 1975; Gundersen, 1987). Assessment of the precise functions of laminin and other ECM components
in supporting axonal outgrowth is complicated by the fact that the molecules exist in multiple forms and have multiple receptors. Several vertebrate laminin homologs have been identified: merosin, which has an alternative A chain, supports axonal extension, while S-laminin appears to be associated with synapses (Reichardt and Tomaselli, 1991). Multiple laminin receptors have been identified, including at least six integrins and as many non-integrin surface proteins can act as laminin receptors (Mecham, 1991). Similarly, fibronectin has multiple splice forms, multiple functional domains, interaction with proteoglycans, and multiple integrin receptors (Reichardt and Tomaselli, 1991; Hynes, 1992). The potential diversity of receptor-substrate interactions is thus very high, and so an interpretation of the function of ECM molecules partly depends upon the use of reagents that are sensitive to different forms of these molecules.

Some spatial and temporal restriction of expression of receptors for ECM components has been observed, suggesting that distinct groups of axons could use generally permissive substrates. The chicken integrin α8 subunit is distributed in axon-rich areas of the spinal cord and periphery in a manner reminiscent of several cell-adhesion molecules described below (Bossy et al., 1991). Some populations of neurons have been shown to regulate the type and activity of their integrin molecules over time. For example, chick retinal neurons express β1-class integrins at a time corresponding to the period of growth along the laminin-lined optic nerve. These integrins lose their ability to bind laminin at the time they would leave the optic nerve in vivo, although they are still present on the cell surface (Neugebauer and Reichardt, 1991). In other retinal neuron populations, the mRNA and protein levels
of the α6 subunit are reduced at the appropriate time (de Curtis et al., 1991).

Do ECM components guide axonal outgrowth? Artificial gradients of laminin in vitro fail to cause oriented axonal outgrowth (McKenna and Raper, 1988). Opinions are mixed as to the ability of vertebrate ECM molecules to function in guidance in vivo, largely as a result of the widespread distribution of these proteins (Bixby and Harris, 1991; Reichardt and Tomaselli, 1991; Letourneau et al., 1992). Some regions in developing vertebrate nervous systems, however, do contain localized deposits of ECM proteins that could provide tracks for ingrowing axons. Laminin is found transiently in pathways along the optic nerve, the spinal ventral longitudinal pathway, developing muscle, and spinal nerve (Sanes, 1989; Reichardt and Tomaselli, 1991). Fibronectin and chondroitin sulfate proteoglycan are found in layers of the developing cortex shortly before the arrival and during the extension of axons (Sanes, 1989; Sheppard et al., 1991). Furthermore, the presence of laminin is correlated with the ability of axons to extend. Peripheral nerve and goldfish optic nerve, which can support regeneration or continued axonal extension, maintain laminin expression, while most CNS pathways express laminin only transiently (Reichardt and Tomaselli, 1991). Laminin is found in regions of the zebrafish central nervous system in which primary motorneurons extend, and fibronectin is in regions avoided by these neurons. These motorneurons avoid laminin-rich regions into which fibronectin has been injected (Eisen, 1991). Recently an ECM molecule that interacts with the chick axonal glycoprotein Fl1, called restrictin, has been shown to be expressed in a
localized pattern in the developing nervous system, suggestive of a role in guidance (Norenberg et al., 1992).

Experiments to test the function of the extracellular matrix in specific axonal guidance functions in vivo have been limited.

Application of an antibody that recognizes a laminin/heparin sulfate proteoglycan complex ('INO') slows the reinnervation of denervated iris but does not block it completely (Sandrock and Matthew, 1987).

Antibodies against thrombospondin, tenascin, integrin $\beta_1$, and laminin/heparin sulfate proteoglycan can inhibit migration of neural crest cells and differentiating neurons (Reichardt and Tomaselli, 1991).

In at least one case, filopodial contacts with the ECM have been observed but found to be unnecessary for normal guidance. The Til pioneer axons in filleted grasshopper limbs grow between a basal lamina and neuroepithelial cells. Despite observed filopodial contact with the basal lamina in normal embryos, enzymatic digestion of the basal lamina did not prevent normally-directed outgrowth (Condic and Bentley, 1989). Axons apparently derived guidance information from the epithelial cells they normally contact. However, evidence that the basal lamina was used for adhesion was provided by the observation that axonal retraction was caused by digestion of the lamina after the axon had extended most of the length of the limb.

*Caenorhabditis elegans* genetic studies have demonstrated a role for a potential ECM molecule in axonal guidance. Mutations in the unc-6 gene prevent axonal extension in the dorsal and ventral directions along the basement membrane (Desai et al., 1988; McIntire et al., 1992; Hedgecock et al., 1990). The gene product shares similarity to the N termini of the laminin B1 and B2 chains; however, it lacks the N
terminal domains with which vertebrate laminin B chains associate with one another and the A chain, and it has a C terminal domain not found in laminin (Ishii et al., 1992). The localization of this protein to the cell surface or extracellular matrix has not been demonstrated. Mutants lacking unc-6 function show characteristic defects in axonal outgrowth along the dorso-ventral axis, a position in which axons grow between hypodermal cells and a basement membrane. Specific mutations in V2 EGF repeat domain of unc-6 disrupt only dorsal-directed growth, while complete loss of gene function blocks all growth in this environment (B. Wadsworth and E. Hedgecock, personal communication). The possible association of this molecule with the hypodermal cell surface, however, is raised by the observation that dorsal-growing pioneer axons, which require unc-6 activity for normal growth, grow along hypodermal cells rather than the basal lamina when forced by cells in their path to choose one or the other (Durbin, 1987). Mutations in unc-6 block the migration of certain mesodermal cells as well as axons, however, and because mesodermal cells contact only the basal lamina and not the hypodermal cells, the possibility exists that the Unc-6 protein is localized in the basal lamina as well (Hedgecock et al., 1990).

The proposed receptor for Unc-6 is the Unc-5 protein, a transmembrane protein with two immunoglobulin-like domains, as well as two domains similar to thrombospondin (Leung-Hagesteijn et al., 1992). Axons in animals lacking unc-5 function are capable of growing ventrally but cannot grow dorsally between the hypodermis and basal lamina (Desai et al., 1988; McIntire et al., 1992; Hedgecock et al., 1990). Ectopic expression of unc-5 in neurons that normally do not express the gene or grow in a dorsal direction causes them to grow dorsally (M. Hamelin and
J. Culotti, personal communication). This dorsal growth is dependent upon a functional unc-6 gene. C. elegans contains multiple collagen genes and several genes with sequence similarity to laminin, but specific roles for these in axonal outgrowth have not been demonstrated.

**Repulsive cues in axonal guidance**

Studies of the interaction of chick retinal axons with tectal membranes and the interaction of central and peripheral neurons *in vitro* have suggested that inhibitory interactions are likely to play a significant role in shaping developing vertebrate nervous systems. Signals that cause growing axons to stop at the appropriate place are clearly necessary. In *C. elegans* embryos, the first motor axons to extend in the ventral nerve cord, the DDs, stop growing anteriorly and leave the ventral cord when they contact the cell bodies of the DB motor neurons (Durbin, 1987). The ends of a number of *C. elegans* neurons abut but do not overlap, suggesting that inhibitory interactions occur frequently in nematode development.

In vertebrates, several molecules that inhibit axonal outgrowth have been identified. Tenascin, an ECM component, inhibits axonal outgrowth in some assays *in vitro* (Faissner and Kruse, 1990) and is present around developing domains of the rodent cortex (Bartsch et al., 1992). Two protein components of the myelin sheath inhibit neurite outgrowth *in vitro* (Caroni and Schwab, 1988), and antibody perturbation studies in developing rats indicate that these molecules serve as "guard rails" to keep corticospinal axons from exiting their fascicles (Schwab and Schnell, 1991). One *C. elegans* gene potentially required for the
inhibition of axonal outgrowth tax-2, the molecular nature of which is not known. In tax-2 mutants, sensory axons in the head sometimes leave the bundle in which they travel and grow posteriorly in the ventral nerve cord, while sensory axons in the tail that normally travel only a short distance anteriorly in the ventral cord extend much further (C. Bargmann, personal communication).

A role for repulsion in fasciculation has been suggested in the case of the zebrafish spinal cord. Hatta (1992) observed that fine processes of floor plate cells appear to avoid contact with longitudinal spinal cord axons. In the absence of these midline cells, resulting either from laser ablation or the cyclops mutation, axons that normally remain bundled on either side of the midline frequently cross and join bundles on the opposite side.

**Chemoattractant molecules in axonal guidance**

The role of soluble chemoattractants in axonal guidance is not well understood. Early observations of the effects of high local concentrations of nerve growth factor (NGF) in chick embryos suggested that this molecule could attract growing axons, and growth cones in vitro are able to reorient toward a NGF-containing pipette (Gundersen and Barret, 1980). The timing of NGF expression in vivo suggests that guidance is unlikely to be a major function of this molecule, however (Davies et al., 1987).

Commissural axons in the developing vertebrate spinal cord have been shown to grow ventrally toward the floor plate by a chemotactic mechanism. In vitro, commissural axons from explanted tissue orient
toward a source of a soluble factor released by floor plate cells (Tessier-Lavigne et al., 1988). This molecule has not yet been identified but may associate with the ECM (cited in Bixby and Harris, 1991). In addition, growth cone chemotaxis toward factors released by the mouse maxillary whisker pad epithelium (Lumsden and Davies, 1986) and the pons (Heffner et al., 1990) has been demonstrated.

While, in principle, diffusible molecules could function in C. elegans to establish the direction of axonal outgrowth, the intimate association with specific cellular structures and the demonstrated effects of ablation of these structures suggests that soluble molecules are not sufficient to provide a major source of guidance information. The ability of axons to grow normally in fillets of grasshopper limb in culture, in which local gradients of soluble molecules have been eliminated, supports this view (Lefcort and Bentley, 1987).

Cell-surface molecules involved in axonal outgrowth and fasciculation

Axons in developing nervous systems, both vertebrate and invertebrate, clearly have selective interactions with other axons. Reversals of sections of developing chick spinal cord along the rostral-caudal axis lead to the formation of spinal nerves in abnormal locations, but the axons in these nerves mix, defasciculate, and refasciculate to produce normal innervation of the limb, suggesting that axons are able to identify the correct neighbors in an abnormal environment (Lance-Jones and Landmesser, 1980). In the developing grasshopper central nervous system, the growth cones of the G, aCC, and
pCC axons contact or come within filopodial reach of close to 100 other axons, and yet they develop extensive contacts with only three specific axons (Raper et al., 1984; Bastiani and Goodman, 1986; du Lac et al., 1986). If the arrival of the G, aCC, or pCC axons precedes the arrival of their partners at the so-called choice point, where contact is normally made, the axons wait until the partners arrive, and if these partners are ablated, the G, aCC, and pCC axons do not extend further (Raper et al., 1984; Bastiani and Goodman, 1986; du Lac et al., 1986). Similarly, axons of the DL1 neurons in the Japanese medaka fish fail to extend if the Rohon-Beard pioneer axons are ablated, despite the presence of other axons within filopodial reach (Kuwada, 1986). The interpretation of experiments such as these is that molecules on the surfaces of these cells mediate the recognition of preexisting axons in "labeled pathways" by ingrowing growth cones.

Interactions of growing axons with the surfaces of other cells are also likely to be very important in both the outgrowth of pioneer axons in the C. elegans ventral nerve cords and in later addition of axons to the bundle. The pioneer axons from the posterior, PVPL and PVQR on the right side and PVPR and PVQL on the left, travel in tight association on both sides of the ventral cord; Durbin (1987) observed that the tips of the PVPL and PVQR growth cones on the right side of the cord were within 0.1 μm of each other. All of the axons growing dorsally along the hypodermis appear to grow along hypodermal cells rather than the basement membrane when they encounter another cell and are forced to leave one or the other substratum, suggesting that these growth cones interacted preferentially with hypodermal cell surfaces (Durbin, 1987). Finally, axons entering the ventral cord postembryonically can grow in
the center of the cord, where they are likely to be in contact only with axons (White et al., 1983, 1986).

The labeled pathways hypothesis, proposed for the development of the insect nervous system but appropriate for early vertebrate nervous system development as well, predicts that pathways established early in development will have distinct ligands on their axonal surfaces recognized by specific receptors on follower growth cones (Raper et al., 1983). Three families of cell-surface molecules that can serve as such receptors, ligands, or both, have been identified on the basis of their ability to mediate cell adhesion or their expression on a restricted set of axons and subsequent biochemical characterization. These are the integrins, receptors primarily for ECM molecules but for cell-surface ligands as well; the cadherins, calcium-dependent homophilic cell adhesion molecules; and cell adhesion molecules of the immunoglobulin superfamily. Several molecules of unusual sequence unrelated to these three families have also been identified.

The integrins comprise a family of over 20 heterodimeric transmembrane proteins (reviewed in Hynes, 1992). Most of the integrins identified in the nervous system contain the β1 subunit and interact with ECM molecules such as fibronectin, laminin, and collagens. However, integrins α4β1 and α4β2 can bind to the cell-surface immunoglobulin superfamily molecules VCAM-1 and ICAM-1 in the immune system, respectively, suggesting that direct cell-cell interactions in the nervous systems could also be mediated by integrins. The localization of β1-class integrins in regions of axonal outgrowth in the nervous system suggests that they are likely to be involved primarily in interactions with ECM (Shiga and Oppenheim, 1991). The adhesive
function of integrin α6β1 on chick retinal axons is down-regulated at approximately the same time in development at which laminin expression in the optic nerve decreases (Neugebauer and Reichardt, 1991). Antibody perturbation of β1-class integrins interferes with extension of retinal axons on astrocytes and of ciliary ganglion axons on muscle cells in vitro (Neugebauer et al., 1988; Tomaselli et al., 1988; Bixby et al., 1987), indicating that integrin ligands can interact with molecules associated with the surfaces of cells with which growing axons interact (probably laminin, in the case of astrocytes). β1-class integrins have been localized to the tips of filopodia of chick dorsal root ganglion cell growth cones extending on polyornithine, which suggests that they are in a position to mediate the rapid responses to ECM components mediated by single filopodial contacts in these cells (Hammarback et al., 1986; Letourneau and Shattuck, 1989). Integrins have been shown to act as signal transduction molecules in fibroblasts, lymphocytes, and platelets, indicating that their functions are more complex than simply providing mechanical linkage of cells to surfaces (Hynes, 1992).

The cadherins, homophilic calcium-dependent adhesion molecules, are believed to function in the separation of tissue layers early in vertebrate development (Takeichi, 1988). N-cadherin expression is widespread on neuronal and glial surfaces throughout the nervous system during times of axonal outgrowth. As is the case with many ECM components and cell adhesion molecules such as N-CAM, its distribution is probably too broad to allow it to function as a specific guidance molecule. Nevertheless, it is expressed on early neuroepithelia and perhaps provides a permissive substratum for outgrowth of pioneer axons (Dodd et al., 1988; Takeichi, 1988). Recent identification of eight
cadherin homologs in the rat brain suggests that cadherins could be involved in more specific adhesion processes, however (Suzuki et al., 1991).

The immunoglobulin (Ig) superfamily contains molecules that can promote cell adhesion by diverse mechanisms. Many, such as N-CAM, associate by homophilic binding, but others can bind via heterophilic interactions with other Ig superfamily molecules, integrins, or a recently-described extracellular matrix molecule (restrictin). Members of this family often have large numbers of alternative splice forms that encode transmembrane, glycosylphosphatidylinositol (GPI)-linked, or secreted variants. The extracellular domains of Ig superfamily molecules implicated in axonal outgrowth usually have five or six repeats of an immunoglobulin C2 domain and two to five repeats of fibronectin type III (FNIII) domains close to the site of membrane linkage (Sonderegger and Rathjen, 1992). Three subgroups within this family are evident based on domain arrangement and mode of membrane attachment: N-CAM and fasciclin II; the GPI-linked molecules F3/F11 and TAG-1/axonin-1; and the transmembrane molecules L1/Ng-CAM, Nr-CAM, neurofascin, and neuroglian (reviewed in (Sondregger and Rathjen, 1992; Bieber, 1991; Dodd and Jessell, 1988).

N-CAM consists of five Ig domains, two FNIII repeats, and either a GPI membrane linkage or a transmembrane segment and two alternative cytoplasmic tails (Walsh and Doherty, 1991). N-CAM molecules can bind in a homophilic manner, independent of calcium. The extracellular domain can be modified by the addition of polysialic acid, the amount of which is correlated with the degree to which N-CAM supports axonal outgrowth and inversely correlated with adhesiveness (Doherty et al.,
1990). In addition, an alternatively-spliced extracellular exon termed VASE is capable of reducing the neurite outgrowth-promoting activity of N-CAM-expressing cells (Doherty et al., 1992a).

N-CAM is widely distributed in neuronal bundles and is unlikely to specify particular axonal pathways. However, antibody perturbation experiments suggest that it is necessary for maintenance of the fascicle structure of vertebrate optic nerves. Anti-N-CAM antibodies applied either in the chick retina or the Xenopus tectum disrupt the bundling of axons within the optic nerve and the subsequent tectal innervation pattern (Fraser et al., 1988; Thanos et al., 1984). Interestingly, application of antibodies in the retina does not disrupt organization of retinal axons until they enter the optic nerve, suggesting that another mechanism maintains axonal organization within the retina (Thanos et al., 1984). The degree of polysialic acid addition to N-CAM appears to affect its ability to support adhesion and axonal outgrowth (Doherty et al., 1990), and different groups of chick motor axons express different levels of sialylation in the plexus region, where axons from the spinal cord defasciculate and sort out into new fascicles (Tang et al., 1992). Endosialidase treatment causes errors in the organization of these fascicles reminiscent of those caused by anti-N-CAM antibody perturbation in the optic nerve (Tang et al., 1992), suggesting that modifications to N-CAM provide additional bundling specificity to axons within a larger fascicle.

The fasciclin II molecule found on axonal surfaces in grasshopper and Drosophila clearly functions in axon-axon interactions during nervous system development (see below). Antibodies against mouse N-CAM have been reported to stain four male-specific C. elegans neurons (S.

Whether this reflects a true nematode N-CAM homolog or fortuitous cross-reactivity is not known, however.

Molecules expressed on the surfaces of subsets of growing axons, candidates for guidance labels under the labeled pathways hypothesis, have been identified by screens of monoclonal antibodies raised against nervous system membranes or membrane glycoproteins in insects, rodents, and chick. The majority of molecules identified in this way comprise the transmembrane- and GPI-linked subgroups of the Ig superfamily, although DM-GRASP, a transmembrane protein expressed in a subset of chick spinal cord neurons, myelin-associated glycoprotein, and fasciclin III, an insect protein with restricted axonal expression, contain only Ig domains (Burns et al., 1991; Patel et al., 1987) Insect studies have also revealed two proteins with novel sequences, fasciclins I and IV, which are both expressed on discrete and partially overlapping sets of central and peripheral fascicles (Bastiani et al., 1987; Kolodkin et al., 1992). Immunostaining of chick or rat spinal cords or insect central nervous systems with antibodies against the appropriate molecules in this group shows that most are expressed during the outgrowth of specific sets of axons (Shiga and Oppenheim, 1991; Harrelson et al., 1988; Snow et al., 1988; Dodd et al., 1988). Some vertebrate molecules appear to be expressed on the same axons simultaneously (L1, neurofascin, F11), while others are expressed sequentially (e.g. TAG-1 and L1), changing when the axons change environments (Shiga and Oppenheim, 1991; Dodd et al., 1988).

Most of these molecules can function as homophilic adhesion molecules in vitro, based in the case of the vertebrate molecules on
antibody perturbation of fasciculation or extension of axons on the surfaces of axons or other cells (Chang et al., 1987; Rathjen et al., 1987a, 1987b). Insect molecules were assayed for adhesion activity by aggregation assays using cultured Drosophila cells expressing each molecule (Harrelson et al., 1988; Snow et al., 1989; Elkins et al., 1990a). L1/NgCAM, TAG-1, neuroglian, and fasciclin II have been demonstrated to mediate homophilic adhesion, as have the Ig family member fasciclin III and the novel protein fasciclin I (Rathjen and Jessell, 1991; Lemmon et al., 1989; Harrelson et al., 1988; Snow et al., 1989; Elkins et al., 1990a). These observations suggest that these molecules could serve as both surface labels and adhesion receptors for demarcating assembling pathways. In both vertebrate and invertebrate nervous systems, there is correlation between expression of certain molecules (TAG-1, fasciclins I, II, III) and elongation in a particular fascicle, indicating that homophilic adhesion could play a role in fascicle formation (Dodd et al., 1988; Harrelson et al., 1988; Snow et al., 1989). Axons in vitro do not show preferential growth or fasciculation on adjacent stripes of laminin, L1, and N-cadherin, however, despite different degrees of adhesion (Lemmon et al., 1992), suggesting that simple adhesive interactions between homophilic or heterophilic ligands are unlikely to produce guided growth. However, the presentation of cell-surface molecules as an immobilized substratum probably does not reflect their function in the membrane of a living cell.

Fasciclin IV appears to function by heterophilic interaction with an unknown ligand (Kolodkin et al., 1992). Heterophilic interactions between L1/NgCAM and axonin-1 (the chick TAG-1 homolog) and between
F11/contactin and restrictin (an ECM molecule with spatially-restricted distribution in the chick CNS; (Norenberg et al., 1992) have also been observed (Sondregger and Rathjen, 1992). While the functional roles of these interactions have not been tested in vivo, the potential for multiple heterophilic interactions among Ig superfamily members increases the possible combinations of surface labels and receptors available for demarcating pathways (Sondregger and Rathjen, 1992).

Indeed, mixtures of immobilized L1 and N-CAM appear to be better substrates for neuronal adhesion and neurite outgrowth than either is alone (Kadmon et al., 1990), and these molecules colocalize on patches of neuroblastoma cell membrane (Thor et al., 1986). Multiple splice forms of several of these Ig family members have been described, creating additional potential diversity by altering cytoplasmic domains, method of surface linkage, or extracellular domain exons (Walsh and Doherty, 1991). TAG-1 and F11 exist in secreted forms (Ruegg et al., 1989). Finally, the discovery that one Ig family member expressed on lymphocytes, ICAM-1, can interact with two distinct integrin types via two different Ig repeats raises the possibility that neuronal Ig family members can also serve as ligands for integrins (Sondregger and Rathjen, 1992). The presence of Arg-Gly-Asp sequences in TAG-1, NgCAM, and neurofascin in positions comparable to the Arg-Gly-Asp that functions in integrin binding to fibronectin further suggests a possible interaction with integrins (Sondregger and Rathjen, 1992). Thus, a variety of cell-cell interactions is made possible by these Ig superfamily members.

Some of these cell-surface molecules have spatially-restricted expression patterns suggestive of a role in regional specification. Axons in a given insect fascicle tend to express the same fasciclin
(Harrelson et al., 1988), and when homologous axons in grasshopper and Drosophila show affinity for different fascicles in the two species, their fasciclin expression correlates with that of the fascicle they join (Grenningloh et al., 1991). Furthermore, when axons leave one bundle to enter another, their fasciclin expression changes accordingly. For example, fasciclin I expression in commissural axons switches to fasciclin II as these axons enter a longitudinal fasciclin-positive bundle (Harrelson et al., 1988). Similarly, commissural axons growing transversely across the floor plate of the rat or chick spinal cord express TAG-1 until they cross the midline, but then TAG-1 expression decreases and is replaced by L1 expression as the axons turn to grow longitudinally (Dodd et al., 1988; Shiga and Oppenheim, 1991). Finally, several molecules are sometimes coexpressed on the same axons (e.g., neurofascin, F11, and N-CAM/G4 on longitudinal chick spinal cord follower axons (Shiga and Oppenheim, 1991). In Drosophila intersegmental nerves, the axons that express fasciclins I and III and neuroglian are more tightly bundled together than other axons that express only fasciclin I and neuroglian (Bieber, 1991). The likely presence of subfascicles within the large bundle of axons in the C. elegans ventral nerve cord (Durbin, 1987) suggests that similar localized expression of adhesion molecules could take place in C. elegans.

Genetic studies in Drosophila and antibody perturbation studies in grasshopper have provided the best opportunity to test the roles of Ig superfamily molecules in mediating directed axonal outgrowth. The most striking result is that null mutations in fasciclins I and III and neuroglian cause relatively minor effects, despite their expression on a
large proportion of the neurons in the developing CNS. Animals lacking each molecule are viable through embryogenesis and have no gross disorganization of their central nervous systems (Elkins et al., 1990b). Neuroglian mutants have subtle cell body positioning defects in the peripheral nervous system (Bieber, 1991), and adult fasI mutants have uncoordinated leg movements (Elkins et al., 1990b), suggesting that small numbers of neurons do require these molecules for normal development.

Antibody perturbation of fasciclin IV and fasciclin I in grasshopper limbs causes greater disruption of axonal outgrowth than does genetic elimination of these molecules in Drosophila. Antibody treatments were performed either by simple application of antibodies to cultured grasshopper limb preparations (in the case of fasciclin IV; (Kolodkin et al., 1992) or by application of chromophore-labeled antibodies that bind fasciclin I followed by laser illumination to heat-inactivate the ligand, a procedure called CALI (chromophore-assisted laser inactivation; (Jay and Keshishian, 1990). Treatment with fasciclin IV antibodies, which label a strip of epithelial cells that serve as a guidance point for the Til axons but not the Til axons themselves, causes these axons to defasciculate and take a variety of abnormal pathways when they encounter the normal fasciclin IV-positive epithelial cells (Kolodkin et al., 1992). CALI inactivation of fasciclin I causes the Til axons, which express fasciclin I, to defasciculate (Jay and Keshishian, 1990). The difference between these clear disruptions of outgrowth in grasshopper and the subtle Drosophila mutant phenotypes for fasI and fasIII could reflect experimental artifacts induced by the antibody or laser treatments. However, it could also suggest either a
difference in fasciclin function in the two species (or a difference between fasciclin function in the central nervous system, studied in *Drosophila*, and in the periphery, studied in grasshopper) or differences in the timing of interference with the molecules. It is possible that other molecules can compensate for those missing in the mutants, while the cells in the grasshopper embryos may not have had sufficient time to up-regulate these other molecules, having already begun growth when they were disrupted. Jacobs and Goodman (1989) speculate that such differences might arise from the anatomical differences between the two species. Compact bundling of axons in the *Drosophila* nerve cords provides growth cones with access to alternative fascicles which are too far apart in the more loosely-assembled grasshopper nerve cords, so elimination of one adhesion molecule does not eliminate all possible pathways in the fly. The degree to which antibody binding and CALI disrupted surface molecules other than the intended targets is difficult to judge, however.

*FasII* null mutants have been reported to have more severe defects, leading the growth cones that normally express the molecule to fasciculate improperly (Goodman and Shatz, 1993; Grenningloh et al., 1991). Antibody perturbation studies of fasciclin II in the grasshopper central nervous system indicated that, like fasciclin I and fasciclin IV, disruption of the function of fasciclin II could lead to defects in axonal extension (Harrelson et al., 1988). In addition, ectopic expression of fasII in *Drosophila* peripheral neurons, which normally do not express it, has been reported to cause abnormal fasciculation between PNS axons and CNS axons that normally express fasciclin II (Lin et al., 1992). Based on these observations, fasciclin II appears likely
to have a role in selective fasciculation as proposed in the labeled pathways hypothesis.

The lack of severe defects in fasI and fasIII mutants suggests that these molecules are not the sole labels for most of the axons that express them. A recent report indicates that fasI;fasIII double mutants show defects in cells that normally express both molecules (Chita et al., 1992). Similarly, double mutant studies showed that animals defective in both fasI and abl, the Drosophila homolog of the Abelson tyrosine kinase proto-oncogene, have severe structural defects in the central nervous system, while double mutants for fasI and fasIII, 1(1)myospheroid, disco, zipper, 1(2)giant larvae, Notch, torpedo, or a c-src deletion show no phenotypic interaction (Elkins et al., 1990b). Animals lacking abl function die as pupae but have normally-formed central nervous systems in the embryo. These results suggest that fasciclin I and fasciclin III (and possibly unknown receptors that act through the abl product) are functionally redundant, although without ultrastructural analysis of fascicles the degree to which these mutant animals are normal cannot be fully ascertained. The relatively normal gross morphology of the nervous system in fasI, fasIII, and abl single mutant embryos in contrast with the severe defects in double mutants suggests that combinatorial mechanisms may exist to fine-tune axon-axon interactions.

The studies of both cell-surface and ECM molecules show that a wide array of molecules can promote axonal outgrowth in vitro, but that few of these provide a unique set of cues for guiding a specific axon. The observation of more severe phenotypes in fasI;abl and fasI;fasIII double mutants than in any single mutant and the observation of a more
severe effect of blocking combinations of cadherin, N-CAM, and integrin function in vitro (Tomaselli et al., 1988; Bixby et al., 1987) all imply that multiple molecules on the same cell surface can share guidance functions. Similarly, elimination of an entire cell or group of cells often does not produce grossly abnormal axonal outgrowth or bundle organization because axons follow cells other than those they normally follow (Chitnis et al., 1991. 1992).

How many molecules are needed to organize a fascicle? The expression of surface molecules in insect nervous systems suggests a relatively large number, perhaps on the order of the number of distinct subfascicles, although combinations of molecules could provide high specificity with a lower number of molecules. The spatially-restricted vertebrate molecules such as TAG-1, however, are expressed on a considerably larger number of axons (Dodd et al., 1988). This may reflect a bias toward abundant molecules inherent in any antibody screen, but the fact that the same molecules have been isolated in multiple independent screens raises the question of whether a large number of molecules with spatially-restricted expression patterns exist in the vertebrate spinal cord. The normal sorting-out of chick spinal axons into small subfascicles as they leave the spinal cord and resort in the plexus (Tosney and Landmesser, 1985) and the ability of axons to sort themselves out after spinal cord reversals (Lance-Jones and Landmesser, 1980) suggests a finer scale of fascicle identity than could be produced by molecules distributed as broadly as TAG-1. As more structural isoforms and heterophilic interactions are identified for individual molecules, however, a higher degree of specificity could be revealed.
The C. elegans nerve cords have relatively small numbers of axons (5-50) and yet are unlikely to be single uniform bundles because the axons within them have highly reproducible positions with respect to their neighbors not consistent with a simple addition of newly-arrived axons to the outside of the bundle (White et al., 1986). Axons of contralateral homologs are often associated in the nerve cords (White et al., 1986; Durbin, 1987). Evidence for the existence of subfascicles of axons within the larger bundles has been derived from observations of ventral nerve cords disrupted by mutation or laser ablation of pioneer axons (Durbin, 1987). While the overall structure of the ventral cord was found to be disrupted in these cases, the local arrangement of several groups of motor axons remained quite constant. The mutants discussed in this thesis affect the formation of bundles and the extension of axons within them, and could shed light on the question of how specific cell-cell interactions are mediated.

Intracellular mechanisms by which extracellular signals regulate axonal outgrowth and fasciculation

It is clear from observations of growth cones in situ that the extracellular environment has a strong influence on growth cone morphology and behavior. Growth cones extending on relatively uniform surfaces in vitro or basement membranes in situ have complex morphologies, with many filopodia covering a wide area, while growth cones extending along axonal surfaces tend to be simpler in shape (e.g., (Bray and Chapman, 1985; Holt, 1959; Durbin, 1987). Growth cones show increased morphological complexity in regions in which they are known to "decide" to change pathways (e.g., Raper et al., 1984; Tosney and
As discussed in the previous section, different environments in developing nervous systems are demarcated by the presence of different cell-surface and extracellular matrix molecules. Recent evidence indicates that axons might use distinct intracellular mechanisms to grow in these different environments. Although the molecular details of these mechanisms are not well understood, and different in vitro assays produce results that are difficult to compare, three themes are emerging. First, axonal outgrowth on cell surfaces appears to use a different mechanism from axonal outgrowth on ECM substrates and immobilized molecules. Second, several cell adhesion molecules in the immunoglobulin superfamily appear to promote axonal outgrowth by the same signalling pathway. Third, the outgrowth-promoting functions of several cell adhesion molecules can be disrupted pharmacologically without affecting their adhesive functions, indicating that the same molecules might serve both as signal transduction molecules in axonal outgrowth and as structural molecules in the formation of fascicles. The environment-specific behavior and directed movements of growth cones might therefore arise from the effects of different signalling mechanisms on the growth cone cytoskeleton.

CAMs and ECM molecules promote axonal outgrowth by different mechanisms

Pharmacological experiments suggest that the cell adhesion molecules N-CAM, L1, and N-cadherin can stimulate axonal elongation by
opening calcium channels via a G-protein-mediated signal (Williams et al., 1992; Schuch et al., 1989). The extension of PC12 neurites on 3T3 cells expressing any of these adhesion molecules (or rat cerebellar neurons on 3T3 cells expressing L1) can be blocked by pertussis toxin, indicating the involvement of heterotrimeric G proteins, or by blockers of L and N-type calcium channels or the intracellular calcium chelator BAPTA-AM. Furthermore, application of the calcium channel agonist Bay K 8644 or depolarization of these neurons with potassium stimulates neurite elongation similar to that mediated by N-cadherin, suggesting that the calcium signal is sufficient to produce neurite elongation (Saffell et al., 1992). The possibility that these drugs act on the 3T3 cells to inhibit neurite outgrowth cannot be ruled out, however. Axons grown on laminin are not affected by these treatments, suggesting that a different mechanism is used for integrin-stimulated elongation (Williams et al., 1992). Interestingly, outgrowth of axons on substrates of immobilized cell adhesion molecules is also insensitive to pertussis toxin and calcium channel blockers, suggesting that the physical association of receptors in the growth cone membrane is an important factor in signalling (cited in Williams et al., 1992). The 180 kDa isoform of N-CAM has reduced lateral mobility in the membrane as compared to the 140 kDa isoform, and the ability of N-CAM-transfected 3T3 cells to support axonal outgrowth is considerably higher when the more mobile isoform is expressed (Doherty et al., 1992b), further implying a role for receptor association in outgrowth stimulation.

Other pharmacological experiments suggest that at least four different signalling pathways can stimulate PC12 outgrowth (Williams et al., 1992): the cell adhesion molecule pathway and integrin-mediated
pathway discussed above, a pathway stimulated by NGF treatment (which
presumably involves the trk tyrosine kinase NGF receptor), and a cholera
toxin-stimulated pathway involving a rise in intracellular cAMP and the
function of protein kinase A. Only the cell adhesion molecule pathway
is affected by pertussis toxin. Saffell et al. (1992) show that all of
these can be blocked by the broad-specificity kinase inhibitor K-252b
and suggest that if the inhibitor acts on a single kinase, it represents
a step common to all of these pathways.

A different set of studies conducted on chick ciliary ganglion
cells grown on immobilized purified substrates has also suggested that
cell adhesion molecules and ECM components promote axonal outgrowth
through different mechanisms, although the relationship of these
mechanisms to those discussed above is not clear. In these studies,
axonal elongation on fibronectin, laminin, or collagen requires protein
kinase C function, while elongation on N-cadherin or L1 appears to be
inhibited initially and later stimulated by protein kinase C (Bixby,

The adhesive and signalling functions of cell adhesion
molecules can be distinguished pharmacologically

In the experiments described above, pharmacological perturbation
of axonal outgrowth did not appear to affect adhesion (Williams et al.,
1992; Bixby and Jhabvala, 1990). This suggests that the cell adhesion
molecules studied have two distinct functions, one in sending signals to
stimulate axon elongation and another in mediating cell-cell adhesion.
This is reminiscent of the dual functions of the T cell receptor complex
and the protein complexes present at focal contact sites in adherent cells, both of which serve both as sites of mechanical attachment and as the source of intracellular signals (Rudd, 1990; Hynes, 1992). In both cases, binding of cell adhesion molecules to their ligands causes the co-clustering of multiple protein species, including tyrosine kinases and, in the case of focal contacts, elements of the cytoskeleton. The concentration of β1 integrins in filopodia of chick dorsal root ganglion growth cones on polyornithine (Letourneau and Shattuck, 1989) suggests that focal contact-like complexes could function in stimulating the growth of these axons on ECM substrates. Similarly, local clustering of L1 with N-CAM or with axonin-1 (Kadmon et al., 1990; Kuhn et al., 1991) and clustering of calcium channels in growth cones have been reported (Silver et al., 1990), suggesting that adhesion and signalling complexes like focal contacts or the T cell receptor complex could function in axons as well.

The adhesive function of cell adhesion molecules, like the axonal outgrowth-promoting function, can be regulated. Alternatively-spliced forms of N-CAM affect adhesion differently, and the shift from the outgrowth-promoting 140 kDa form to the adhesion-promoting, spectrin-associated 180 kDa form occurs when axons in the optic nerve are completing their elongation (Walsh and Doherty, 1991). A common protein kinase C-based mechanism of stimulating the adhesive functions of L1, N-CAM, and P11 has been proposed (Cervello et al., 1991). Retinal axons grown on immobilized L1 or laminin normally fasciculate tightly, but the application of antibodies or Fab fragments against any of these cell adhesion molecules is sufficient to cause complete defasciculation (Chang et al., 1987; Rathjen et al., 1987a, 1987b). Phorbol esters,
which activate protein kinase C, and okadaic acid, a serine/threonine phosphatase inhibitor, promote fasciculation in these cells and antagonize the defasciculating action of the antibodies (Cervello et al., 1991). Because cell adhesion molecule levels are not affected by the drug treatments, and antibodies against the three molecules act in an additive manner to offset the effects of the drugs, the authors propose that the three molecules control adhesion by a common phosphorylation mechanism. Whether drug treatments actually increase the adhesivity of cell adhesion molecules or weaken competing interactions of axons with the immobilized substrate is not clear from these experiments, however.

Comparison and interpretation of pharmacological experiments such as these are complicated by questions of drug specificity, the responsiveness of the cell type under study, and the nature of the assay for axonal outgrowth. Differentiating PC12 cells are different from regenerating cerebellar, retinal, or ciliary ganglion cells, and immobilized purified molecules are probably different from the same molecules on the surfaces of living cells or in isolated membranes. The drugs studied are likely to influence multiple targets (e.g., calcium-sensitive molecules and kinase substrates). Nevertheless, the consistent pattern is that in multiple assays, cell adhesion molecules appear to behave similarly to one another and differently from extracellular matrix components in axonal outgrowth and fasciculation.

If different cell adhesion molecules do employ the same signalling systems, they must do so by means of additional molecules that link them to the common signalling molecules. N-CAM, L1, and N-cadherin do not share a common cytoplasmic sequence, and F11 is an extracellular protein
linked to the membrane by a glycosylphosphatidylinositol (GPI) linkage, so at least one and possibly more adaptor molecules are needed. Such adaptors are analogous to, but probably distinct from, the SH2/SH3 domain-containing adaptors that link growth factor receptors to a variety of intracellular signalling processes (Pawson and Gish, 1992). The cytoplasmic tails of cadherins are known to associate with three molecules, α, β, and γ-catenin, which are believed to mediate interactions with the actin cytoskeleton (Hirano et al., 1992). No signalling role has yet been assigned to catenins, however.

**Structural proteins in axonal elongation and stabilization**

What are the effector molecules that convert outgrowth-promoting signals into directed growth cone movement? Growth cones resemble other motile cells in that they have a dynamic actin cytoskeleton at their leading edge, and experiments with actin-depolymerizing drugs *in vitro* and *in vivo* have shown that an intact actin cytoskeleton is essential for directed outgrowth but not for elongation *per se* (Marsh and Letourneau, 1984; Bentley and Toroian-Raymond, 1986). Single filopodial contacts can cause growth cones to change direction (Hammarback et al., 1986; O'Connor et al., 1990) or retract (Bandtlow et al., 1990), and these changes are probably mediated by a variety of growth cone proteins capable of crosslinking actin (e.g., filamin and α-actinin) or producing force in association with actin (e.g., type I myosin) (Letourneau and Shattuck, 1989; Sobue and Kanda, 1989; Cypher et al., 1991).
Selection of a new direction by actin-based structures is closely followed by a rearrangement of growth cone microtubules. Observations of fluorescent tubulin movements in living grasshopper limb pioneer growth cones in situ or growth cones of cultured Xenopus neural tube explants suggest that turning growth cones selectively assemble or stabilize microtubules in growth cone branches oriented in the direction of growth (Sabry et al., 1991; Tanaka and Kirschner, 1991). Finally, axonal elongation is accompanied by forward movement of growth cone cytoplasm, bundling of microtubules into parallel arrays, and subsequent condensation of the plasma membrane around the microtubules (Sabry et al., 1991; Tanaka and Kirschner, 1991). Any of these cytoskeletal reorganizations is a potential site for regulation of axonal outgrowth.

In addition, cytoskeletal structures in the newly-assembled axon shaft are also important for formation of a stable axon. Microtubule-associated proteins (MAPs) such as MAP2 and tau are found in neurites but not in growth cones and are thus likely to function in microtubule bundling and stabilization (Letourneau and Shattuck, 1989). Elimination of either MAP2 or tau by antisense oligonucleotides prevents neurite outgrowth from cerebellar cells in culture, suggesting an essential role in outgrowth (Caceres et al., 1992; Caceres and Kosik, 1990). MAP2 appears to be required for initial phases of outgrowth of minor neurites from these cells, while tau is required for later extension of one of these as the major axon. Intermediate filaments are highly stable structures in vitro that are assembled in axons during and after outgrowth. Recent evidence has suggested that they are capable of more dynamic behavior than originally believed (reviewed in Nixon and Shea, 1992), and a phosphorylated form of neurofilament NF-M has been
localized to a region of chick spinal axons at points at which they are believed to make specific pathway decisions (Landmesser and Swain, 1992). This phosphorylation correlates well with the onset of decision-making, unlike modifications to MAP-1B (Sato-Yoshitake et al., 1989), tau (Papasozomenos et al., 1987), and other epitopes on neurofilaments NF-M and NF-H (Carden et al., 1985), which are generally associated with distal regions of all axons. Interestingly, treatment with the protein kinase C-activating drug PMA, the calcium ionophore A23187, and cGMP can induce region-specific expression of the phosphorylated NF-M epitope in vitro, while cAMP reduces its expression (Landmesser and Swain, 1992). The role of intermediate filaments in axonal outgrowth is not clear. Antisense oligonucleotide-mediated depletion of most peripherin, an intermediate filament present in growing axons, does not impair extension of PC12 neurites in vitro (Troy et al., 1992). However, the differential modification of a number of components of the axonal cytoskeleton, some in apparent response to axonal guidance cues, suggests that these proteins could influence axonal structure during outgrowth.

Finally, insertion of new membrane into growing axons is a potential point of regulation. The Drosophila gene shibire, which encodes the microtubule-associated motor protein dynamin, is required for membrane insertion into axons and subsequent axonal elongation (van der Bliek and Meyerowitz, 1991). Mutations in the C. elegans genes unc-14 and unc-51 (the latter of which encodes a serine/threonine kinase; Y. Ohshima and H. Tobler, personal communication) cause axonal extension defects accumulation of membrane vesicles similar to those observed in Drosophila shibire mutants (McIntire et al., 1992).
The connections between second messengers implicated in axonal elongation and bundling and the many proteins involved in axonal assembly and movement have not been clearly established. Manipulation of calcium concentration in cultured cells can have profound influences on growth cone morphology and behavior. Outgrowth of identified Helisoma neurons in culture is blocked if the intracellular calcium concentration is increased by exposure of the cell to serotonin, electrical stimulation, or calcium ionophores (Kater and Mills, 1991). Measurement of calcium in locomoting growth cones has suggested that there is an optimal range of calcium concentrations for axonal growth, and calcium levels above or below this inhibit growth, while subtle changes within this range alter growth cone morphology and behavior (Kater and Mills, 1991). Rapid changes in calcium concentration by relatively small amounts (30-50 nM), rather than absolute concentration, have been suggested as the major factor in determining the effect of calcium on growth cone behavior (Rehder and Kater, 1992). Clustering of calcium channels in regions of the growth cone periphery has been observed, suggesting that localized calcium "hotspots" could lead to local remodeling of the cytoskeleton (Silver et al., 1990).

Many growth cone molecules are potential targets of calcium-regulated signals, perhaps accounting for the diversity of responses to calcium manipulations in different experimental systems. Actin filaments and microtubules both appear to depolymerize when growth cone advance is halted by ionophore-mediated calcium influx (Lankford et al., 1989). Tropomyosin, which in muscles binds actin and prevents interaction with myosin unless released by calcium, is found in filopodia of cultured chick dorsal root ganglion growth cones and could
serve a similar regulatory role (Letourneau and Shattuck, 1989). A
calcium-sensitive form of the actin-bundling protein α-actinin is also
found at the leading edges of differentiated PC12 cells (Sobue and
Kanda, 1988), providing a direct mechanism by which calcium could affect
the state of actin organization.

The substrates of protein kinase C (PKC) important in axonal
extension are not known. The microtubule-associated proteins MAP2 and
tau and the abundant growth cone protein GAP-43 are all PKC substrates
(Bixby and Harris, 1991). The abundant growth cone protein GAP-43
appears able to stimulate the G protein G_0 (Strittmater et al., 1990)
and has been implicated in calmodulin binding and sequestration of the
actin-binding protein profilin (Forscher, 1989). Which of these roles
is important for axonal outgrowth is unclear, although the apparent role
of G proteins in cell adhesion-mediated axonal outgrowth suggests a
possible connection (Williams et al., 1992; Saffell et al., 1992). Recent experiments suggest that the phosphorylated form of GAP-43 can
induce extensive cell spreading in nonneuronal cells, while a
nonphosphorylatable mutant form inhibits spreading and promotes
formation of filopodia (Widmer and Caroni, 1993). The *C. elegans* unc-51
gene product, a putative serine/threonine kinase, is required for normal
axonal extension, probably by regulation of membrane assembly (Y.
Ohshima and H. Tobler, personal communication).

A number of tyrosine kinases are present in growth cones and have
been implicated in axonal outgrowth. Neurite extension by PC12 cells
can be stimulated by the injection of *src*, which is perhaps a reflection
of the effects of the stimulation of PC12 neurite outgrowth by nerve
growth factor, the receptor for which is the receptor tyrosine kinase
trk (Hempstead, et al., 1991). Locally-applied NGF rapidly increases the number of filopodia at the site of application to cultured growth cones, and this can perhaps be attributed to trk kinase activity (Gundersen and Barret, 1980). The Drosophila trk homolog, Dtrk, contains extracellular immunoglobulin repeats, can act as a cell adhesion molecule in vitro, and is found in developing axons, suggesting a direct link between adhesion and signalling (Pulido et al., 1992). Furthermore, the severe defects in axonal outgrowth seen in Drosophila mutants lacking both fasciclin I and the tyrosine kinase abl suggest a central role for this tyrosine kinase in CNS assembly (Elkins et al., 1990b). Two protein tyrosine phosphatases (PTPases) expressed transiently in growing axons have been described in Drosophila, and both have fibronectin type III repeats in their extracellular domains, suggesting that they could serve as coreceptors for cell adhesion molecules (Yang et al., 1991).

The precise mechanisms by which tyrosine phosphorylation regulates growth cone behavior and axonal outgrowth, and whether these effects are on the direction or simply the extent of outgrowth, are unclear. The primary targets of tyrosine kinases in the growth cone are the actin-binding protein vinculin and membrane-associated tubulin subunits (Bixby and Harris, 1991). src-mediated tubulin phosphorylation is inhibited by antibodies to the cell-surface receptors L1 and N-CAM (Atashi et al., 1992), and because phosphorylated tubulin cannot assemble into microtubules in vitro (Bixby and Harris, 1991) fasciculation via these surface molecules could promote microtubule assembly.

Relatively few experiments address the roles of specific second messengers in directing axonal outgrowth along specific pathways.
One recent report of studies in cultured grasshopper embryos showed that manipulation of calcium levels, which altered rates of outgrowth and axon diameter, did not cause pathfinding errors, while application of 8-bromo-cGMP, which raises intracellular cGMP levels, did cause pathfinding errors while leaving axon outgrowth rates normal (Lankford, 1992). The target of cGMP in these axons is not known.

*C. elegans* genetic studies have provided a provocative link between signalling mechanisms and directed axon outgrowth. Mutations in the gene unc-73 cause pathfinding errors in fascicles and along hypodermal cells (McIntire et al., 1992). This gene has recently been reported to show significant similarity to the *Saccharomyces cerevisiae* gene CDC24, believed to be a guanine nucleotide exchange factor for the CDC42 gene product, a small ras-like GTPase of the rho family (J. Culotti, personal communication). These GTPases are associated with organized actin structures in motile cells. Antibody injection studies have shown that rac is required for leading-edge ruffling in fibroblasts and rho regulates stress fiber assembly (Ridley and Hall, 1992; Ridley et al., 1992). While the effect of rho on the directed movements of motile cells is not known, the yeast CDC24 protein is required for localized actin deposition in budding (Adams and Pringle, 1984). Mutants lacking CDC42 or CDC24, the unc-73 homolog, fail to form buds because they do not assemble the localized actin structures that precede formation of a bud site (Johnson and Pringle, 1990). The analogy to the localized rearrangements of actin following filopodial or lamellipodial contact with a favorable substrate is appealing, although no observations of growth cone morphology have been made in unc-73 mutants.
A common theme to the studies of both intracellular and extracellular molecules involved in axonal outgrowth is that many molecules appear to have overlapping functions. Different cell adhesion molecules are often coexpressed on the same axon (e.g., fasciclin I and III, or β1 integrins and N-cadherin; Patel et al., 1987; Tomaselli et al., 1988), and in many cases, if one is disrupted by experimental manipulation, the others can still support some degree of axonal outgrowth. In most cases, the measurements of axonal outgrowth are relatively crude—measurements of total neurite length in vitro or light microscopic observation of insect central nervous system structure—and so the roles of these molecules in the precise growth cone-axon interactions likely to underlie axonal growth along specific pathways remain unclear. Electron microscopic analysis of individual growth cones in developing animals (e.g., Bastiani et al., 1983) is needed to assess the roles of specific molecules in generating nervous system fine structure.

On a gross scale, however, multiple molecules often appear to provide overlapping functions in axonal outgrowth. Perhaps this reflects a mechanism for allowing subtle distinctions to be made among cells of similar structure. The presence of redundant systems also could serve to minimize developmental errors. However, the complexity of cellular architecture that allows closely-spaced regions of a highly motile growth cone to behave quite differently also probably leads to the presence of multiple mechanisms for providing axons with adhesiveness, locomotory ability, and mechanical strength. Under certain conditions in vitro, major axonal cytoskeletal components (actin and intermediate filaments) can be eliminated without preventing axonal
extension. While these observations do not imply that neurons in vivo do not use all of their cell-surface and cytoskeletal proteins in normal growth, it serves as a reminder that multiple mechanisms are at work to produce a fully-formed axon, and that the genetic studies discussed below are likely to explore the functions of individual molecules that are not shared with other molecules.

Genetic approaches to the understanding of axon elongation and guidance

The biochemical approach to the identification of molecules involved in axonal outgrowth and guidance has produced a large array of molecules capable of supporting axonal outgrowth in vitro, and strong evidence for a function in vivo for some of these molecules has been obtained. Often, however, the axonal outgrowth processes for which a given molecule is used are difficult to determine. In addition, most of these studies have focused on extracellular molecules, and the connections between these molecules and the cytoskeletal processes needed for directed axonal outgrowth and fasciculation remain uncertain. The genetic approach, because it requires only that alteration of single genes will lead to observable defects in axonal morphology, provides access to intracellular events as well as those occurring at the cell surface.

While the genetic approach does not presuppose specific biochemical properties or subcellular localization of the molecules under study, it does have its own inherent biases. Molecules with functions in cellular processes in addition to axonal outgrowth would
probably not be identified in screens for mutants with defective axonal morphology because the effect of loss of gene function would not be limited to nervous system development. Conversely, if multiple molecules can perform the same function (as is evidently the case with fasciclin I and fasciclin III in Drosophila; Elkins et al., 1990b; Chiba et al., 1992), elimination of one molecule with this function would probably not cause an observable defect, and so such molecules would be difficult to detect by mutation. Nevertheless, the genetic approach provides access to processes that might otherwise be difficult to dissect.

Genetic screens for mutants defective in the outgrowth of specific axons, conducted in Drosophila, C. elegans, or, to a limited extent, in zebrafish, generally take one of two forms. If a specific set of axons can be labeled, a direct search for mutants with defects in the morphology of these axons can be undertaken. As this often involves antibody staining, this can be a laborious procedure and limits the numbers of mutagenized animals that can be analyzed. More typical screens involve searches for mutants with behavioral defects that are predicted to result from abnormal outgrowth of specific axons.

Mutations obtained by direct screening for defective axonal outgrowth

Several small-scale direct screens for mutants with abnormal axonal morphology have been undertaken in C. elegans. These techniques have identified mutants in several genes already known on the basis of other behavioral mutant phenotypes, and mutations in several new genes found in these screens cause behavioral defects that would have
permitted isolation in behavioral screens. However, several mutants had no readily-detectable behavioral defects, suggesting that such direct screens can provide access to genes not otherwise available.

The endings of several sensory neurons in the head and tail of *C. elegans* are exposed to the environment and take up the dyes FITC, diO, or diI (Hedgecock et al., 1985 and personal communication). Living animals can be stained with these dyes and their axonal morphology determined by fluorescence microscopy. Several mutations affecting the outgrowth of the PHA and PHB sensory axons were obtained by such an observation of F2 progeny of mutagenized animals. One of these was an allele of the unc-6 gene, which encodes a laminin-related protein, as discussed above. Two additional genes, unc-107 and *enu-1* (*enhancer of unc-107*), were also obtained from the same screen (J. Culotti, personal communication). The elongation of the PHA and PHB axons in the ventral nerve cord is blocked in unc-107 and *enu-1;unc-107* mutants in a way reminiscent of the defects observed in other neuronal bundle-specific mutants (see below). While the *enu-1* mutation increases the severity of *unc-107* defects, it does not confer apparent defects in locomotion or phasmid axon outgrowth alone, and so would not have been found by screens for such mutations. Mutations in *enu-1* cause defects in HSN axon outgrowth but do not impair HSN function, however (see chapter 2).

Hedgecock and co-workers identified a monoclonal antibody that labels axons that pioneer the *C. elegans* ventral nerve cord, the PVP axons (C. Norris, H. Bhatt, and E. Hedgecock, personal communication). Direct screens for mutants altered in PVP staining patterns produced at least one mutant with no behavioral abnormalities and PVP cell bodies that appear normal except for the absence of antibody staining.
Additional uncoordinated mutants not previously identified also showed abnormalities in staining patterns. These genes have not been characterized molecularly.

Behavioral screens for mutants with defective axonal outgrowth

The identification of mutants with behavioral defects often eliminates the need for labor-intensive staining procedures and detailed microscopic studies in the initial round of screening. If the neurons involved in that behavior are known and can be visualized, a collection of behavioral mutants is a likely source of axonal outgrowth mutants. In *C. elegans*, behavioral screens are facilitated by the fact that hermaphrodites can self-fertilize, allowing even paralyzed mutants to reproduce. Laser ablation studies have revealed only three neurons necessary for viability in *C. elegans* (the two CAN cells, implicated in osmoregulation, and the M4 cell, needed for coordinated feeding), suggesting that mutants with severe structural defects in the nervous system could be viable (Avery and Horvitz, 1987; J. Sulston, personal communication).

Redundancy on any of several levels can be problematic for the isolation of defects in small numbers of neurons. For example, *C. elegans* animals in which the ventral cord pioneer AVG is ablated have disorganized nerve cords but are remarkably normal in locomotion (Durbin, 1987), and a mutant with this defect would probably be difficult to distinguish from wild-type in a behavioral screen. The subtle abnormalities infasI, abl, fasIII, and nrg mutants in Drosophila
suggest that these mutants, too, might have been overlooked in a
behavioral screen (Elkins et al., 1990b; Bieber, 1991).

Screens have been conducted in C. elegans based on the defects
observed when small numbers of neurons are killed with a laser
microbeam. Clear behavioral alterations are observed in worms lacking
the two HSN motorneurons (egg-laying defects; Trent et al., 1983; Desai
et al., 1988; Desai and Horvitz, 1989), the six neurons with unusual 15-
protofilament microtubules (defects in mechanosensation; Chalfie and
Sulston, 1981), the six DD and 13 VD motorneurons (a characteristic
"shrinking" locomotion; S. McIntire, E. Jorgensen, and H.R. Horvitz, in
preparation), or the motorneurons AVL and DVB (defects in defecation; S.
McIntire, E. Jorgensen, and H.R. Horvitz, in preparation). Genetic
screens have been conducted for mutants causing each of these defects.
In addition, the functions of a number of C. elegans sensory neurons
have been determined by laser ablation studies (Bargmann and Horvitz,
1991a,b; J. Thomas, J. Kaplan, and H.R. Horvitz, personal
communication). While the neural circuits for responses to
chemoattractants, high-osmolarity solutions, touch to the tip of the
nose, and chemical stimuli leading to the formation of an alternative
larval stage (dauer) are not well understood, mutations affecting each
of these behaviors have been identified. Some of these chemosensory
mutations cause defects in the uptake of dyes by sensory cells in the
nose, and electron microscopic analysis shows ultrastructural defects in
sensory structures.

A striking result of these screens is that among the mutants
characterized at the level of axonal morphology, few cause defects
primarily in axonal outgrowth. Screens for mechanosensory-defective
mutants did not produce any mutants with shortened or misdirected mechanosensory neurons (Chalfie and Sulston, 1981), suggesting either that any such mutations also produced defects in locomotion or viability, preventing an assay of their mechanosensory response, or that abnormally-positioned neurons still provided wild-type mechanosensation. Likewise, none of the "shrinker" mutations, indicative motorneuron defects specific to the VD and DD cells, causes defects in VD and DD axon outgrowth (S. McIntire, E. Jorgensen, and H.R. Horvitz, in preparation; McIntire et al., 1992). Both of these results suggest that unique molecules do not direct the outgrowth of these specific classes of neurons, but rather that multiple classes of neurons probably utilize the same molecules. The axons of many mutants obtained from screens focused on single neuronal classes have not been examined, and so the degree to which this is a general limitation of behavioral screens is not certain. The chemotaxis-defective mutant tax-2 shows abnormal outgrowth of multiple sensory neurons but no locomotory defects (C. Bargmann, personal communication). Behavioral assays that can be performed on uncoordinated mutants, such as analyses of egg laying or defection patterns, should permit isolation of genes affecting multiple neuronal classes in addition to the specific class under study.

C. elegans mutants affecting axon outgrowth in specific environments

The use of behavioral mutants to study axonal outgrowth in C. elegans has depended on the availability of reagents that allowed the visualization of specific neurons without interference from neighboring
cells. Three types of reagents have been particularly useful: vital dyes that label sensory neurons exposed to the environment (FITC, diO, diI; Hedgecock et al., 1985 and personal communication), antibodies against proteins expressed in small numbers of neurons (e.g., acetylated tubulin; Siddiqui et al., 1989), and antibodies against neurotransmitters produced by small numbers of neurons (e.g., serotonin, GABA, and FMRFamide; Desai et al., 1988; McIntire et al., 1992; Schinkmann and Li, 1992). While these reagents do not label axons that have not completed outgrowth, they do permit detailed examination of the morphologies of fully-formed axons. They have been used to survey the collection of over 100 unc (uncoordinated) mutants initially isolated by Sydney Brenner (Brenner, 1974) and supplemented by other workers in the C. elegans community (Hedgecock et al., 1985; Siddiqui, 1990; Siddiqui and Culotti, 1991; McIntire et al., 1992).

Three general classes of mutants have emerged from these surveys. One class affects outgrowth of axons circumferentially around the body wall in the dorso-ventral axons, but not outgrowth of axons in bundles. A second class shows the opposite effects, causing abnormal growth in fascicles but normal growth around the body wall. The third class of mutations affects outgrowth of axons in both environments and may define genes necessary for axonal elongation in general. The screen for unc mutants has thus yielded molecules potentially involved in axonal structure and in the information pathway that regulates pathfinding.

Mutations in three genes were found to affect outgrowth around the body wall. Neither dorsally-directed nor ventrally-directed axonal growth occur normally in unc-6 mutants, while only ventrally-directed growth was blocked in unc-40 mutants and dorsally-directed growth is
blocked in *unc-5* mutants (Hedgecock *et al.*, 1990; McIntire *et al.*, 1992). Migration of mesodermal cells such as the distal tip cells of the gonad is similarly affected in these mutants. Because the mesodermal cells migrate on the opposite side of a basement membrane from neurons, these genes are believed to affect the presentation and interpretation of guidance cues contained in the basement membrane. As discussed above, sequence analysis has shown that the *unc-6* gene encodes a protein related to the ECM molecule laminin (Ishii *et al.*, 1992), while the *unc-5* gene encodes a putative transmembrane receptor for the *Unc-6* protein (Leung-Hagesteijn *et al.*, 1992). Specific *unc-6* mutant alleles cause only dorsal-directed growth defects, similar to those caused by *unc-5* mutations, further supporting the view that *unc-5* encodes an *Unc-6* receptor used in dorsal-directed guidance (Hedgecock *et al.*, 1990). The *unc-40* gene has not yet been molecularly characterized. Although the distribution of *Unc-6* protein is unknown, models for its function suggest that a gradient of *Unc-6* exists along the dorso-ventral axis. Receptors (*Unc-5* and a putative "ventral-directed" receptor, possibly the *Unc-40* protein) are proposed to be capable of reading this gradient to produce opposite effects.

Mutations in several genes cause defects in axonal outgrowth in both the neuronal bundles and along the body wall (Hedgecock *et al.*, 1985; McIntire *et al.*, 1992). A combination of ultrastructural and sequence analysis has suggested that these genes are likely to affect intracellular processes in axonal assembly or outgrowth. The *unc-73* gene, as discussed above, encodes a molecule implicated in the regulation of actin cytoskeleton dynamics (R. Steven, A. Ruiz, J. Mancillas, and J. Culotti, personal communication). Mutations in this
gene cause misrouting of axons and some shortening, as well as migration
defects of the HSN cell bodies (Siddiqui, 1990; Siddiqui and Culotti,
1991; McIntire et al., 1992)

Mutations in two genes, unc-14 and unc-51, cause severely
shortened axons, and electron microscopic examination has shown that
axons contain abnormal large membranous vesicles (McIntire et al.,
1992). A Drosophila gene with a similar mutant phenotype, shibire, has
been implicated in the regulation of addition of membranes to the
growing axon, and sequence analysis has shown that it encodes a homolog
of the microtubule motor protein dynamin (van der Bliek and Meyerowitz,
1991). The unc-51 gene has been reported to encode a serine/threonine
kinase (Y. Ohshima and H. Tobler, personal communication), but its
precise function in axonal assembly is not known.

Mutations in two genes, unc-33 and unc-44, cause abnormal
shortening of microtubule-containing sensory cilia. Sensory cilia in
unc-33 mutants contain higher numbers of microtubules than normal, and
some of these have abnormal hook, doublet, or triplet structures,
suggesting a role for this gene in microtubule organization (Hedgecock
et al., 1985). The Unc-33 protein, which lacks sequence similarities to
other known proteins, is present along the length of growing and mature
axons (Li et al., 1992; W. Li and J. Shaw, personal communication).
The product of the unc-44 gene is a protein with strong similarity to
ankyrin, a mammalian protein involved in linking the actin-binding
protein spectrin to the plasma membrane (Otsuka et al., submitted).
While the localization of the Unc-44 protein is unknown, a functional
link between the Unc-44 and Unc-33 proteins has been suggested by the
observation that Unc-33 protein is not transported from neuronal cell
bodies to axons in unc-44 mutants (W. Li and J. Shaw, personal communication).

The genes under consideration in this thesis are required for formation of axonal bundles in C. elegans. Animals carrying mutations in unc-34, unc-71, or unc-76 have normal axonal extension along the dorso-ventral axis. However, a number of axons fail to elongate normally in the ventral nerve cord (McIntire et al., 1992; Hedgecock et al., 1985; Garriga personal communication). In particular, the HSN motorneurons, which in adults can be visualized by staining with anti-serotonin antibodies, appear shorter than normal, stopping at variable positions along their normal trajectory in the ventral cord (McIntire et al., 1992). The number of longitudinal axonal profiles visible in electron micrographs is lower than normal in these mutants, suggesting that large numbers of axons fail to reach their normal length (McIntire et al., 1992). A second class of defects common to this group of mutants is that axons that normally fasciculate fail to do so. Observations of amphid sensory axons stained with FITC and of VD and DD motor axons stained with anti-GABA antibodies reveals defasciculation of two specific groups of axons that normally run as tight bundles (Hedgecock et al., 1985; McIntire et al., 1992). Electron microscopy shows that the ventral and dorsal cords are defasciculated on a larger scale, in that these bundles are broken up into several smaller bundles (McIntire et al., 1992).

In principle, the defects in these mutants could arise from either problems in cellular structure, such as abnormalities in cytoskeletal or membrane assembly, or problems specifically in pathfinding. Evidence from McIntire et al. (1992) suggests that interactions of specific axons
with other cells in the fascicle, and not intrinsic defects in axonal elongation, are responsible for the observed defects in *unc-34*, *unc-71*, and *unc-76* mutants: the HSN axons in these mutants are capable of extending to the wild-type length if caused by a second mutation (*unc-40*) to grow on lateral axons that are not part of the ventral cord.

Possible functions for bundle-specific axonal outgrowth genes include mediation of cell-surface interactions of axons within the fascicles or of axons with surrounding hypodermal tissue or basement membranes. Alternatively, these genes could function in an intracellular response to extracellular cues present in the bundle, possibly related to the differences in growth cone structure that have been observed between growth cones growing on axons and on ECM substrates in *C. elegans* (Durbin, 1987) and elsewhere (Bovolenta and Dodd, 1990; Holt, 1989). Evidence suggesting that a number of vertebrate cell adhesion molecules use a common signalling pathway to stimulate outgrowth along cell surfaces, while different mechanisms are used to elongate along ECM components (Williams et al., 1992; Bixby and Jhabvala, 1990) further indicates that mutants with widespread defects in fascicle formation could have roles in signalling.

This thesis first presents a discussion of the genetic analysis of the two genes with the strongest mutant phenotypes, *unc-34* and *unc-76*, and a determination of their null phenotypes. Interactions of these genes with each other, with other genes with bundle-specific mutant defects, and with potential suppressor genes are discussed. A molecular analysis of *unc-76* is presented in Chapter 3, and preliminary work toward the cloning of *unc-34* is presented in the Appendix. In Chapter 4, experiments to produce new labels for the study of growing axcns in
C. elegans are discussed, with the goal of generating tools for achieving a better understanding of how the products of unc-34, unc-76, and other genes function during axonal outgrowth. Chapter 4 also contains the report of the development of a technique for C. elegans primary neuronal culture, an effort to facilitate the study of single molecules by allowing experiments in defined conditions comparable to those possible in vertebrate cells. The implications of the results described in Chapters 2-4, a model for unc-34 and unc-76 function, and future experiments to explore these functions, are discussed in Chapter 5.

References


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Chapter 2. Genetic Analysis of unc-34 and unc-76
Summary

Mutations in the C. elegans genes unc-34 and unc-76 cause defects in two aspects of axonal outgrowth, elongation in contact with other axons and formation or maintenance of axon bundles. Isolation of additional mutant alleles and analysis of these mutations placed in trans to deficiency chromosomes indicated that these axonal outgrowth defects result from complete loss of function of either gene. Double unc-34 unc-76 mutants showed more severe defects than either single mutant, suggesting that the two genes provide independent functions in axonal outgrowth. Null mutants in the unc-34 gene showed temperature-sensitive defects in axonal outgrowth, suggesting that an axonal outgrowth process occurring in the absence of unc-34 function is itself temperature-sensitive. Attempts to identify genes involved in this process gave rise to several mutations that act as extragenic suppressors of unc-34 without causing evident nervous system defects on their own. Suppressor analysis of unc-76 produced informational suppressors but no nervous-system specific mutations; however, a mutation previously shown to be capable of suppressing the unc-76 defects in one group of neurons was found to suppress additional unc-76 defects.
Introduction

The formation of neuronal bundles is an important process in the assembly of nervous systems. Evidence from vertebrate and invertebrate species suggests that selective association of specific axons in fascicles is an active process of cell recognition and attachment. Growth cones in developing grasshopper and fish nervous systems show precise association with specific axons and fail to extend if these axons are removed, despite the presence of up to 100 neighboring axons within filopodial reach (Raper et al., 1984; Bastiani and Goodman, 1986; du Lac et al., 1986).

What molecular events lead to this specific fasciculation? A large number of cell-surface glycoproteins in insects and vertebrates, mostly but not exclusively members of the immunoglobulin superfamily, have been shown to mediate fasciculation in vitro, either by homophilic or heterophilic association. Some of these, such as the vertebrate TAG-1/axonin-1 protein (Dodd et al., 1988; Ruegg et al., 1989) and the insect proteins fasciclin I-IV (Harrelson et al., 1988; Patel et al., 1987; Snow et al., 1988; Kolodkin et al., 1992) are expressed on subsets of axons in developing nervous systems, suggesting that they play a role in organizing fascicles. Analysis of Drosophila mutants lacking fasciclin I, II or III and intact grasshopper embryos treated with antibodies against fasciclin I, II, and IV has shown that these molecules are required to different degrees for the outgrowth of specific axons in fascicles and that several play redundant roles (Raper et al., 1983; Goodman and Shatz, 1993; Grenningloh et al., 1991; Elkins

These studies have focused on the roles of cell-surface proteins in fascicle formation. Relatively little is known about the intracellular events that lead to specific association of axons. The nematode Caenorhabditis elegans is a convenient organism in which to approach this question for a number of reasons. Its nervous system is small, consisting of 302 neurons in adult hermaphrodites, and mutants with severe defects in nervous system development or function can be easily isolated and propagated. Reconstruction of the entire nervous system from serial section electron micrographs has indicated that the arrangement of axons within the three major longitudinal fascicles and the circumpharyngeal nerve ring is generally highly reproducible from animal to animal (White et al., 1986; Hall and Russell, 1991). That these fascicles are assembled by the active association of ingrowing axons with previously-existing axons is suggested by the observation that late-developing axons can extend along pathways deep within a fascicle of 50 axons (White et al., 1983).

Mutations in over 100 genes can lead to uncoordinated locomotion (Brenner, 1974; Babu and Brenner, 1981; Wood, 1988). Analysis of mutant animals with stains that allow the visualization of specific subsets of axons has led to the identification of at least 35 genes with abnormal axonal morphology (Wood, 1988; Hedgecock et al., 1985; Desai et al., 1988; McIntire et al., 1992; Siddiqui, 1990; Siddiqui and Culotti, 1991; Hekimi, 1990; C. Norris, H. Bhatt, and E. Hedgecock, personal communication). These mutants can be divided into three groups, based on the body region in which axonal defects are observed. Mutations in
the circumferential outgrowth genes, *unc-5*, *unc-6*, and *unc-40*, cause
defective axonal trajectories along the dorsoventral axis, where axons
extend in contact with the body wall epithelium, known as the
hypodermis, and a basement membrane (Hedgecock et al., 1990; McIntire et
al., 1992). Mutations in genes required for longitudinal extension,
*unc-34*, *unc-69*, *unc-71*, and *unc-76*, cause variable defects in axon
morphology in longitudinal bundles but permit normal dorsoventral
outgrowth (McIntire et al., 1992; G. Garriga, personal communication).
Mutations in a third group of genes, *unc-14*, *unc-33*, *unc-44*, *unc-51*, and
*unc-73*, cause defects in axonal trajectory in both of these regions and
are therefore believed to be involved in general processes of axonal
elongation.

The genes required for longitudinal axonal extension are of
particular interest because mutants in these genes consistently display
two classes of axonal defects suggestive of defective axon-axon
interactions. First, many axons in the longitudinal bundles of adult
*unc-34*, *unc-69*, *unc-71*, and *unc-76* mutants are not full-length, as
determined by immunocytochemical analysis of specific neurons and an
electron microscopic survey of all axons reaching a specific point in
the mid-body region (McIntire et al., 1992) Second, axons that are
normally bundled together within the dorsal and ventral cord fascicles
and the nerve ring fail to remain tightly associated in these mutants,
and the dorsal and ventral cords themselves are frequently split into
several smaller fascicles (McIntire et al., 1992). Studies of
longitudinal extension have focused primarily on the morphology of a
pair of serotonergic motorneurons, the HSNs, which can be visualized
with an anti-serotonin antibody. In wild-type worms, the HSN
motorneurons extend processes from their cell bodies, located in a lateral position just posterior to the vulva, ventrally into the ventral nerve cord in the second larval stage (L2), and during the L4 stage, the processes extend anteriorly in the ventral nerve cord until they enter the nerve ring. In unc-34, unc-69, unc-71, and unc-76 mutant worms, the HSN axons are found in the ventral nerve cord, but they terminate before they reach the head. Labeling of exposed sensory neurons by soaking animals in the lipophilic dyes FITC or diO reveals similar defects in the PHA and PHB axons in the tail (Hedgcock et al., 1985). In wild-type worms, these axons extend ventrally from lateral cell bodies and then grow anteriorly for a short distance in the ventral nerve cord during embryogenesis, and in these mutants, the axons reach the ventral cord but are reduced in length. Fasciculation can be measured by immunostaining of the VD and DD motorneurons with antisera against the neurotransmitter GABA (McIntire et al., 1992). These axons are normally bundled tightly in the ventral nerve cord but are often spread apart in unc-34, unc-71, and unc-76 mutants. None of these axons can be visualized until they have completed outgrowth, and so the precise events leading to the aberrant axonal morphologies have not been determined. Nevertheless, the localization of observed defects to regions of axon-axon contact suggests that these genes are involved in either fascicle formation or maintenance. The two genes with the strongest mutant phenotypes, unc-34 and unc-76, were chosen for further analysis.

Two general types of defects could be responsible for the abnormal neuronal morphologies observed in the longitudinal extension mutants. First, structural abnormalities could prevent normal axonal extension.
Abnormalities in the cytoskeleton or membrane of the growing axon, such as those observed in unc-33, unc-14, and unc-51, could prevent the assembly of full-length axons, or they could slow axon growth such that the axon fails to complete outgrowth before its pathway is no longer permissive for elongation. Loose attachment of axons to their substrates could cause the axons to be pulled loose as the animal grows or as other axons enter the region. Structural abnormalities in cells near the growing axons could also account for the observed defects: misplaced cells could provide a physical barrier that prevents HSN axonal extension or growth of VD axons onto their proper DD targets.

The second kind of defect that could account for the failure of HSN extension in the ventral nerve cord in unc-34 and unc-76 mutant worms is a defect in information. The absence of specific guidance cues or the molecules needed to respond to these cues could cause aberrant growth, as has been observed when axons or molecules used for guidance in other species are missing (Raper et al., 1983; Goodman and Shatz, 1993; Grenningloh et al., 1991; Harrelson et al., 1988; Kuwada, 1986).

Alternatively, the inappropriate placement of molecules that serve as stop signals for growing axons (Kapfhammer and Raper, 1987; Faissner and Kruse, 1990) could also cause the observed defects.

Several lines of evidence suggest that the defects in unc-34 and unc-76 are more likely to be informational than structural. First, the defects caused by these mutations appear to be specific to the neuronal bundle environment, while in mutant animals with axons with clearly defective structures (e.g., unc-33, unc-14, and unc-51), defects in outgrowth occur in neuronal bundles and along the basement membrane. Second, experiments reported by McIntire et al. (1992) and Hedgecock et
al. (1985) indicate that several axons that fail to extend properly in unc-34 and unc-76 mutants are capable of doing so if a second mutation alters their position. Mutations in unc-6 and unc-40 prevent the normal ventral extension of the HSN axons, causing them to extend along a lateral pathway instead. They reach the nerve ring in these mutants by extending anteriorly until they approach the posterior bulb of the pharynx and then extending ventrally (probably along the amphid commissure) into the ventral nerve cord, where they almost immediately enter the nerve ring. McIntire et al. (1992) argued that if the HSN axons in unc-34 and unc-76 mutants were shortened as a result of an inability to assemble a complete axon, laterally-positioned HSNs in unc-40;unc-34 or unc-40;unc-76 mutants should show the same failure to extend as the HSNs in unc-34 and unc-76 mutants alone. However, if the problem resulted from an abnormal interaction with the ventral nerve cord environment, placement of the axons into a different environment might alleviate the outgrowth defect. The latter result was observed: HSN axons in unc-40;unc-34 and unc-40;unc-76 double mutants extended to the nerve ring in the same manner as HSNs in unc-40 single mutants, indicating that the unc-34 and unc-76 mutations do not themselves prevent the assembly of full-length HSNs. Similarly, PHA and PHE axons also fail to extend in unc-76 mutants in the ventral cord environment, but do extend to a normal length when moved to a lateral position by mutations in unc-6 (Hedgecock et al., 1985).

Two sets of observations indicate that the ventral cord-specific outgrowth defect in the HSNs does not result from a barrier at a single position in the outgrowth path. First, HSNs can end anywhere between the vulva and the head (e.g., Fig. 2-5). Second, McIntire et al. (1992)
showed that in egl-43;unc-34 and egl-43;unc-76 double mutants, in which the HSN cell bodies are located far posterior to their normal positions, the HSNs tend to end further posteriorly than they do in unc-34 and unc-76 single mutants. This observation also suggests that a single structure does not cause HSN axon termination.

While these data do not rule out the possibilities that abnormal HSN axon structure or multiple ventral cord structures could cause the observed HSN outgrowth defects, they are at least consistent with the model that the cause of the abnormal HSN outgrowth in unc-34 and unc-76 mutants is an informational defect. The goal of these experiments described below was to achieve a detailed understanding of the axonal outgrowth defects in these mutants, to observe the effects of the complete loss of function of these genes, and to identify additional genes with which unc-34 and unc-76 interact.

**Experimental Procedures**

Genetic methods

Culture of *C. elegans* on agar plates seeded with the *E. coli* strain OP50 was performed as described by Brenner (1974). Worms were maintained at 20°C unless otherwise noted.

Strains of the genotype dpy-18(e346)/+;unc-34 (e566 or e315) + dpy-11(e224)/sDf32 unc-46(e177) + were constructed by crossing unc-34 dpy-11(e224) homozygotes with males of the genotype dpy-18(e346)/+;unc-46(e177);dpy-18(e346)/+;unc-46(e177). Deficiency heterozygotes (non-Dpy) were grown on 9 cm plates for fixation. Prior to fixation, all

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obviously Dpy animals were removed to remove unc-34 homozygotes. Most deficiencies in the unc-34 region of LGV suppress recombination to the right of unc-34, and sDf32 has been reported to reduce recombination in the 16 map unit sDf32-unc-46 interval by 160-fold (Rosenbluth et al., 1990), indicating that nearly all of the Unc nonDpy animals in the strain above were likely to be deficiency heterozygotes. Heterozygotes of the genotype dpy-11(e224)unc-76(e911)/unc-42(e270)yDf8 were constructed by crossing dpy-11(e224)unc-76(e911)/++ males to unc-42(e270)yDf8/dpy-11(e225) unc-42(e270);xol-1(y9) and choosing non-Dpy animals. All Dpy animals were removed from the plates before fixation. Heterozygotes of the genotype sma-1(e30)unc-76/yDf8 were constructed by crossing males of the genotype +/-eT1 unc(n754dm); yDf8/eT1 to sma-1(e30)unc-76 hermaphrodites. Deficiency heterozygotes were the Unc non Sma cross progeny, scored on the basis of the tapered nose morphology of non-Sma L3 and L4 animals. Non-Sma F2 progeny of deficiency heterozygotes from the above cross were transferred to a separate plate at the L3 and L4 stages, and these populations were fixed when the animals reached adulthood.

Screens for mutations that fail to complement unc-34(e566) were conducted as follows. Males carrying the him-5(e1490) mutation were mutagenized with EMS (Wood, 1988) or gamma irradiation (8000 rads from a 60Co source) and mated with hermaphrodites of the genotype unc-34(e566) egl-8(n488)dpy-11(e224);lon-2(e678)xol-1(y9). From the progeny of this cross, Unc hermaphrodites that were not Egl or Dpy were isolated. If these animals produced only Unc progeny, Unc non-Egl-non-Dpy lines were established from the F2 generation. Unc mutants were outcrossed three times with wild-type males. Putative deficiencies generated by gamma
mutagenesis were tested for complementation of sDf28 by crossing males of the genotype dpy-18(e364)/et1; sDf28 unc-46(e177)/et1 let(s2165) with balanced hermaphrodites of the genotype +/-et1; m/et1 let(s2165), where m is the newly-induced mutation. Individual wild-type progeny of this cross were allowed to self-fertilize, and their broods were examined for the presence of Dpy animals. The absence of Dpy animals indicated that /dpy-18(e364); m/sDf28 heterozygotes were not viable, suggesting that the mutation was a deficiency failing to complement both unc-34 and sDf28.

A similar screen was conducted for new unc-76 mutations. EMS-mutagenized wild-type or egl-1(n986dm) males were mated with hermaphrodites of the genotype + sdc-3(y52) unc-76(e911)/unc-61(e228) ++; dpy-3(e27). All experiments were carried out at 15°C to maximize the proportion of sdc-3(y52) unc-76(e911); dpy-3(e27) homozygotes that were transformed into males by the sdc-3(y52) mutation (Klein and Meyer, 1993), and parental animals were moved to fresh plates every 12 hours to avoid exhaustion of the food supply before slow-growing mutants were old enough to score. Non-Dpy uncoordinated hermaphrodites with the characteristic unc-76 coiling movement were isolated from the F1 generation, and individual Unc non-Dpy hermaphrodites from their F2 progeny were isolated to produce strains homozygous for the new unc-76 mutations as judged by the absence of males (indicating the absence of the parental sdc-3(y52) unc-76(e911) chromosome). Mutant strains were outcrossed three times with N2 males. The unc-76 allele n2457 was separated from the closely-linked marker egl-1(n986dm) by crossing the double mutant with males of the genotype dpy-11(e224)/+. Dpy Unc non-Egl recombinant F2 progeny were isolated, and the dpy-11 mutation was removed by outcrossing with N2 males.
Suppressors of *unc-34(e566)* and *unc-76(e911)* were isolated by inspection of the F2 progeny of EMS-mutagenized homozygous unc hermaphrodites. Non-Unc animals were placed on individual plates and kept if the F3 broods contained animals with distinctly better locomotion than the parental strain. Screens were conducted at 20°C for *unc-76(e911)* suppressors and at either 20°C or 25°C for *unc-34(e566)* suppressors.

HSN axon measurements

Worms were stained with affinity-purified polyclonal rabbit anti-serotonin antisera (H. Steinbusch, Free University, the Netherlands) by methods described in Desai et al. (1988). Most preparations were stained with both FITC-conjugated goat-anti-rabbit secondary antibodies and FITC-conjugated rabbit-anti-goat tertiary antibodies (Cappell, Jackson ImmunoResearch). In this case, nonspecific labeling was blocked by treatment with 1% bovine serum albumin instead of goat serum. Animals were observed on a Zeiss Axiophot microscope equipped with epifluorescence optics, and photographs were taken with Kodak Technical Pan or T-Max 3200 film.

HSN morphologies were recorded as freehand sketches of each axon and cell body. The HSN axon length was estimated with the use of an ocular micrometer and noted on each sketch. In these measurements, the distance between the vulva and the posterior end of the posterior pharyngeal bulb was divided into tenths, and each HSN axon length was expressed to the nearest tenth of this distance. When mean HSN lengths are compared, differences of less than 10% of the vulva-head distance
are probably not significant. Entry of the HSN axons into the nerve ring could not be scored unambiguously because other serotonergic axons in the head follow a similar path. HSNs were therefore scored as 100% of their normal length if they reached the posterior end of the posterior pharyngeal bulb.

HSN lengths in a population were analyzed according to several criteria. Individual HSN axons were grouped by the position of the cell body (migration normal or abnormal) and position of the longitudinal portion of the axon (ventral cord or lateral). The side of the animal on which the cell body was located was noted but was not taken into account in comparisons among strains because no statistically significant differences between left and right sides of normally- or abnormally-migrating HSNs were noted for unc-34(e566), unc-76(e911), unc-34(e566) unc-76(e911), or unc-76(n2457) analyzed by the Statview statistics package. When both axons in an animal could be scored, any crossing of one HSN to the contralateral side was recorded. In animals in which an HSN axon did cross, axons on the same side of the ventral cord could not usually be distinguished, and so only one axon per animal was counted in calculations of mean axon length unless the ends of both axons could be seen clearly. This would tend to increase the average scored HSN length, because the shorter of two HSN axons running together was not included. However, left and right HSNs that remained separate tended to have the same average length, and animals in which a crossover had occurred did not have significantly different average HSN lengths from animals in which the HSNs remained separate. Crossover animals were therefore not separated for determination of average HSN length. HSN lengths are reported as mean ± 95% confidence limits, calculated as
± 1.65 s/n^{0.5}, where s = standard deviation and n = number of HSNs (Rosner, 1986).

The morphologies of the PHA and PHB axons were determined by soaking living animals in FITC or diO solutions (Hedgcock et al., 1985). diO staining was done by washing worms into a 1.5 ml eppendorf tube containing 400 μl of 10 μg/ml diO in M9 and incubating the closed, foil-covered tube with gentle rocking for two hours at room temperature (E. Hedgcock, personal communication). Worms were washed twice in M9 and returned to a plate with bacteria for 1 hour before observation by epifluorescence and Nomarski optics. PHA and PHB axons were scored as "short" if the processes appeared to stop at the point of their entry into the ventral cord, "normal" if they appeared to have roughly normal length, "intermediate" if they clearly were shorter than normal but had entered the ventral nerve cord, and "long" (in the case of tax-2) if they were distinctly longer than normal.

Electron microscopic analysis of nerve rings

All electron microscopy was performed by Erika Hartwieg, and the tracings of electron micrographs and axon counts were done by Lisa Delissio. Animals were fixed in 0.8% glutaraldehyde/0.8% osmium tetroxide in 0.1 M cacodylate buffer for 1 hr on ice, cut in half, and fixed overnight at 4°C in 1% osmium tetroxide in 0.1 M cacodylate. Fixed worms were placed in blocks of 1% agar, dehydrated, and embedded in an epon-araldite mixture, which was polymerized at 55-60°C. Thin sections (about 600 angstroms) were cut with a diamond knife, mounted on formvar-coated slot grids, and viewed at 80 kV on a JEOL 1200 EXII
electron microscope. Because the nerve ring lies in a plane slightly oblique to the dorsoventral axis, blocks were oriented at an angle such that the nerve ring was likely to be perpendicular to the plane of section and readjusted if initial sections did not appear to be at the correct angle. Sections were taken at several levels through the nerve ring of each worm, and nerve ring profiles on each side were photographed and the axonal profiles traced onto acetate sheets. Axons clearly entering or exiting the nerve ring were not counted. The two sides of the nerve ring were counted separately, but because of ambiguities in orientation of the worms prior to sectioning, it was not possible to determine which were left and right sides. Axon profile counts were analyzed with the Statview statistics package.

Results

Longitudinal axon outgrowth defects in unc-34 and unc-76 mutants

Several studies have demonstrated axonal outgrowth defects in unc-34 and unc-76 mutants. Most defects are in longitudinal axonal extension, and while the two mutants are not identical in the range of affected cells, there are a number of defects in common. The strongest defects occur in the extension of the axons of the phasmid sensory cells PHA and PHB and of the HSN moto neurons in the ventral nerve cord. Both cell types send axons ventrally and then anteriorly in the ventral nerve cord, and in unc-34 and unc-76 mutants, the ventral extension is normal while the axons do not reach their full length in the ventral cord (Figs. 2-1, 2-7) (Hedgecock et al., 1985; McIntire et al., 1992).
Similarly, axons of the PDE sensory neurons grow from a laterally-positioned cell body into the ventral nerve cord, branch, and send long processes anteriorly and posteriorly (White et al., 1986), and in unc-76 mutants, the ventral-directed extension is normal while the ventral cord processes, particularly those extending posteriorly, are shorter than normal (Hedgecock et al., 1985). PDE morphology in unc-34 mutants has not been scored. The longitudinal portions of the DD motorneurons in the dorsal nerve cord are also shorter than normal in unc-34 mutants, while they appear to be normal in unc-76 mutants (McIntire et al., 1992).

Other longitudinal outgrowth defects that have been observed in unc-34 mutants include relatively rare defects in the direction of outgrowth of the NSM axons in the pharynx (defective in 1-8% of cells) and of the CP1 axon in the male ventral cord (defective in up to 30% of animals; Fig. 2-3) and failure of the excretory canals to extend fully (Hedgecock et al., 1987) Although they are not neuronal structures, the excretory canals are long processes with a central lumen that extend the length of the animal in association with the CAN axons. Defects in the excretory canals and NSMs have not been observed in unc-76 mutants, and CP axons have not been tested. Electron microscopic examination of the ventral and dorsal nerve cords in unc-34 and unc-76 mutants has shown that the number of axonal profiles is lower in these mutants than in wild-type animals (McIntire et al., 1992). This indicates that axons other than those described above fail to reach their normal length, consistent with the uncoordinated movements of unc-34 and unc-76 mutant animals. The morphological defects known for specific neurons in these
mutants are not sufficient to account for the impaired movement observed.

Several classes of axons are defective in extension in the nerve rings of unc-34 and unc-76 mutants. Axons of the amphid sensory neurons (ADF, ASH, ASI, ASJ, ASK, and ADL) can be visualized with the dyes FITC, diO, and diII, and in wild-type animals these axons extend ventrally together from their laterally-positioned cell bodies in the head to the ventral surface of the nerve ring, where they turn anteriorly and then run dorsally in the ring. Normally these axons end when they meet their contralateral homologs in the dorsal portion of the nerve ring, but in unc-34 and unc-76 mutants, these axons terminate in the nerve ring before reaching the top (Hedgecock et al., 1985; this work).

To characterize further the nerve ring defects in unc-34 and unc-76 mutants, cross sections of nerve rings were examined in the electron microscope (Fig. 2-3). While specific positions in the ring could not be compared directly, the average number of axonal profiles in unc-34 and unc-76 mutant adults was clearly lower than in wild-type adults (Fig. 2-4). While the five wild-type worms examined had an average of 142 ± 5 axon profiles per section of either the left or the right side of the nerve ring (n = 44 sections), the two unc-34(e566) animals had an average of 98 ± 5 axons per section (n = 8), and five unc-76(e911) had an average of 108 ± 4 axons per section (n = 36). The two unc-75(n2457) animals examined had slightly more axons than did the other mutants, 125 ± 8 axons per section (n = 16), but all were significantly different from wild-type (p < .01 for n2457, p < .0001 for e566, e911). These observations suggest that, like axons in the ventral nerve cord, a
significant proportion of axons in the nerve rings in unc-34 and unc-76 mutants fail to reach their full lengths.

An additional defect in longitudinal cell movements that has been observed for unc-34 and unc-76 mutants is the mispositioning of several neuronal cell bodies along the anterior-posterior axis. In both mutants, the cell bodies of the VD and DD ventral cord motorneurons are abnormally positioned in the ventral cord (McIntire et al., 1992). In addition, approximately one-third of the HSN cell bodies failed to complete their migration in unc-34 mutants (Table 2-1; see below), while in unc-76 mutants, HSN migration was nearly normal. The HSNs in migration-defective unc-34 mutants migrated approximately 75% of their normal distance, comparable to the defects observed in other mutants that also affect axonal outgrowth (unc-71, unc-73, and mig-2; Desai et al., 1988).

Fasciculation defects in unc-34 and unc-76 mutants

All of the major axonal outgrowth defects in unc-34 and unc-76 mutants occur in regions of axon-axon contact. Several defects in circumferential extension of axons along the lateral hypodermis have been observed (failure of HSN axons to extend ventrally, failure of VD and DD axons to reach the dorsal cord by their normal routes), but these are considerably lower in penetrance than defects observed in the same axons in the nerve cords (McIntire et al., 1992). Mutations in unc-34 and unc-76 have been observed to cause a second type of defect in axon-axon interactions, failure to form or maintain fascicles. Several defects in this category have been observed. First, two groups of axons
that are normally bundled in wild-type animals, the amphid sensory axons and the VD and DD motor axons in the ventral and dorsal nerve cords, have been observed by fluorescence microscopy to separate and run parallel to one another (Hedgecock et al., 1985; McIntire et al., 1992). Second, electron microscopy of the nerve cords has shown that the major bundles of the right and left ventral cords and the dorsal cord are often split into two or three smaller bundles (McIntire et al., 1992). The nerve rings of unc-34 and unc-76 mutants, by contrast, do not appear to split (8 sections from two unc-34(e566) nerve rings, 36 sections from five unc-76(e911) nerve rings, and 16 sections from two unc-76(n2457) nerve rings). Third, the left and right HSN axons frequently fail to remain on separate sides of the ventral nerve cord, with the left HSN ususally crossing to the right side (Tables 2-1, 2-2). This defect is characteristic of the behavior of HSN axons in the absence of two other axons in the left ventral nerve cord, PVPR and PVQL (Garriga et al., 1993), and in other mutants has been shown to reflect either disorganization of large portions of the ventral cord or the specific exiting of the left HSN from the fascicle in which it normally extends (G. Garriga, personal communication).

HSN axon morphologies in unc-34 and unc-76 mutants

The HSN axons were used as the primary assay of the effects of unc-34 and unc-76 mutations because they can be visualized by immunofluorescence microscopy for nearly their entire length without interference from neighboring axons. In addition, many of the cell-cell interactions by which the left HSN axon is guided have been identified
(Garriga et al., 1993). (A potential behavioral consequence of abnormal HSN extension, "constitutive" egg-laying unaffected by the absence of food, was too variable and too similar to wild-type behavior for use as an indicator of defective HSN outgrowth.) To facilitate comparison of different unc-34 and unc-76 mutant alleles, the morphologies of the HSN axons in animals carrying the strongest of the originally-identified alleles, unc-34(e566) (examined at 25°C) and unc-76(e911) (examined at 20°C) were characterized in detail.

Examination of HSN axon morphologies in these mutant animals showed that four features were variably defective: longitudinal extension, circumferential extension, cell body position, and separation of the left and right HSNs. Fig. 2-1 illustrates several of these defects in unc-34 animals. Longitudinal extension was the most frequently defective aspect of HSN development in these mutants, with 87% of unc-34(e566) HSN axons (n=85) and 90% of unc-76(e911) HSN axons (n=68) failing to reach the head. In both mutants, the HSNs frequently joined and ran together on the same side of the midline (usually the right). This was observed in 72% (n=29) of unc-34(e566) and in 40% (n=30) of unc-76(e911) animals in which both HSNs could be observed. 47% of HSN cell bodies failed to migrate to their appropriate position in unc-34(e566) (n=209) animals, while only 5% (n=73) of unc-76(e911) HSNs were migration-defective. In both mutants, defects in circumferential extension (ventral-directed growth from the cell body to the ventral cord) were comparatively rare, occurring in 6% (n=49) of unc-34(e566) HSNs (with normally-positioned cell bodies) and 5% (n=58) of unc-76(e911) HSNs. Circumferential extension defects in unc-34 animals were much more frequent in HSNs that had failed to migrate to
their normal position: 31% (n=58) of migration-defective e566 HSNs failed to enter the ventral nerve cord.

Inspection of the distributions of HSN axon endpoints in unc-34 and unc-76 mutants indicated that the axons can end anywhere between the vulva and the head (Fig. 2-5). When subdivided by the position of the HSN cell body and the position of the axon (ventral cord vs. lateral), unc-34(e566) mutant axons showed clear differences in the distribution of lengths. In unc-34(e566) mutants, axons growing from normally-positioned cell bodies in the ventral nerve cord tended to be longer relative to total body length than were ventral axons from migration-defective HSN cell bodies, and HSNs that grew in a lateral position from migration-defective cell bodies were still shorter. Too few axons grew in a lateral position from normally-placed cell bodies to compare.

Lateral axons arising from migration-defective HSN cell bodies often wandered and looped on the lateral body wall (Fig. 2-1), unlike lateral HSN axons from normally-positioned cell bodies in other mutants (unc-6, unc-40; McIntire et al., 1992). Lateral HSN axons were considered separately from ventral axons in all subsequent analyses.

The position of the cell body has been shown to influence the length of HSN axons in unc-34 and unc-76 mutants (McIntire et al., 1992). The HSN axons in double mutant combinations of either unc-34 or unc-76 with egl-43, which causes HSN cell bodies to remain close to their starting positions in the tail, terminate at more posterior positions than do HSN axons in unc-34 or unc-76 mutants alone (McIntire et al., 1992). Statistical analysis of the ventral unc-34(e566) HSNs shown in Fig. 2-5 showed a significant difference based on cell body position. HSNs with normally-positioned cell bodies extended axons in
the ventral cord to an average of 66% (n=42) of the normal HSN length (defined for purposes of unambiguous scoring as the distance from the vulva to the posterior bulb of the pharynx; see Experimental Procedures). Migration-defective HSNs in the same population extended axons only to a mean length of 48% (n=27), a statistically significant difference (p=.011). For this reason, migration-defective HSNs were considered separately in subsequent comparisons of mutant alleles.

The effects of the failure of HSNs to remain on separate sides of the ventral cord were more difficult to determine because the lengths of the two axons could not be scored unambiguously when they grew together (see Experimental Procedures). In animals in which two distinct HSNs could be detected on the same side of the ventral nerve cord, the lengths did not usually appear grossly different. Among unc-34(e566) HSNs with normal cell body positions, the mean axon lengths in animals in which a crossover occurred (69%, n=14) and animals in which the axons remained separate (59%, n=16) were not significantly different. Similarly, unc-76(e911) animals with normally-positioned HSN cell bodies did not have different mean HSN lengths in animals in which crossovers did (68%, n=31) and did not (64%, n=12) occur. For comparison of mutants, HSNs were not distinguished on the basis of crossing over.

The side of the ventral cord in which an HSN axon grew did not appear to affect its length. Comparison of left and right HSNs arising from normally-positioned cell bodies in animals in which no crossover took place showed no significant differences in either unc-34 or unc-76 mutants. HSN axons in unc-76(e911) animals grew to a mean length of 66% (n=21) on the left and 67% (n=28) on the right, while unc-34(e566) HSN axons grew to a mean length of 54% (n=5) on the left and 58% (n=12) on
the right. The side of the ventral cord on which an HSN axon grew was not considered in further comparison of mutants.

**Null phenotypes of unc-34 and unc-76**

In order to infer the function of a gene from the phenotype of mutants, it is important to know that the mutants completely lack gene function. Mutations that cause only partial loss of gene function can cause defects that do not reflect the full set of cells and processes in which the gene is involved. Therefore a set of experiments was designed to determine the phenotypes of null unc-34 and unc-76 mutants. For both genes, the evidence for the existence of null mutants relied on several observations: the similarity of the HSN defects of homozygous mutants with those in animals carrying mutations in trans to deficiencies; the frequency with which mutations were obtained in a screen in which deficiencies could also have been obtained; the similarity of defects caused by all alleles of each gene; and, for unc-76, molecular evidence.

**Determination of the unc-34 null phenotype**

Phenotype of existing alleles in trans to deficiency

The HSN defects of animals carrying unc-34(e315) or unc-34(e566) in trans to the deficiency sDf32 were quantitated by measuring the lengths of HSNs visualized by immunofluorescence microscopy with an anti-serotonin antibody. The deficiency sDf32 is the smallest known deficiency that deletes unc-34 (Rosenbluth et al., 1988). Strains of
the genotype unc-34 + dpy-11(e224)/sdF32 unc-46(e177) + were grown, and all obviously Dpy animals were removed to remove unc-34 homozygotes. sdF32 suppresses recombination nearly completely in the sdF32-dpy-11 interval (Rosenbluth et al., 1990), indicating that nearly all of the Unc nonDpy animals in the strain above were likely to be deficiency heterozygotes. Animals on plates from which the Dpy animals had been removed were fixed and stained with anti-serotonin antibodies.

Animals homozygous for either e566 or e315 were viable and healthy and showed HSN defects that were similar to those of animals carrying either allele in trans to sdF32 (Table 2-1). HSN lengths and severity of the migration defect were not significantly increased in the deficiency heterozygotes. Because the two trans-heterozygotes were different from each other in severity but similar to the appropriate homozygote, a two-fold reduction in unc-34 gene dosage did not appear to have a significant effect on the severity of unc-34 mutant defects.

Isolation of new unc-34 mutations

In order to identify new unc-34 mutations, a screen was designed based on the observation that unc-34(e566)/sdF32 heterozygotes are uncoordinated and healthy. Males carrying the him-5(e1490) mutation were mutagenized with EMS or gamma irradiation and mated with hermaphrodites of the genotype unc-34(e566) egl-8(dpy-11(e224);lon-2(e678)xol-1(y9). From the progeny of this cross, Unc hermaphrodites that were not Dpy were isolated as potential heterozygotes carrying a new unc-34 mutation in trans to e566. If these animals produced only Unc progeny, several Unc non Egl nonDpy hermaphrodites were placed on
individual plates, and those with no Egl or Dpy progeny in the next
generation were selected as clones homozygous for the new mutation.

A total of 13,500 F1 hermaphrodite progeny of EMS-mutagenized
males were analyzed in this manner, and four mutations were isolated.
This frequency, $3 \times 10^{-4}$, is comparable to the average mutation
frequency of $5 \times 10^{-4}$ obtained from EMS mutagenesis (Brenner, 1974).

A total of 12,900 hermaphrodite progeny of males mutagenized with
gamma radiation (8000 rads from a $^{60}$Co source) were analyzed using the
scheme described above, and six mutations that failed to complement unc-
34(e566) were identified. One of these, n1890, was viable in homozygous
form. The remaining five could not be isolated as homozygotes and were
thus considered candidates for lethal mutations. They were balanced by
the translocation chromosome eT1 le(t2165) and tested for their ability
to complement sDf28, a deficiency that deletes genes to the right of
unc-34 but not unc-34 itself. All five strains failed to produce viable
progeny of the genotype m/sDf28 (where m is the new mutation),
indicating that the new mutations were deficiencies that failed to
complement both unc-34 and at least one essential gene deleted by sDf28.
These deficiencies were named nDf35-nDf39. Thus, deficiencies for the
unc-34 locus were readily obtained from this mutant screen.

Comparison of HSN defects caused by different unc-34 alleles

When the HSN growth defects in animals mutant for all unc-34
alleles were compared, two striking observations were made (Table 2-1).
First, all mutations caused defects that were approximately equal in
severity. Second, the defects caused by all alleles were temperature-
sensitive (ts). Animals raised at 25°C had consistently shorter HSN axons than did animals raised at 20°C or 15°C, and the cell body migration defects were generally more severe at high temperatures. Ventral-directed HSN extension defects and failure to remain on separate sides of the ventral cord also tended to increase with temperature. Extension of phasmids in the ventral nerve cord was also temperature-sensitive in the three alleles examined (e315, e566, and e951; Table 2-3), and the low-penetrance defects (anteriorly-directed NSM processes) also increased with temparture in the four alleles scored (Table 2-3).

The ability of either of two informational suppressors to suppress the Unc phenotype of the unc-34 alleles e566, e951, and s138 was examined. Neither the amber suppressor sup-7(st5) nor the suppressor smg-1(e1228), one of a class of mutations believed to elevate the levels of unstable RNA (Hodgkin et al., 1989), reduced the severity of the Uncoordinated phenotype conferred by any of these mutations (Table 2-5).

Taken together, the similarity of the effects of the strong allele e566 to those of e566 in trans to sDf32, the frequency with which new unc-34 mutations were obtained from a screen that also readily yielded deficiencies, and the similarity of defects caused by all unc-34 mutations (except the weaker allele e315) indicates that most are likely to be null alleles. The allele e566 has been studied most extensively.

**Temperature-shift experiments**

In order to determine the developmental stage at which the unc-34 gene product is required, temperature-shift experiments were performed. The temperature-sensitive period for the locomotory defect in animals
homozygous for the weak allele e315, which are distinctly less uncoordinated when raised at 15°C than they are when raised at 20°C or 25°C, was tested first. Adult hermaphrodites raised at 15°C or 25°C were allowed to lay eggs for short periods (2 hr. at 15°C, 1 hr. at 25°C), and plates of synchronized broods were shifted to the opposite temperature after hatching and each molt. They were scored for locomotory ability when they reached adulthood. The temperature-sensitive period for e315 as judged by locomotion was during oogenesis or embryogenesis (Fig. 2-6). Populations kept at 25°C during oogenesis and embryogenesis had roughly the same proportion of uncoordinated adult animals (60-80%), irrespective of the time at which they were shifted to 15°C. Similarly, populations kept at 15°C during oogenesis and embryogenesis gave rise to the same low proportion of uncoordinated animals (10-20%), regardless of the time of shift to 25°C. These observations imply that the unc-34 gene product is required during early development for the proper assembly of certain motor circuits, and that a return to the permissive temperature after this period is not sufficient to allow the assembly of these circuits. In addition, the lack of major effects of temperature shift during the L1 stage suggests that defects in embryonically-derived motor circuits are responsible for most of the locomotory defects observed in unc-34(e315). However, because the degree of uncoordination in individual animals was not quantitated, it is possible that additional neuronal defects acquired as a result of defects during L1 axonal outgrowth would not have been included in these data.

Temperature-shift experiments were also performed to determine the developmental stage during which the unc-34 gene product is required for
normal HSN outgrowth. Because the HSNs extend their axons in the ventral cord during the L4 stage, while the remainder of the axons in the ventral cord grow out in the embryo or L1 stages (with the exception of the postdeirid neurons PDE and PVD, which are born in the late L2), a requirement for the unc-34 product during HSN outgrowth should be easily distinguishable from a requirement for this product during the period in which the ventral cord is assembled.

Adult e566 or e315 homozygotes that had been raised at 25°C or 15°C were allowed to lay eggs on fresh plates for one and two hours, respectively, and then removed. After hatching and each subsequent molt, one set of plates at 15°C was shifted to 25°C, and vice versa, and worms were fixed and stained with anti-serotonin antibodies after they reached adulthood.

The results of the temperature-shift experiments were not clearcut for either allele, possibly as a result of small sample size at each time point. However, HSN outgrowth appeared to be more sensitive to the temperature during larval development than during embryogenesis for both alleles (Fig. 2-6). Animals raised at 25°C and shifted to 15°C during larval development tended to have fairly normal HSN lengths except when the shift occurred late in larval development, suggesting that the HSN defects were not caused primarily by defects in early assembly of the nervous system. Conversely, embryogenesis and early larval development at 15°C was insufficient to prevent later shifts to 25°C from causing HSN defects. The precise larval period in which temperature was critical was difficult to determine from these data. The critical period for e315 animals appeared to be later than for e566 animals, but this is difficult to interpret because it is not known whether e315
encodes a temperature-sensitive **unc-34** product or is simply a weak allele.

**Determination of the null phenotype of **unc-76**

For reasons similar to those discussed above in the case of **unc-34**, experiments to determine the phenotype of **unc-76** null mutants were performed. The defects observed in e911 appear to be those of a null mutant, based on comparison of the HSN defects of homozygous mutants to those of animals carrying **unc-76** mutant alleles in trans to a deficiency and the results of a screen for mutations that fail to complement e911. Molecular analysis of **unc-76** mutants, however, suggests that e911 retains more gene function than most of the other mutants (see below).

Animals of the genotype **unc-76(e911)/unc-42(e270) yDf8** are viable and resemble e911 homozygotes in their locomotion, although their growth rate is somewhat slower. Because **unc-76** null mutants in trans to e911 were therefore expected to be viable, a screen was performed for new mutations that failed to complement e911. Wild-type or **egl-1(n986)** males were mutagenized with EMS and crossed with hermaphrodites of the genotype **+ sdc-3(y52) unc-76(e911)/unc-61(e228) + ;dpy-3(e27)**. The hermaphrodites are phenotypically non-Unc, and unlike the marked **unc-76** homozygotes used in pilot screens, they distribute their progeny around the plate, facilitating the scoring of the Dpy phenotype. Approximately 98% of animals homozygous for the **+ sdc-3(y52) unc-76(e911)** chromosome are males at 15°C, due to the effects of the **sdc-3** mutation (J. Plenefisch, B. Klein, and B. Meyer, personal communication), and the few hermaphrodites homozygous for this chromosome can easily be scored as
self progeny by their Dpy phenotype. Animals heterozygous for the + sdc-3(y52) unc-76(e911) chromosome and a new unc-76 mutation were non-Dpy Unc hermaphrodites.

Five new alleles were isolated by this technique, one (n2457) from a screen of 1800 F1 animals sired by eg1-1(n986) males and four (n2367, n2397, n2398, and n2399) from a screen of 30,000 F1 animals sired by N2 males. The total frequency, 3 x 10^{-4}, is only slightly lower than the average mutation frequency of 5 x 10^{-4} from the standard C. elegans EMS mutagenesis procedure (Brenner, 1974).

A comparison of the HSN defects in all of these mutant alleles, as well as two others identified in other laboratories, showed that all were similar in severity (Table 2-3). Only n2398, with a mean HSN length of 89 ± 2 % (n=75), was substantially weaker than e911 (mean length 66 ± 3 %, n=55), and no allele was stronger than e911. The proportions of HSNs with cell body migration defects or laterally-positioned axons and the proportion of animals with both HSNs on the same side of the ventral cord were similar in all mutant strains. The mean HSN lengths in animals carrying n2367, n2397, n2457, or ev424 in trans to the deficiency yDf8 were all similar to one another (73-77%) and to those observed in animals for each mutant allele.

The Unc phenotype caused by one allele, n2399, was suppressible by the amber suppressor sup-7(st5), suggesting that this mutant encoded a truncated protein product. The other alleles tested, e911, ev424, n2397, and n2398, were not suppressed (Table 2-4). Furthermore, of the eight unc-76 alleles, only two (e911 and n2398) were suppressed by the informational suppressor smg-1(e1228), believed to elevate the level of
unstable RNA (Hodgkin et al., 1989). (See Table 2-4 and the unc-76 suppression section below for details.)

The experiments above are consistent with the model that the alleles ev424, rh116, n2397, n2367, n2399, and n2457 are severe loss-of-function or null alleles. Molecular analysis of the DNA sequence from the coding regions of these mutant alleles and analysis of Unc-76 protein from extracts of mutant worms, discussed at length in Chapter 3, support this conclusion. Briefly, the sites of seven of the eight mutations were defined. All of these are predicted to lead to the synthesis of truncated proteins, either by frameshift, nonsense, or splice acceptor mutations. DNA sequence analysis and immunoblotting showed that the smg-suppressible alleles e911 and n2398 encode the largest of the mutant forms of Unc-76 protein. The alleles n2457, ev424, n2397, and n2367 encode the smallest predicted proteins.

The effects of mutations in unc-34 and unc-76 are additive.

The similarity of HSN outgrowth defects in putative null mutants of unc-34 and unc-76 raised the question of whether the two genes function in the same process or independent processes. McIntire et al. (1992) addressed this question by analyzing the HSN defects in double mutant combinations of the two genes. If the genes function in the same biochemical process, double mutant combinations of null alleles would not be expected to cause a more severe defect than either null allele alone. However, if the genes function in independent processes, a double mutant would be expected to have defects more severe than either single mutant. McIntire et al. (1992) observed that the unc-34(e566)
unc-76(e911) double mutant had HSN defects more severe than either single mutant. This strain was analyzed further.

The effects of unc-34 and unc-76 on HSN axon outgrowth appeared to be roughly additive (Table 2-5). HSNs with cell bodies in the wild-type position and axons growing in the ventral nerve cord had axons that grew to $67 \pm 6\%$ of the distance from vulva to nerve ring in unc-34(e566) animals (n=42), to $78 \pm 6\%$ in unc-76(e911) animals (n=17), and to only $35 \pm 7\%$ of the distance in unc-34(e566) unc-76(e911) double mutant animals (n=28). While the proportion of HSNs with migration-defective cell bodies in the double mutant (39%) was similar to that in unc-34(e566) alone (44%)--unc-76(e911) had only 5% migration-defective HSNs--the outgrowth of migration-defective HSNs in the double mutant was significantly poorer than in either single mutant. Migration-defective HSNs in unc-34(e566) mutants grew to an average of $53 \pm 9\%$ (n=23), while in the double mutant they reached $12 \pm 8\%$ (n=17) on average. The proportion of animals with HSNs that joined on one side of the ventral cord was roughly intermediate between that of the two single mutants.

**Identification of suppressors of unc-34 and unc-76**

The experiments described above suggested that unc-34 and unc-76 function independently in axonal outgrowth. In order to define the molecules involved in the specific axon outgrowth processes defined by unc-34 and unc-76, it was of considerable interest to identify genes that function in the same process as unc-34 and unc-76. The experiments described below were designed to isolate extragenic suppressors of the Unc phenotypes of unc-34 and unc-76 mutants. Because neither the
biochemical function of these gene products nor the mutant lesions in these genes were known, the nature of genes capable of mutating to suppress unc-34 and unc-76 mutant defects was not possible to predict. The temperature-sensitive null phenotype of unc-34, as discussed above, implied the existence of other axonal guidance molecules capable of functioning in the absence of the unc-34 product (see below). Genes capable of mutating to forms that suppressed a null allele of unc-34 would be likely candidates for genes encoding these molecules or molecules with which they interact. Therefore the putative null allele e566 was used for suppression screens. This strategy was judged unlikely to yield genes with products that interact with the unc-34 product, and so a small-scale screen was also conducted with the weak, visibly temperature-sensitive allele e315. A screen for suppressors of unc-76, conducted prior to the identification of the molecular defects in the mutant unc-76 alleles, was conducted with the allele e911.

Suppressors of unc-34

The F2 progeny of mutagenized unc-34(e566) hermaphrodites representing 17,500 haploid genomes (14,000 at 25°C and 3,500 at 20°C) were inspected for the presence of animals with normal locomotion. One strain at 20°C, and five strains at 25°C were identified as containing candidate suppressors. (Nine potential suppressed strains were identified in a screen of 2500 haploid genome equivalents of mutagenized e315 animals, but the weak uncoordinated phenotype of the parent strain often made distinguishing mutant from suppressed individuals difficult. These suppressors were not pursued). Staining of suppressed e566
strains with anti-serotonin antibodies indicated that the 20°C suppressed strain, *unc-34(e566);n1959*, had an average HSN length of 98 ± 2% (n=22), compared to an average length of 83 ± 4% (n=74) (Table 2-6). Only cell body positions could be scored for the 25°C suppressors. In all six strains, 13-32% of the HSN cell bodies were mispositioned posteriorly, as compared to 47% (n=209) for *unc-34(e566)* (Table 2-6). Staining with DiO indicated that the phasmid defects were also suppressed.

Animals of the putative genotype *sup;unc-34(e566)* were crossed with wild-type males, and the broods of F1 hermaphrodites were scored for animals with the locomotory pattern characteristic of *unc-34* mutants. All segregated approximately one-fourth Unc-34 animals, indicating that all of the strains carried recessive extragenic suppressors. No additional behavioral phenotypes were detected in these broods, with one exception, suggesting that most of the suppressor mutations alone did not confer behavioral defects. The exceptional case was that of the suppressor *n1963*, which appeared to confer a weak egg-laying defect (see below).

Broods of animals of the genotype *sup/+;unc-34(e566)/+* were also stained with the vital dye DiO in order to determine whether axcn outgrowth defects in the phasmid or amphid neurons, which are stained with DiO in living animals, could be detected. Only non-Unc animals, of which 4/13 should be homozygous for the suppressor mutation, were examined, and any animals with defects in amphid or phasmid structure were recovered and analyzed in the next generation. The few animals with defects were *sup;unc-34(e566)* homozygotes, based on the slightly uncoordinated phenotype of animals in the next generation. Therefore,
the suppressor mutations alone were judged not to cause amphid or phasmid defects. Because these suppressors lacked clearcut defects in either locomotory neurons, as indicated by the absence of any Unc phenotype, or amphid and phasmid neurons, it was not clear whether they have a specific role in nervous system development, and they were not analyzed further.

The suppressor mutation n1963 appeared to confer a very weak recessive egg-laying defect which was stronger at 25°C than at 20°C. Egl animals from the broods of n1963/+;unc-34(e566)/+ hermaphrodites were backcrossed three times with wild-type males, and to determine whether the Egl mutation was linked to the suppressor mutation, suppressed unc-34(e566) strains were genetically reconstructed. Egl hermpahrodites were crossed with unc-34(e566)/+ males, and among the hermaphrodite progeny of this cross, broods containing both Egl and Unc animals were selected. Several Egl (non-Unc) animals were transferred to individual plates, and the broods of the Egl non-Unc animals were scored for the presence of the slightly Unc animals characteristic of n1963;unc-34(e566) animals. All of these Unc animals proved to be suppressed unc-34(e566) homozygotes, indicating that the Egl phenotype cosegregated with the suppressor mutation.

The HSNs of n1963 homozygotes were examined by anti-serotonin antibodies. The longitudinal extension of the HSNs in the ventral nerve cord appeared normal (n=30), as did the migration of the HSN cell bodies. Among 22 animals in which both HSNs could be examined, two animals were found in which the left HSN crossed to the right side just anterior to the vulva and ran together with the right HSN. In one animal, the right HSN sent an axon normally to the nerve ring but also
had a branch that wandered extensively on the lateral body wall in a posterior direction for a short distance. Other HSNs appeared to branch normally.

By a technique similar to that used for reconstructing \textit{n1963;unc-34(e566)} strains, strains of the genotype \textit{n1963; unc-34(e315)} and \textit{n1963; unc-34(e951)} were constructed. Both showed suppression of the Unc phenotype, indicating that \textit{n1963} functions as an allele-nonspecific suppressor of \textit{unc-34} mutations. This result was not surprising, since a putative null allele was used in the original screen, and is consistent with a role for \textit{n1963} as a bypass suppressor of \textit{unc-34}.

The ability of either of two informational suppressors to suppress the Unc phenotypes conferred by the \textit{unc-34} alleles \textit{e566}, \textit{e951}, and \textit{s138} was examined. Neither the amber suppressor \textit{sup-7(st5)} nor the putative RNA-elevating suppressor \textit{smg-1(e1228)} reduced the uncoordination conferred by any of these mutations (Table 2-5).

**Identification of \textit{unc-76} suppressors**

Mutations that suppress the Unc phenotype of \textit{unc-76(e911)} were identified by inspection of the F2 progeny of EMS-mutagenized \textit{unc-76(e911)} hermaphrodites. From 96,000 mutagenized haploid genome equivalents, nine weakly suppressed strains were obtained. All animals from each of these strains were uncoordinated to some degree, and the most Unc individual animals were often difficult to distinguish from \textit{unc-76(e911)} animals alone. However, populations of \textit{unc-76(e911)} suppressed strains always contained individuals that moved more normally than \textit{unc-76(e911)}. This variability was characteristic of each
suppressed strain: the progeny of the most Unc and least Unc animals of a given brood gave rise to broods with the same range of locomotory ability.

All of the suppressed strains restored the HSNs to near-normal lengths, 89-99% of the normal vulva-head distance, in contrast with 66% of the distance in unc-76(e911) animals (Table 2-7). All of these suppressors were recessive and extragenic. Strains of the genotype sup;unc-76(e911) were crossed with wild-type males, and approximately one-fourth Unc animals were observed among the progeny of the resulting wild-type hermaphrodites, indicating that the suppressor mutations could segregate independently of unc-76(e911). Although no animals with abnormal behavior other than the characteristic unc-76 locomotion were observed among the progeny of crosses by wild-type, all of the suppressed strains gave rise to animals with slightly protruding vulvae (Pvul phenotype). This phenotype is characteristic of the smg class of suppressors, which are believed to elevate the levels of unstable RNA produced by mutant alleles of a number of genes (Hodgkin et al., 1989). These suppressors were determined to be smg suppressors based on several observations described below: the Pvul phenotype cosegregated with the unc-76(e911) suppressors tested; these five suppressors of unc-76(e911) were members of the smg class; and smg-1(e1228) suppressed unc-76(e911) in a manner similar to that of the suppressors isolated in the screen described above.

Reconstruction experiments:

Pvul animals from n1736, n1738, n1739, n1740, and n1742 were backcrossed once to wild-type males. Males of the genotype unc-76(e911)
were mated to Pvul hermaphrodites, and the broods of the wild-type progeny of this cross were examined for the presence of Pvul animals and Unc animals. Several (4-5) Pvul hermaphrodites from these broods (progeny of a parent of the presumed genotype pvul/+;unc-76(e911)/+) were transferred to individual plates, and individual Unc progeny of Pvul parents were analyzed. The broods of these Unc animals clearly resembled the broods of suppressed unc-76(e911) animals obtained in the original screen, and contained animals whose locomotion was distinctly more normal than that of unc-76(e911) homozygotes alone. This indicates that the suppressor mutations cosegregated with the protruding vulva mutations, suggesting that n1736, n1738, n1739, n1740, and n1742 are suppressors of the smg class.

The function of n1736, n1738, n1739, n1740, and n1742 as smg suppressors was confirmed by construction of double mutant combinations between each suppressor and lin-29(n546), a smg-suppressible heterochronic mutation causing egg-laying defects and abnormalities in vulval morphology (Ambros and Horvitz, 1984; Hodgkin et al., 1989). Males of the genotype lin-29(n546)/+ were mated to each of the Pvul suppressor strains, and from broods of the wild-type progeny of this cross, animals with the Lin-29 phenotype were transferred to individual plates. Some broods gave rise to Pvul progeny that were considerably healthier than lin-29(n546) homozygotes, and these subsequent generations of these Pvul animals continued to appear healthy. The presence of lin-29(n546) was confirmed by crossing the putative suppressed hermaphrodites with wild-type males and observing animals with the Lin-29 phenotype in the broods of the cross progeny. Thus, the Pvul suppressors of unc-76(e911) also appeared to suppress the smg-
suppressible mutation lin-29(n546), further suggesting that they are smg suppressors.

One of the originally-defined smg suppressors (Hodgkin et al., 1989) was shown to suppress unc-76(e911). unc-76(e911)/+ males were crossed with smg-1(e1228) him-2(e1065) hermaphrodites, and from the broods of wild-type progeny of this cross, Unc animals and Pvl animals were transferred to individual plates. Among the progeny of several Pvl F2 animals were Unc F3 worms that produced broods with the range of locomotory ability observed in other smg-suppressed unc-76(e911) strains. Four Unc F2 hermaphrodites gave rise to Pvl suppressed Unc worms. Thus, smg-1(e1228) suppressed the unc-76(e911) Unc phenotype to an extent similar to that observed with smg suppressors obtained from the unc-76(e911) suppression screen. As mentioned above, subsequent examination of all unc-76 mutant alleles showed that only one other, n2398, was suppressed by smg-1(e1228), while six other alleles were not. e911 and n2398 encode the most nearly complete of the truncated mutant forms of the Unc-76 protein (Chapter 3).

An independent screen in which suppressors of the unc-76(e911) Unc phenotype could have been obtained has also produced only smg suppressors. Nick Rhind and Barbara Meyer (personal communication) used a strain carrying unc-76(e911) and an extrachromosomal array including a plasmid that rescues unc-76 mutants, p76-16B (Chapter 3), and a plasmid carrying a dpy-30/xol-1 fusion that is toxic only in hermaphrodites, to identify mutations that abolish this plasmid's toxicity. They selected non-Unc hermaphrodites because such mutations were expected to allow the survival of non-Unc (presumably array-bearing) hermaphrodites, which otherwise would die. In addition, they expected to isolate suppressors
of unc-76(e911), and in a screen of under 1000 haploid genomes isolated two weak suppressors that confer the protruding vulva characteristic of smg suppressors.

The isolation of smg suppressors of unc-76(e911) suggests that this mutation does not cause a complete loss of unc-76 function. Evidence from the effect of smg suppressors on other genes suggests that RNA levels are increased by smg suppressors, and this also appears to be true for the unc-76(e911) transcript (Chapter 3). If elevated unc-76(e911) RNA levels directly cause the partial suppression of the Unc phenotype found in smg;unc-76(e911) animals, it is likely that some small amount of function is retained by the mutant gene product such that increased concentration of the product increases the amount of gene function available. The failure of smg-1(e1228) to suppress unc-76 alleles encoding more severely truncated proteins (e.g., n2397, n2367, ev424; see Chapter 3) indicates that smg suppression is not the result of elevated levels of transcript from another gene.

One specific neuronal defect of unc-76(e911) mutants, the failure of the phasmid axons PHA and PHB to extend in the ventral nerve cord, was found to be suppressed by a mutation in the gene tax-2 (C. Bargmann, personal communication). Mutants in tax-2 fail to perform chemotaxis normally, and Bargmann demonstrated that they have axonal outgrowth defects in their amphid and phasmid axons. While the phasmid axons are abnormally short in unc-76 mutants, they are longer than normal in a high proportion of tax-2(p691) animals (Fig. 2-7). The PHA and PHB axons in tax-2(p691);unc-76(e911) double mutants were often found to grow to their normal length. The HSN axons in these double mutant animals were likewise much more normal than those in e911 homozygotes.
(Table 2-7). Although tax-2 alone caused no obvious alteration in HSN outgrowth, HSN axons in the double mutant extended to 95 ± 2% of their normal length, compared with 66 ± 3% in unc-76(e911) animals alone. Despite the striking suppression of the HSN defect, tax-2(p691); unc-76(e911) double mutants showed no apparent suppression of the unc-76(e911) locomotory defect. The phasmid defect in unc-34(e315) animals is not suppressed by tax-2(p691) (C. Bargmann, personal communication).

The outgrowth of the phasmid and HSN axons can be disrupted independently

The fact that both unc-34 and unc-76 mutations cause similar defects in the phasmid and HSN axons, a failure to elongate in the ventral nerve cord, suggested that the outgrowth of these classes of axon might share a common mechanism. Both classes are defective in unc-69 mutants as well, but only HSN defects are observed in unc-71 mutants (G. Garriga, personal communication). Mutations in the gene unc-107 cause a failure of the phasmid axons PHA and PHB to elongate in a way reminiscent of the defects in unc-34 and unc-76 (J. Culotti, personal communication). The HSN processes in unc-107(ev411) mutant animals, however, have been found to be normal (G. Garriga, personal communication).

The unc-107 gene was originally identified in a double mutant combination with enu-1(ev419). The enu-1(ev419); unc-107(ev411) double mutant is more severely uncoordinated and has a more extreme phasmid defect than does unc-107(ev411) alone (J. Culotti, personal communication). However, enu-1(ev419) homozygotes are not uncoordinated
and have normal phasmid axons, and an enu-1(ev419);unc-107(ev411) strain reconstructed from the backcrossed single mutants did not show more severely defective phasmids than did unc-107(ev411) animals alone, although the Unc phenotype was more severe (J. Culotti, personal communication). Despite its apparent lack of effect on phasmid axonal outgrowth, enu-1(ev419) did cause an outgrowth defect of the HSN axons. The left HSN axon in 48% of enu-1(ev419) homozygotes crossed to the right side of the ventral nerve cord, a phenomenon seen in unc-34 and unc-76 mutants but not in wild-type animals. This defect was more severe in enu-1(ev419);unc-107(ev411) double mutants, occurring in 94% of animals. Extension to the nerve ring appeared normal, however, in contrast with the shortened axons observed in unc-34 or unc-76 mutants. The crossing of HSNL to the right side of the ventral cord resembles the growth of HSNL in animals in which the axons of the pioneers of the left side of the ventral cord, PVPR and PVQL, have been ablated (G. Garriga, personal communication).

Discussion

What do unc-34 and unc-76 do? Null mutations for either gene cause two classes of defects, a shortening of axons in longitudinal fascicles, and defasciculation of axons that are normally tightly bundled. These axonal defects are largely confined to axon bundles, and relatively few defects are observed where the same axons grow in contact primarily with hypodermal cells. The environment-specificity of these defects as well as the apparent restoration of normal growth to axons removed from their ventral cord environment (McIntire et al., 1992)
suggest a role for these genes in mediating cell-cell interactions within axonal bundles.

The cellular events leading to the observed morphological defects in these mutants are unknown because current techniques do not permit labeling of growing C. elegans axons. However, the morphologies of defective longitudinal axons are reminiscent of those of axons deprived of their normal cellular interactions in a variety of systems. Ablation of axons required for guidance of the G, aCC, and pCC axons in the grasshopper and the DL-1 axons in the Japanese medaka fish leads to stunted extension of the follower axons (Raper et al., 1984; Bastiani and Goodman, 1986; du Lac et al., 1986; Kuwada, 1986). In these studies, the growth of the follower axons slows or ceases despite the presence of neighboring axons within filopodial reach. Grasshopper axons deprived of a single molecule, fasciclin II, have been reported to show behavior similar to that observed when the entire guiding axon is ablated (Harrelson et al., 1988). In addition, the frequent crossing of HSNs from the left side of the ventral cord to the right in unc-34 and unc-76 mutants is reminiscent of the behavior of Mauthner axons in the zebrafish spinal cord when midline cells are absent (Hatta, 1992).

By analogy with these experiments, the defects observed in unc-34 and unc-76 mutants could occur because the absence of pioneer axons or surface cues slows or stops extension of longitudinal axons or reduces their affinity for the bundle in which they extend. In insect and vertebrate embryos, absence of a specific cell can cause variable defects in follower axons, implying that multiple alternative pathways can be used. Alternatively, the observed longitudinal extension defects could arise from the unmasking of a signal that stops axonal extension,
from defects in axonal assembly that slow or prevent axonal extension, or from defects in cell-cell adhesion that lead to detachment of axons as the animal grows. Similarly, fasciculation defects could arise from a failure of follower axons to recognize their partners, from active avoidance of normal partners, from defective axonal assembly, or from poor adhesion.

It is difficult to assess how well these models fit the unc-34 and unc-76 phenotypes without observation of the behavior of growing axons. The possibility that mutant axons reach their normal targets but fail to adhere and are pulled loose as the animal grows, analogous to the Drosophila disconnected mutant defect (Steller et al., 1987), cannot be addressed. In addition, the apparently normal circumferential growth of axons could result from the shorter distances these axons must extend relative to longitudinal axons. However, models concerning intrinsic structural defects and defective cell-cell interactions can be considered in light of other C. elegans mutants and the results of laser ablation and antibody studies.

Are longitudinal axons in unc-34 and unc-76 mutants defective in establishing or following longitudinal tracts, as suggested by the similarity of final neuronal morphologies in these mutants and the operated grasshoppers and fish described above? In principle, defects could arise from defects in pioneer axons, follower axons, surrounding nonneuronal cells, or a combination of these. Genetic mosaic experiments which would indicate whether the genes are required in the axons known to be affected by unc-34 or unc-76 mutations or in their neighbors or both are technically impractical. In unc-76 mutants, the HSN axons and the pioneer PVP axons known to be important for HSN
guidance are both defective, while the pioneers appear to be normal in
\textit{unc-34} mutants (C. Norris, H. Bhatt, and E. Hedgecock, personal
communication). No defects in the nonneuronal cells surrounding the
ventral cord have been demonstrated in these mutants.

The cell-cell interactions required for normal HSN growth have
been explored by laser ablation studies (Durbin, 1987; Garriga \textit{et al.},
1993). Laser ablation experiments have shown that the HSN on the left
side of the ventral cord, which has only four potential axonal partners,
requires two of them for normal guidance (Garriga \textit{et al.}, 1993). The
PVPR and PVQL axons pioneer the left ventral cord during embryogenesis,
growing anteriorly from the tail, and the AVKR and RMEV axons extend
posteriorly along PVPR and PVQL (Durbin, 1987). Laser ablation studies
have shown that PVPR must be present to establish a left ventral cord;
in its absence, PVQL crosses to the right side of the ventral cord, and
AVKR and RMEV do not extend on the left side (Durbin, 1987). The HSN
axons arrive in the ventral nerve cord in the L2 stage but do not grow
further until the L4 stage, a period of about 24 hours (Garriga \textit{et al.},
1993). Ablation of AVKR in the L2 stage has no apparent effect on HSNL
outgrowth, but ablation of PVPR, PVQL, or both in the L2 stage (well
after the left ventral cord is established) causes the left HSN axon to
cross to the right side of the ventral cord and run in close proximity
to the right HSN, as detected by immunofluorescence microscopy.

Studies with a PVP-specific monoclonal antibody have shown that
this axon appears normal in \textit{unc-34}, \textit{unc-69}, and \textit{unc-71} mutants, but that
in \textit{unc-76(rh116)} and \textit{unc-76(e911)}, the left and right PVP axons
frequently run together on the same side of the ventral nerve cord (C.
Norris, H. Bhatt, and E. Hedgecock, personal communication).
Approximately 36% of HSNL axons in unc-76(e911) mutants cross to the right side of the ventral nerve cord, which could be explained by a partially-pentetrant PVP outgrowth defect. However, 90% of HSNs in unc-76 mutant animals fail to extend to the head, indicating that at least some of the short axons cannot be attributed to PVP guidance defects. Furthermore, while premature PVP termination is observed in unc-51 and unc-73 mutants, the PVP axons appear to be full-length in unc-34, unc-76, unc-69, and unc-71 mutants. Premature HSN termination in these mutants thus does not arise from premature termination of the pioneer axons. Likewise, the frequent shift of HSNL to the right side of unc-34 ventral cords cannot be explained by a shift of PVPR to the right side. The morphology of the PVQ axons in unc-34 and unc-76 mutants has not been determined. A failure of PVQ to follow PVP properly in the same way proposed for the HSN above could also account for the HSN defects if the HSN follows a defective PVQ. However, laser ablations suggest that at least at the point of entry to the ventral cord, the HSN is more dependent upon PVP than PVQ (Garriga et al., 1993).

The removal of PVPL from the right side of the ventral nerve cord during embryogenesis does not alter the outgrowth of PVQR, in contrast with the results obtained with the contralateral homologs (Durbin, 1987). Similarly, ablation of PVPL and PVQR in the L2 stage does not impair HSNR outgrowth, again in contrast with the effects of PVPR and PVQL on HSNL. It appears likely that if the HSN outgrowth defects are related to defects in PVP and PVQ, the left HSN is likely to be more severely affected. However, in both unc-34 and unc-76 mutants, left and right HSNs appear to be approximately the same length on average, suggesting that the HSN defects are independent of the pioneer axons.
Ablation of PVPR and PVQL does not cause premature termination of HSNL, so the unc-34 and unc-76 mutations do not simply cause the HSNs to behave as if their pathway is completely absent. A pathway that is still present but that lacks specific guidance information could support axonal extension by (nonspecific) adhesive interactions, perhaps leading to slower axonal extension or termination. Observation of axons in grasshopper and Japanese medaka fish suggest that some follower axons whose pathways have been ablated do extend on other substrates but not as quickly or as far as they do in normal bundles (Raper et al., 1984; Bastiani and Goodman, 1986; du Lac et al., 1986; Kuwada, 1986).

Fasciclin II mutants in Drosophila show improper association of axons in fascicles, but the axons are able to extend (Goodman and Shatz, 1993). Thus, the absence of specific labels on the HSN pathway but the presence of nonspecific or secondary labels could produce the HSN morphology observed in unc-34 and unc-76 mutants.

The timing of axonal outgrowth has been shown to be important for specific fascicle formation in the grasshopper central nervous system. Growth cones that do not meet with their normal fascicles until later than normal sometimes extend normally, but in some cases the positions of glial cells along the pathway changes and the axons no longer serve as a permissive substrate. Populations of chick axons downregulate their surface expression of TAG-1 independently of the substratum on which they are growing (Karagogeos et al., 1991), suggesting that the timing is cell-autonomous. Delayed outgrowth of such an axon might cause it to reach its target after the appropriate cell-surface molecule was no longer present.
Experiments in vertebrate embryos suggest that one consequence of failure to follow a normal substrate is a tendency for growing axons to respond to cues that cause them to leave their pathway. About half of the axons of the zebrafish brain nucPC cluster are capable of extending along nearby axonal bundles if their preferred pathway, the tPOC axons, is removed, but they frequently pause or leave these alternative fascicles at specific points, as if the absence of their normal pathway leaves them prone to follow certain extracellular cues that remain (Chitnis et al., 1992). If unc-34 and unc-76 axons do stop prematurely, it is possible that features of the terrain provide signals to do so that they normally ignore. However, there must be multiple stop sites if this is true, because HSN axons in these mutants appear able to stop at positions throughout the ventral nerve cord. In addition, left and right HSNs are exposed to relatively different neighbors aside from PVP and PVQ, suggesting that the potential sources of stop signals are different in the two fascicles, but the average lengths of HSNL and HSNR axons arising from normally-positioned cell bodies are similar in unc-34 and unc-76 mutants. The existence of small numbers of specific stop signals thus appears unlikely.

Implications of independent gene functions

Analysis of double mutant combinations of unc-34, unc-71, and unc-76 suggest that each gene provides an independent contribution to the overall process of axonal extension (McIntire et al., 1992). Although molecular confirmation of the loss of gene function is only available for unc-76, the unc-34 alleles studied appear to be null by genetic
criteria, and the alleles of unc-71 studied confer similar HSN defects in homozygotes and deficiency heterozygotes, suggesting that they are severe loss-of-function mutations (McIntire et al., 1992). Mutations in any pair of these genes cause more severe defects in HSN elongation than mutations in any single mutant. This is reminiscent of experiments in vitro in which multiple adhesion molecules appear responsible for supporting axonal extension. Antibody perturbation of cadherin, N-CAM, and β1 integrin reduced axonal extension on astrocyte or muscle surfaces more than application of antibodies to any of these molecules alone (Tomaselli et al., 1988; Bixby et al., 1987). These studies, together with observations that many axons are capable of following alternative pathways in vivo if their normal pathways are missing, suggest that the genetic identification of independent processes in axonal outgrowth is not surprising.

The temperature sensitivity of axonal outgrowth in worms apparently lacking in unc-34 activity implies that the outgrowth of axons is not strictly dependent on the unc-34 gene product. Rather, axons must use the products of other genes to navigate, but this process is less efficient or less thermostable than that mediated by unc-34. The functions represented by unc-76, unc-69, and unc-71 are candidates for involvement in this temperature-sensitive process. Assessment of the temperature sensitivity of double mutant combinations between unc-34 and unc-76, unc-71, and unc-69 could indicate if any of these is involved in the temperature-sensitive process. Elimination of such a process would be expected to eliminate the temperature sensitivity in the double mutant. unc-71;unc-34 double mutants are extremely unhealthy at higher temperatures, while unc-34 unc-76 double mutants and all of
the single mutants are not (G. Garriga, personal communication),
suggesting that unc-76 could be involved in the thermolabile process.

The isolation of suppressor mutations that compensate for the
absence of unc-34 might provide candidates for genes involved in this
process. However, suppressor mutations resulting from loss of gene
function must make the thermolabile process more stable or replace it
entirely, suggesting that this approach is unlikely to generate
mutations in genes directly responsible for the thermolabile process.
Overexpression of the wild-type unc-76 gene does not appear to suppress
unc-34 (e566). The extreme subtlety of phenotype exhibited by unc-
34(e566) suppressor mutations alone has made analysis of their function
difficult. Further investigation of the weak Egl suppressor, n1963,
which can suppress multiple unc-34 alleles, is warranted.

Longitudinal outgrowth of different axons can be affected independently.

While the genes studied in this chapter share many common
features, it is clear that not all longitudinal axonal elongation is
affected equally by all of these genes. Elongation of the HSNs is
impaired by mutations in unc-34, unc-69, unc-71, and unc-76 but not by
mutations in unc-107. Mutations in unc-34, unc-75, and enu-1, but not in
unc-107, cause some degree of HSNL crossover to the right side of the
ventral cord. Elongation of PHA and PHB is impaired by mutations in
unc-34, unc-69, unc-76, and unc-107 but not by unc-71 mutations. Of the
genes in this group, only unc-76 mutations affect PVP outgrowth.
Furthermore, unc-34 and unc-71 cause defects in HSN cell body migration
rare in the other mutants. Evidence from the patterns of expression of
cell-surface adhesion molecules in vertebrate and invertebrate nervous systems supports the notion that overlapping combinations of surface cues, rather than cues unique to individual sets of cells, are likely to specify pathways. The identification of mutations that affect similar but not completely overlapping subsets of neurons suggests that the same could be true in *C. elegans*.

Implications of tax-2 suppression

No strong suppressors of the *unc-76(e911)* Unc phenotype were obtained from screens of 96,000 haploid genome equivalents, and the strongest of the weak suppressors that were obtained all appear to be informational suppressors of the *smg* class. These observations suggest that mutations that bypass the requirement for *unc-76* in axonal outgrowth, at least for axons required for normal locomotion, are difficult or impossible to obtain. The loss of function of another gene is therefore unlikely to compensate for the loss of *unc-76* function.

However, specific defects in *unc-76* mutants, the HSN, PHA, and PHB elongation defects, can largely be overcome by mutations in *tax-2* without apparent effect on *unc-76* locomotory defects. In *tax-2* mutant animals, PHA and PHB axons extend farther than normal in the ventral nerve cord, and amphid sensory axons leave their commissure at aberrant locations (C. Bargmann, personal communication), but the HSN axons appear normal. It is possible that *tax-2* normally provides a restraint on the extension of certain axons and that this restraint is responsible for the shortened axons observed in *unc-76* mutants. The absence of this restraint in *tax-2* mutants then restores the ability of HSN, PHA, and
PHB axons to elongate. Like the observation of full-length extension of HSN axons in unc-40;unc-76 double mutants, this result suggests that the HSNs in unc-76 mutants are not intrinsically incapable of extending full-length axons.

What might this restraint be? A surface adhesion molecule could constrain axons to stay in specific paths, and one consequence of its elimination could be the spreading of axons into incorrect paths, such as is observed when N-CAM function is impaired in vivo by injection of antibodies or endosialidase (Thanos et al., 1984; Tang et al., 1992; Fraser et al., 1988). Inhibition of fasciculation of retinal axons in vitro with antibodies against cell adhesion molecules can actually increase axon length, suggesting that adhesion with other axons in a fascicle impedes extension (Cervello et al., 1991; Rathjen et al., 1987a, 1987b). Loss of an adhesion molecule encoded by tax-2 could allow longer extension. An inhibitor of axonal extension could also demarcate boundaries, as demonstrated by experiments in vitro with vertebrate tectal membrane components and in vivo with myelin-associated proteins (Walter et al., 1987; Schwab and Schnell, 1991). Such restraining molecules in C. elegans would presumably be located at the boundary of the amphid commissure, at the termination point of the PHA and PHB axons, and, in unc-76 mutants, at points at which the PHA, PHB, and HSN axons terminate. Alternatively, the suppression of unc-76 by tax-2 could indicate that novel permissive substrates for HSN, PHA, and PHB elongation, such as additional axons, are introduced into the ventral cord. Ultrastructural examination of ventral nerve cords in tax-2 and tax-2;unc-76 could shed light on this issue.
The existence of a suppressor that affects only a subset of the neurons that are abnormal in unc-76(e911) mutants suggests that not all of the axonal outgrowth processes for which unc-76 is required use the same set of molecules. In addition, it suggests that future screens for unc-76 suppressors might be more successful if they focus on smaller subsets of neurons affected by unc-76 mutations. The phasmid neurons are particularly attractive candidates for such a screen because they can be visualized in living animals with the vital dye DiO, allowing rapid screening of large numbers of animals. Mutations in at least one suppressor gene, tax-2, would be expected to be obtained in such a screen.

The failure of tax-2 mutations to suppress the defects in PHA and PHB caused by unc-34(e315) and unc-69(e602) (C. Bargmann, personal communication) suggests that in these mutants, the premature phasmid axon termination results from a different mechanism than the similar defect in unc-76 mutants. The effect of tax-2 on HSN outgrowth in these mutants has not been determined.

Conclusions

The identification of mutants with defective HSN, PHA, and PHB morphologies has led to the recognition that certain genes function in specific environments and others are necessary for axonal extension irrespective of environment. Among the genes necessary for neuronal bundle assembly, at least three, unc-34, unc-71, and unc-76, appear to function independently and to affect distinct but overlapping subsets of cells. These properties are reminiscent of those predicted for the
multiple cell adhesion systems present in vertebrate axons, and a molecular analysis of these genes should indicate the degree to which the parallels exist between vertebrates and C. elegans.

Acknowledgements

The hard work of Erika Hartwig and Lisa Delissio in preparing and analyzing over 100 electron micrographs of nerve rings was greatly appreciated. I thank Steve McIntire, Gian Garriga, Michael Basson, Dcrit Ginsberg, Chand Desai, and Joe Culocti for sharing data and ideas.

References


Table 2-1

HSN morphology in unc-34 mutants

HSN lengths were measured as described in Experimental Procedures. Axons growing from normally-positioned cell bodies were considered separately from axons growing from migration-defective cell bodies. HSN length was expressed as a percentage of the distance from the vulva to the posterior of the posterior pharyngeal bulb ± 95% confidence limit (calculated assuming a normal distribution). Many samples contained too few axons for significance, but all of the data were included to illustrate trends. % Mig HSNs, percentage of HSNs that migrated 80% of the normal distance or less; % lateral axons, percentage of HSNs with axons that did not grow ventrally into the ventral nerve cord; % animals with HSN crossover, percentage of animals in which one of the HSN axons crossed to the contralateral side of the ventral cord. Only animals in which both axons and cell bodies were visible were included in this last category. Axons that were not visible for their entire lengths were included in the % Mig and % lateral categories. e315, e566, e951, s138, n1877, n1888, n1889, and n1890 are alleles of unc-34. e1490 is an allele of the him-5 gene, which produces males at high frequency but does not affect the nervous system. e224 is a recessive allele of dpy-11. sDf32 is a genetic deficiency deleting unc-34 and at least one gene on either side (Rosenbluth et al., 1988).
<table>
<thead>
<tr>
<th>Genotype</th>
<th>Temp (°C)</th>
<th>% Mig HSNS</th>
<th>% Lateral axons</th>
<th>% Animals with HSN crossover</th>
<th>HSN length</th>
<th>% Lateral axons</th>
<th>% Animals with HSN crossover</th>
</tr>
</thead>
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<td>e315</td>
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<td>14 (22)</td>
<td>96 ± 3 (31)</td>
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<td>--</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td>20°</td>
<td>7 (60)</td>
<td>85 ± 4 (48)</td>
<td>45 (22)</td>
<td>43 ± 38 (3)</td>
<td>0 (7)</td>
<td>100 (3)</td>
</tr>
<tr>
<td></td>
<td>25°</td>
<td>20 (76)</td>
<td>70 ± 5 (44)</td>
<td>100 (17)</td>
<td>33 ± 23 (7)</td>
<td>0 (7)</td>
<td>100 (17)</td>
</tr>
<tr>
<td>e224e315/sDf32</td>
<td>15°</td>
<td>20 (50)</td>
<td>99 ± 2 (26)</td>
<td>0 (6)</td>
<td>85 ± 24 (2)</td>
<td>0 (3)</td>
<td>100 (1)</td>
</tr>
<tr>
<td></td>
<td>20°</td>
<td>33 (43)</td>
<td>89 ± 11 (8)</td>
<td>83 (6)</td>
<td>73 ± 14 (4)</td>
<td>0 (7)</td>
<td>100 (3)</td>
</tr>
<tr>
<td></td>
<td>25°</td>
<td>43 (40)</td>
<td>68 ± 8 (17)</td>
<td>100 (5)</td>
<td>47 ± 14 (7)</td>
<td>7 (15)</td>
<td>78 (9)</td>
</tr>
<tr>
<td>e566</td>
<td>15°</td>
<td>21 (309)</td>
<td>98 ± 2 (39)</td>
<td>20 (5)</td>
<td>91 ± 9 (8)</td>
<td>14 (14)</td>
<td>67 (6)</td>
</tr>
<tr>
<td></td>
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<td>26 (135)</td>
<td>83 ± 4 (74)</td>
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<td>67 ± 15 (14)</td>
<td>0 (31)</td>
<td>77 (22)</td>
</tr>
<tr>
<td></td>
<td>25°</td>
<td>47 (209)</td>
<td>76 ± 6 (42)</td>
<td>100 (4)</td>
<td>53 ± 9 (23)</td>
<td>53 (57)</td>
<td>81 (21)</td>
</tr>
<tr>
<td>e224e566/sDf32</td>
<td>15°</td>
<td>21 (106)</td>
<td>88 ± 4 (70)</td>
<td>62 (26)</td>
<td>74 ± 24 (7)</td>
<td>22 (22)</td>
<td>68 (19)</td>
</tr>
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<td>33 (58)</td>
<td>57 ± 9 (29)</td>
<td>50 (10)</td>
<td>57 ± 30 (6)</td>
<td>25 (16)</td>
<td>100 (6)</td>
</tr>
<tr>
<td>e951</td>
<td>20°</td>
<td>34 (68)</td>
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<td>100 (5)</td>
<td>61 ± 16 (11)</td>
<td>7 (14)</td>
<td>50 (4)</td>
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<td></td>
<td>25°</td>
<td>41 (93)</td>
<td>51 ± 9 (29)</td>
<td>50 (4)</td>
<td>38 ± 20 (8)</td>
<td>33 (27)</td>
<td>86 (14)</td>
</tr>
<tr>
<td>s138</td>
<td>20°</td>
<td>41 (230)</td>
<td>86 ± 5 (28)</td>
<td>50 (8)</td>
<td>93 ± 8 (8)</td>
<td>5 (22)</td>
<td>93 (14)</td>
</tr>
<tr>
<td></td>
<td>25°</td>
<td>30 (74)</td>
<td>70 ± 12 (10)</td>
<td>50 (4)</td>
<td>40 ± 39 (4)</td>
<td>50 (16)</td>
<td>80 (5)</td>
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<td>82 (11)</td>
<td>62 ± 14 (6)</td>
<td>0 (9)</td>
<td>60 (5)</td>
</tr>
<tr>
<td></td>
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<td>30 (74)</td>
<td>72 ± 7 (25)</td>
<td>50 (8)</td>
<td>53 ± 12 (6)</td>
<td>56 (18)</td>
<td>50 (4)</td>
</tr>
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<td>89 ± 6 (17)</td>
<td>40 (5)</td>
<td>58 ± 55 (3)</td>
<td>27 (11)</td>
<td>100 (5)</td>
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<tr>
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<td>25°</td>
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<td>69 ± 7 (23)</td>
<td>75 (4)</td>
<td>53 ± 11 (3)</td>
<td>24 (18)</td>
<td>88 (8)</td>
</tr>
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<td>38 (237)</td>
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<td>0 (16)</td>
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<td>25°</td>
<td>47 (219)</td>
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<td>47 (14)</td>
<td>43 (28)</td>
<td>90 (10)</td>
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<tr>
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<td>50 (4)</td>
<td>75 ± 8 (2)</td>
<td>0 (8)</td>
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<td></td>
<td>25°</td>
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<td>48 ± 14 (5)</td>
<td>50 (20)</td>
<td>71 (7)</td>
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Table 2-2

HSN axon morphology in unc-76 mutants

HSN axon lengths were analyzed in the same way used for the data in Table 2-1. Too few migration-defective axons were visible to allow interpretation of their axon morphologies. Deficiency heterozygotes were constructed as described in Experimental Procedures. e30 is an allele of *sma-1*, *e224* is an allele of *dpy-11*, and *e270* is an allele of *unc-42*; all three markers are fully recessive. The deficiency *yDf8* deletes *unc-76* and genes both to the left (*unc-61*) and right (*dpy-21*; L. DeLong and B. Meyer, personal communication).
## Table 2-2

HSN axon morphology in unc-76 mutants

<table>
<thead>
<tr>
<th>genotype</th>
<th>temp (°C)</th>
<th>% Mig HSNs</th>
<th>HSN length (μm)</th>
<th>% lateral axons</th>
<th>% animals with HSN crossover</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>% (n)</td>
<td>± (n)</td>
<td>% (n)</td>
<td>± (n)</td>
</tr>
<tr>
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<td>20°</td>
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<td>66 ± 3 (55)</td>
<td>5 (58)</td>
<td>36 (28)</td>
</tr>
<tr>
<td></td>
<td>25°</td>
<td>13 (23)</td>
<td>78 ± 2 (17)</td>
<td>6 (18)</td>
<td>29 (7)</td>
</tr>
<tr>
<td>e224/e911</td>
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<td>0 (46)</td>
<td>73 ± 3 (46)</td>
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<td>29 (21)</td>
</tr>
<tr>
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<td></td>
<td></td>
<td></td>
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<tr>
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<td>2 (55)</td>
<td>64 ± 4 (47)</td>
<td>13 (54)</td>
<td>25 (12)</td>
</tr>
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<tr>
<td>rh116</td>
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<td>4 (69)</td>
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<td>7 (60)</td>
<td>15 (20)</td>
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<td>1 (84)</td>
<td>79 ± 4 (57)</td>
<td>8 (62)</td>
<td>36 (22)</td>
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</tr>
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<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>n2397</td>
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<td>77 ± 3 (90)</td>
<td>6 (96)</td>
<td>29 (35)</td>
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<td>76 ± 6 (42)</td>
<td>0 (54)</td>
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</tr>
<tr>
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<tr>
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</tr>
<tr>
<td>e30 n2457</td>
<td>20°</td>
<td>0 (55)</td>
<td>77 ± 5 (44)</td>
<td>0 (57)</td>
<td>44 (25)</td>
</tr>
<tr>
<td>+ yDf8</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 2-3

Phasmid defects in unc-34 mutants

Animals were stained with FITC (see Experimental Procedures) and their phasmid axons in the ventral cord were observed. Axons were roughly categorized on the basis of length: "normal" axons were similar in length to wild-type axon, "short" axons appeared to end at their point of entry into the ventral nerve cord, and "intermediate" axons were clearly shorter than those in wild-type but did extend for a short distance in the ventral nerve cord. Intermediate axons often ended in a large swelling.
<table>
<thead>
<tr>
<th>Genotype</th>
<th>Temperature</th>
<th>Phasmid Axon Length</th>
<th>Number</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Normal (%)</td>
<td>Intermediate (%)</td>
</tr>
<tr>
<td>unc-34</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>e315</td>
<td>15°C</td>
<td>59</td>
<td>34</td>
</tr>
<tr>
<td></td>
<td>25°C</td>
<td>13</td>
<td>60</td>
</tr>
<tr>
<td>e566</td>
<td>15°C</td>
<td>30</td>
<td>48</td>
</tr>
<tr>
<td></td>
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<td>5</td>
<td>13</td>
</tr>
<tr>
<td>e951</td>
<td>15°C</td>
<td>33</td>
<td>54</td>
</tr>
<tr>
<td></td>
<td>25°C</td>
<td>22</td>
<td>13</td>
</tr>
<tr>
<td>n1877</td>
<td>25°C</td>
<td>35</td>
<td>12</td>
</tr>
<tr>
<td>unc-76</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>e911</td>
<td>15°C</td>
<td>0</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>20°C</td>
<td>0</td>
<td>23</td>
</tr>
</tbody>
</table>
Table 2-4

Amber and smg suppression of unc-76 mutant alleles.

Double mutant combinations were constructed between the mutants listed and either dpy-7(e1324ts)sup-7(st5ts) or smg-1(e1228)him-2(e1065). Mutants were considered suppressed (+) if their locomotion was clearly more normal than that of the homozygous mutants alone. sup-7 suppression was scored at 22.5°C, and smg-1 suppression was scored at 20°C. nd, not determined
Table 2-4  Amber and smg suppression of unc-76 and unc-34 mutant alleles

<table>
<thead>
<tr>
<th>mutant allele</th>
<th>sup-7(st5)</th>
<th>smg-1(e1228)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>unc-76 mutants</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>e911</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>ev424</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>rh116</td>
<td>nd</td>
<td>-</td>
</tr>
<tr>
<td>n2367</td>
<td>nd</td>
<td>-</td>
</tr>
<tr>
<td>n2397</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>n2398</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>n2399</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>n2457</td>
<td>nd</td>
<td>-</td>
</tr>
<tr>
<td><strong>unc-34 mutants</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>e566</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>e951</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>s138</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
Table 2-5

HSN morphology in unc-34(e566)unc-76(e911) double mutants

HSNs were measured and scored as described in the legend to Table 2-1. Animals were raised at 25°C.
**Table 2-5**  HSN morphology in *unc-34(e566) unc-76(e911)* double mutants

<table>
<thead>
<tr>
<th>genotype</th>
<th>Normally-positioned HSNs</th>
<th>Migration-defective HSNs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% Mig HSNs</td>
<td>% lateral with HSN crossover</td>
</tr>
<tr>
<td></td>
<td>n</td>
<td>(n)</td>
</tr>
<tr>
<td><em>unc-34(e566)</em></td>
<td>47 (209)</td>
<td>76 ± 6 (42)</td>
</tr>
<tr>
<td><em>unc-76(e911)</em></td>
<td>13 (23)</td>
<td>78 ± 2 (17)</td>
</tr>
<tr>
<td><em>unc-34(e566) unc-76(e911)</em></td>
<td>39 (72)</td>
<td>35 ± 6 (39)</td>
</tr>
</tbody>
</table>
Table 2-6

Suppression of *unc-34(e566)* HSN defects

Strains isolated in a screen for suppressors of the *unc-34(e566)* Unc phenotype were stained with anti-serotonin antibodies. HSNs in *e566;n1959* were analyzed as described in the legend to Table 2-1. Poor staining precluded accurate measurement of the lengths of the axons in the remaining strains, but cell bodies stained relatively brightly and could be scored unambiguously. n, number of cells scored (for % Mig and HSN length), number of animals scored (for % crossover).
Table 2-6

Suppression of unc-34(e566) HSN defects

<table>
<thead>
<tr>
<th>genotype</th>
<th>temp (°C)</th>
<th>% Mig HSNs</th>
<th>HSN length (n)</th>
<th>% animals with HSN crossover</th>
</tr>
</thead>
<tbody>
<tr>
<td>e566</td>
<td>20°</td>
<td>28 (135)</td>
<td>84 ± 4 (74)</td>
<td>60 (30)</td>
</tr>
<tr>
<td>e566;n1959</td>
<td>20°</td>
<td>7 (27)</td>
<td>98 ± 2 (22)</td>
<td>38 (8)</td>
</tr>
<tr>
<td>e566</td>
<td>25°</td>
<td>47 (209)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>e566;n1960</td>
<td>25°</td>
<td>20 (50)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>e566;n1961</td>
<td>25°</td>
<td>18 (82)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>e566;n1962</td>
<td>25°</td>
<td>32 (53)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>e566;n1963</td>
<td>25°</td>
<td>16 (87)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>e566;n1964</td>
<td>25°</td>
<td>13 (110)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 2-7

HSN axon morphology in suppressed unc-76 strains

Animals were stained with anti-serotonin antibodies, and the cell morphologies and axon lengths were analyzed as described in the legend to Table 2-1. The strains e911;n1734-e911;n1742 were identified in a screen for suppressors of the unc-76 locomotory defects. The tax-2;unc-76 double mutant was constructed by C. Bargmann to test the effects of tax-2 mutations on amphid and phasmid morphology.
Table 2-7

HSN axon morphology in suppressed unc-76 strains

<table>
<thead>
<tr>
<th>genotype</th>
<th>temp (°C)</th>
<th>% Mig HSNs</th>
<th>HSN length (n)</th>
<th>% lateral axons</th>
<th>% animals with HSN crossover</th>
</tr>
</thead>
<tbody>
<tr>
<td>e911</td>
<td>20°</td>
<td>5 (73)</td>
<td>66 ± 3 (55)</td>
<td>5 (58)</td>
<td>36 (28)</td>
</tr>
<tr>
<td>e911; n1734</td>
<td>20°</td>
<td>3 (35)</td>
<td>94 ± 3 (24)</td>
<td>3 (35)</td>
<td>54 (13)</td>
</tr>
<tr>
<td>e911; n1735</td>
<td>20°</td>
<td>3 (32)</td>
<td>89 ± 7 (24)</td>
<td>0 (32)</td>
<td>67 (12)</td>
</tr>
<tr>
<td>e911; n1737</td>
<td>20°</td>
<td>19 (26)</td>
<td>97 ± 3 (21)</td>
<td>0 (29)</td>
<td>70 (10)</td>
</tr>
<tr>
<td>e911; n1738</td>
<td>20°</td>
<td>3 (66)</td>
<td>90 ± 6 (51)</td>
<td>4 (53)</td>
<td>50 (26)</td>
</tr>
<tr>
<td>e911; n1739</td>
<td>20°</td>
<td>0 (25)</td>
<td>97 ± 3 (18)</td>
<td>0 (25)</td>
<td>75 (12)</td>
</tr>
<tr>
<td>e911; n1740</td>
<td>20°</td>
<td>3 (36)</td>
<td>91 ± 5 (32)</td>
<td>0 (36)</td>
<td>20 (15)</td>
</tr>
<tr>
<td>e911; n1742</td>
<td>20°</td>
<td>8 (12)</td>
<td>99 ± 3 (9)</td>
<td>8 (12)</td>
<td>40 (5)</td>
</tr>
<tr>
<td>tax-2(p691)</td>
<td>20°</td>
<td>0 (38)</td>
<td>100 ± 1 (35)</td>
<td>0 (30)</td>
<td>0 (16)</td>
</tr>
<tr>
<td>tax-2(p691); 20°</td>
<td>0 (75)</td>
<td>92 ± 2 (66)</td>
<td>1 (74)</td>
<td>0 (22)</td>
<td></td>
</tr>
<tr>
<td>unc-76(e911)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 2-8

HSN axon morphology in mutants with abnormal phasmd axon outgrowth

Animals were stained with anti-serotonin antibodies and their HSNs measured as described in the legend to Table 2-1. The strain enu-1(ev419);unc-107(ev411) was identified by J. Culotti (personal communication) on the basis of its shortened phasmd axons, similar to those seen in unc-76 mutants. a: unc-107 was stained by Gian Garriga (personal communication) with antibodies against serotonin and tubulin. The HSNs were reported to grow to their normal length and to remain on the correct side of the ventral nerve cord, and no gross disorganization of the ventral cord was observed.
Table 2-8

HSN axon morphology in mutants with abnormal phasmid axon outgrowth

<table>
<thead>
<tr>
<th>genotype</th>
<th>temp (°C)</th>
<th>% Mig HSNs (n)</th>
<th>HSN length (n)</th>
<th>% lateral axons (n)</th>
<th>% animals with HSN crossover (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>unc-76(e911)</td>
<td>20°</td>
<td>5 (73)</td>
<td>66 ± 3 (55)</td>
<td>5 (58)</td>
<td>36 (28)</td>
</tr>
<tr>
<td>unc-34(e566)</td>
<td>20°</td>
<td>28 (135)</td>
<td>84 ± 4 (74)</td>
<td>0 (74)</td>
<td>60 (30)</td>
</tr>
<tr>
<td>enu-1(ev419);</td>
<td>20°</td>
<td>2 (51)</td>
<td>89 ± 5 (25)</td>
<td>4 (46)</td>
<td>94 (17)</td>
</tr>
<tr>
<td>unc-107(ev411)</td>
<td>20°</td>
<td>0 (47)</td>
<td>99 ± 1 (38)</td>
<td>0 (51)</td>
<td>48 (21)</td>
</tr>
<tr>
<td>unc-107(ev411)</td>
<td>20°</td>
<td>-</td>
<td>100&lt;sup&gt;a&lt;/sup&gt;</td>
<td>-</td>
<td>0&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>
HSN axons in wild-type and unc-34 animals

HSN axons were visualized by staining with anti-serotonin antisera. Anterior is to the left in all panels. In wild-type animals (A), the HSNs extend from the cell bodies (HSNL is visible at the far right) ventrally into the ventral nerve cord, and then grow anteriorly into the nerve ring (arrow). Left and right HSNs are visible in this slightly ventral view. B-D. Several characteristic abnormalities in unc-34(e566) animals are visible. The animal in B, seen from the ventral side, has a migration-defective HSNL and a normally-positioned HSNR. The axons join anterior to the vulva and terminate before reaching the head. C. A ventral view of an animal in which both HSNs remain on the proper side of the ventral cord but terminate (arrowheads) before reaching the head. D. An unc-34(e566) animal with a migration-defective HSN that failed to extend its axon into the ventral cord. It makes a branch at the vulva (arrowhead) but wanders on the lateral hypodermis and terminates before reaching the head. Scale: A, B: 1 cm = 34 μm; C: 1 cm = 34 μm; D: 1 cm = 23 μm.
Fig. 2-2

NSM and CP defects in unc-34(n1890)him-5(e1490). A. In wild-type animals stained with anti-serotonin antisera, the two NSM axons extend posteriorly in the pharynx. B. In rare unc-34 mutant animals, one process extends anteriorly. C. Wild-type males have six serotonergic neurons in the ventral cord, all of which extend processes posteriorly. D. The anteriormost CP neuron in this unc-34(n1890)him-5(e1490) male extended anteriorly into the nerve ring (arrow). This defect was observed in 10-30% of unc-34 males examined. Scale: A,B: 1 cm = 24 μm; C,D: 1 cm = 29 μm.
Fig. 2-3.

Electron micrographs of nerve rings in cross section. Animals were fixed and embedded for electron microscopy and sectioned at an oblique angle to the main body axis so that the nerve ring axons were visible in profile. Axonal membranes were traced on acetate sheets and the profiles counted. A., a wild-type nerve ring. B, a nerve ring from unc-76(e911). The pharyngeal isthmus is visible at the bottom of each micrograph, and the axons form a semicircle on the pharyngeal surface. Scale: 1 cm = 625 nm.
Figure 2-4.

Number of nerve ring axonal profiles in wild-type, unc-34, and unc-76 mutants. Axons were counted in multiple sections from several animals of each genotype, and the mean for each animal is indicated as a separate bar. Error bars = 95 percent confidence limits. Five animals each from N2 and unc-76(e911) were observed, and two animals each from unc-34(e566) and unc-76(n2457).
Figure 2-4

Number of nerve ring axonal profiles in wild-type, unc-34, and unc-76 mutants
Figure 2-5.

Distributions of HSN endpoints in unc-34(e566) and unc-76(e911) animals.

HSN lengths were measured to the nearest tenth of the distance from the vulva to the head, as described in Experimental Procedures. The data are presented in histogram form to indicate the range of positions at which the axons end. The lengths of HSN axons arising from normally-positioned and migration-defective cell bodies are considered separately, as are the lengths of HSN axons growing in the ventral nerve cord and in lateral positions. unc-34(e566) animals were grown at 25°C. B. HSN axon lengths in unc-76(e911) animals grown at 20°C. Few HSNs had laterally-positioned axons or migration-defective cell bodies, and these were not included.
Figure 2-5
Distributions of HSN endpoints in \textit{unc-34(e566)} and \textit{unc-76(e911)} animals

\textbf{A. \textit{unc-34(e566)}, 25°C}

\textit{ventral HSN axons, normally-positioned cell bodies}

\textit{ventral HSN axons, migration-defective cell bodies}

\textit{lateral HSN axons, migration-defective cell bodies}

\textbf{B. \textit{unc-76(e911)}, 20°C}

\textit{ventral HSN axons, normally-positioned cell bodies}
unc-34 temperature shift experiments

Adults were allowed to lay eggs for brief periods at either 15\degree C or 25\degree C, and the resulting synchronized broods were shifted to the opposite temperature at the times indicated. Animals were scored for their locomotory phenotype (A) or stained with anti-serotonin antisera and the length of their HSN axons were measured (B). Only HSNs arising from normally-positioned cell bodies and growing in the ventral nerve cord were considered. 15-43 individual axons were scored for each time point. Error bars are 95\% confidences limits.
Figure 2-6  *unc-34* temperature shift experiments

A. *unc-34* temperature shift experiment: Unc phenotype of *e315ts*

\[\text{Graph showing the percentage of unc animals over time of shift.} \]

- **25→15**
- **15→25**

B. *unc-34* temperature-shift experiments: HSN length

- **e315**
- **e566**

\[\text{Graphs showing the mean HSN length over time of shift.} \]
Suppression of the unc-76 phasmid defect by tax-2

Phasmid axons were stained with diO and observed by epifluorescence microscopy. Phasmid axons terminate at the arrowhead in each photograph. A. Lateral view of a wild-type tail. B. Phasmids in unc-76(e911) mutants terminate as soon as they reach the ventral nerve cord, as seen in this dorsal view. C. Phasmids in tax-2 animals are often longer than normal. D tax-2(p691);unc-76(e911) animals frequently have phasmid axons of normal length. Scale: A: 1 cm = 11.5 μm. B,C,D: 1 cm = 9 μm.
Chapter 3  Molecular analysis of unc-76
Summary

The gene unc-76 is necessary for normal axonal bundling and elongation of axons within bundles in the nematode C. elegans. We describe the cloning of unc-76 and the analysis of its expression pattern. The gene encodes a 385 amino acid polypeptide that has no similarity to other known proteins, and thus represents a new class of axonal outgrowth molecules. The protein appears in the cell bodies and processes of all neurons during initial axonal outgrowth and persists throughout the life of the animal, but its presence does not appear to be sufficient to induce axon bundling. We propose that the protein plays a role either in signal transduction or formation of axon structures required specifically within fascicles.
Introduction

Association of axons in specific fascicles is believed to play a major role in the assembly of both vertebrate and invertebrate nervous systems (Bastiani et al., 1984; Raper et al., 1983a, 1983b; Kuwada, 1986; Chitnis et al., 1991, 1992). Candidates for molecules involved in this process have been identified by screens for antibodies that label specific subsets of axons in rat, chick, grasshopper, or Drosophila nervous systems (Bastiani et al., 1987; Dodd et al., 1988; Rathjen et al., 1987a; Patel, 1987; Kolodkin et al., 1992). These antibodies have led to the identification of a number of cell-surface glycoproteins, many of them members of the immunoglobulin superfamily, that can mediate cell-cell adhesion or support axonal extension in vitro. Antibody perturbation of several of these molecules in vitro (F11 and L1; Rathjen et al., 1987b; Chang et al., 1987) and in vivo (fasciclins I, II, and IV; Jay and Keshishian, 1990; Harrelson et al., 1988; Kolodkin et al., 1992) or elimination of molecular function by mutation (fasciclins I, II, and III; Grenningloh et al., 1991; Elkins et al., 1990) indicates that these adhesion molecules can function in the formation of fascicles.

Antibody screens to identify molecules involved in fasciculation have focused on cell-surface molecules, and relatively little is known about the intracellular mechanisms by which surface interactions can lead to elongation of axons specifically on other axonal surfaces. Genetic screens for fasciculation-defective mutants can in principle identify molecules involved in fasciculation without presuppositions as
to the biochemical nature or subcellular localization of these molecules. The nematode *C. elegans* is a particularly useful system for a genetic approach to the study of axonal pathfinding because it has a small, well-described nervous system and it is easily manipulated genetically, making possible the isolation of mutations affecting axonal outgrowth.

Studies of the existing set of behavioral mutants with reagents that allow the observation of the morphology of a small set of neurons have identified several groups of genes required for extension of axons in distinct environments (McIntire et al., 1992). One of these groups, consisting of the genes *unc-5*, *unc-6*, and *unc-40*, is necessary for extension of axons between the cells of the lateral hypodermis (body wall epidermis) and the basement membrane secreted by these cells (Hedgecock et al., 1990). Two of these genes have been cloned and show similarity to components of the extracellular matrix or their receptors (Ishii et al., 1992; Leung-Hagesteijn et al., 1992). The second group, defined by the genes *unc-34*, *unc-71*, and *unc-76*, is required for axonal extension within fascicles. Mutants in this second class have normal axon growth along the lateral hypodermis; thus, the two sets of genes define two independently-controlled environments for axon outgrowth. Mutants in a third class of genes (*unc-14*, *unc-33*, *unc-44*, *unc-51*, and *unc-73*) show more general defects in axonal outgrowth and are likely to define molecules important in axonal elongation per se (McIntire et al., 1992; Li et al., 1992; Otsuka, in preparation; Oshima, R. Steven, A. Ruiz, J. Mancillas, and J. Culotti, personal communication).

Detailed studies of the morphologies of a small number of neurons that can be visualized by histochemical stains have revealed two types
of defects observed in the fascicle-specific group: several axons fail to extend fully within the axon bundles of the dorsal and ventral nerve cords, and several axons fail to remain in their normal fascicles (McIntire et al., 1992; Hedgecock et al., 1985). It has been proposed that the fasciculation and extension defects are two manifestations of an underlying defect in axon guidance or adhesion. Experiments using the axons of one neuronal type, the HSN motorneurons, have indicated that the block to HSN axon extension in these mutants can occur anywhere in the ventral nerve cord, and is thus not simply the result of a physical barrier to axon outgrowth (McIntire et al., 1992). In addition, removal of the HSN axon from the ventral nerve cord by a second mutation allows the axon to grow to its normal length, indicating that the unc-34 and unc-76 genes affect the interaction of this axon with its ventral cord environment and not simply the ability of this axon to grow beyond a certain length.

The most severely defective of the fasciculation mutants is unc-76. The HSNs in unc-76 mutant animals are only about 67 percent of their normal length, and axons of sensory cells in the tail, PHA and PHB, end almost as soon as they reach the ventral nerve cord (Desai et al., 1988; McIntire et al., 1992; Hedgecock et al., 1985). The total number of axons in the ventral cord is lower than in wild-type, indicating that the phenomenon of premature axon termination is widespread in these mutants. In addition, defasciculation of specific groups of motor and sensory axons as well as the breakup of the ventral cord into several small bundles has been described (McIntire et al., 1992).
To understand the basis of this environment-specific effect on axonal outgrowth, we have undertaken a molecular analysis of the unc-76 gene. We report here that unc-76 encodes a protein that is localized to neuronal cell bodies and axons and bears no resemblance to previously-identified proteins.

Results

Isolation of unc-76 genomic clones

In order to understand the role of the unc-76 gene product in axonal outgrowth, we cloned the gene by germline transformation, taking advantage of the severe uncoordination of unc-76 mutants. We identified clones containing the unc-76 gene by testing cosmid clones for their ability to restore the locomotion of unc-76(e911) animals to wild-type in germline transformation experiments. The cosmid C25D7 had previously been shown to correspond to a region of DNA rearranged by the deficiency yDf11, which has a breakpoint less than 0.1 map units to the left of unc-76 (B. Klein and B. Meyer, personal communication). We examined clones in the region of C25D7 on the C. elegans physical map (Coulson et al., 1986) and found that each of five overlapping cosmids, C56C4, T25A9, C08C1, C01G10, and C13G10, independently rescued the Unc phenotype of unc-76(e911) animals, while C25D7 and B0289 on the left of this group and T06H10, T01G5, and C28G7 on the right did not (Fig. 3-1). From the 27 kb region common to these cosmids, a 10.7 kb XbaI fragment, represented in clones p76-16A and p76-16B, gave complete rescue of unc-76(e911) animals, returning locomotion to wild-type. Lines carrying any
of several subclones as small as 5.5 kb (p76-14) showed partial rescue, with wild-type movement evident among young larvae but increasing uncoordination in older animals. Smaller subclones (p76-8AHind, p76-7) failed to rescue unc-76(e911) mutants, as did a derivative of the rescuing cosmid C01G10 from which a 1.4 kb KpnI fragment was deleted. These experiments identified a minimal rescuing region of 10.7 kb, with an internal 5.5 kb SacII-HindIII region sufficient for partial rescue. In subsequent experiments, this 10.7 kb region rescued unc-76(n2397), which encodes a shorter form of the Unc-76 protein than does unc-76(e911)(see below), but p76-10, which contains a 7 kb SacII-BamHI insert, rescued unc-76(n2397) animals much more poorly than it did unc-76(e911) animals.

**Identification of cDNA clones**

An 8.6 kb fragment of genomic DNA from the rescuing region was used as a probe of 220,000 plaques from a mixed-stage C. elegans cDNA library (Kim and Horvitz, 1990). Of the five cDNA clones isolated, three hybridized to a 1.4 kb region of the 8.6 kb genomic clone not necessary for rescue. The remaining two clones, p76-c4 and p76-c7, hybridized to sequences throughout the rescuing clone and were thus considered to be likely candidates for unc-76 cDNA clones. When the insert from either p76-c4 (2.6 kb) or p76-c7 (1.7 kb) was used as a probe of Northern blots of poly-A(+) RNA from wild-type embryos, a major transcript of 1.8 kb was observed (Fig. 3-2). This transcript was reduced in abundance in unc-76(e911) embryos, suggesting that it does represent the unc-76 transcript. A faint band at approximately 2.5 kb

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in samples of wild-type RNA hybridized to either the p76-c4 or the p76-c7 inserts was observed in long exposures (Fig. 3-2).

**Sequence analysis of unc-76**

We determined the sequences of both cDNA clones and the 10.7 kb genomic clone (Fig. 3-3). Each cDNA contained a single large open reading frame beginning at the 5′ end. The p76-c7 clone contained a 39 nucleotide poly-A stretch at the 5′ end not found in genomic DNA, possibly an artifact of primers used in cDNA cloning, as well as a 53-residue poly-A tail at the 3′ end, starting at nucleotide 9377 of the genomic sequence, 16 nucleotides after an ATAAA sequence. The 5′ end of p76-c4 began 119 nucleotides before the inferred start of p76-c7 and matched the genomic DNA sequence beginning at nucleotide 1002. The open reading frame following the p76-c7 5′ poly-A sequence matched exactly that of p76-c4 for 347 codons, and comparison of the cDNA and genomic sequences showed that the two cDNA clones differed primarily by the inclusion or exclusion of an intron near the 3′ end of the gene (Fig. 3-4).

The inferred p76-c7 sequence, excluding the 5′ poly-A sequence, was 1636 nucleotides long. This was slightly smaller than the observed 1.8 kb transcript, and its identity to the longer p76-c4 sequence at the 5′ end suggested that the major unc-76 transcript began approximately 100-200 nucleotides before the 5′ end of p76-c7. Although the genomic sequence upstream of the p76-c7 start site contained a potential 62-codon open reading frame that continued into that shared by p76-c4 and p76-c7, the first possible ATG codon within that open reading frame
began within p76-c4, at nucleotide 1089, 18 nucleotides before the inferred start of p76-c7. Based on these sequence data and the observed transcript sizes, we inferred that the major unc-76 transcript encodes a polypeptide of 385 amino acids corresponding to the p76-c7 3' end splice pattern, while p76-c4 represents a minor 376-amino acid species.

A search of the the Swissprot and PIR databases using the BLAST and fasta programs revealed no significant similarity of either predicted unc-76 gene product to any other protein. The predicted proteins are highly hydrophilic overall. Hydrophobicity plots showed no hydrophobic stretches long enough to serve as membrane-spanning or signal-sequence domains. No motifs for calcium or nucleotide binding were found. Consensus protein kinase C phosphorylation sites (1-5 basic-S/T-uncharged-R/K) were identified at amino acids 30, 137, 143, and 144. The unc-76 protein appears to define a new class of molecules involved in axonal outgrowth.

To determine whether the two alternative C termini had different functions, we constructed derivatives of p76-16B that lacked coding sequences unique to one or the other of the 3' ends. p76-22 substituted the last two exons of p76-c7 for the equivalent region of genomic DNA, eliminating the final 22 codons of p76-c4. p76-24 was a derivative of p76-16B from which a 337 bp KpnI–SpeI fragment (containing the last exon of p76-c7 including its splice acceptor and stop codon) was removed. Germline transformation of unc-76(e911) with either construct produced lines with non-Unc animals rescued as well as animals carrying the intact p76-16B. However, the range of locomotion among transgenic animals was more normal in lines carrying p76-22 than in those carrying p76-24, suggesting that the C terminus represented in p76-c7 is
necessary and sufficient for full rescue of unc-76(e911). This is consistent with the observation that transcripts of the 2.6 kb size predicted by p76-c4 were present in considerably lower abundance than the 1.8 kb (p76-c7-sized) transcripts in mixed-stage RNA.

Reverse transcriptase-PCR analysis confirmed the existence of RNA corresponding to both the p76-c4 and p76-c7 patterns. Two primers, one corresponding to the intron spliced out of p76-c7 and retained in p76-c4 (primer c4B) and the other to the last exon of p76-c7 (exon 9; primer c4D), were annealed to separate populations of total C. elegans RNA and used to produce specific cDNA species (Fig. 3-3). These were amplified by PCR using pairs of primers on opposite sides of each splice junction, i.e. primer c4B with a primer (c4A) in exon 8, and primer c4D with primer c4A or a primer in the alternatively-spliced intron (c4C). All pairs gave rise to amplified products of the size predicted if the intron is present, indicating that the intron is retained in at least some transcripts. The c4A-c4D pair gave rise to an amplified product of the size predicted from the p76-c7 splice pattern.

RNase protection experiments showed that the p76-c7 splice pattern is the major splice form. Total RNA was annealed to a radiolabeled probe complementary to exon 8 and overlapping the potential alternative splice site, and the hybrids were digested with RNase A and RNase T1. The major 212-nucleotide product was observed, consistent with the product expected if the p76-c7 splice site was used (data not shown). Only a faint band at 300 nucleotides, expected from the p76-c4 pattern, was observed.

**Determination of the unc-76 transcriptional start site**
The cDNA clone p76-c4 contained no in-frame stop codons upstream of the first ATG codon, and inspection of the unc-76 genomic sequence showed that a potential open reading frame extended as far as 56 codons before the start of p76-c4. This potential open reading frame lacked an ATG codon before the start of p76-c4, but contained a site (CCTCAG/TTT) with partial similarity to the consensus C.elegans splice acceptor sequence (TTTCAG/NNN). To determine the beginning of the unc-76 coding region, the transcriptional start site of the unc-76 gene was investigated by a combination of primer extension and PCR. Primers corresponding to nucleotides 1108-1137 (76-PE-1) and 1027-1057 (76-PE-2) in the genomic sequence were end-labeled with $\gamma^{32}$P-ATP, annealed to total or poly-A (+) fractions of N2 RNA, and extended with AMV reverse transcriptase, and the products were analyzed on a sequencing gel (Fig. 3-6). No extension products were observed with primer 76-PE-2. Extension products of 38-46, 57, 75, and 119 nucleotides, corresponding to sites within the region represented in the cDNA clone, p76-c4, were observed in two independent experiments with primer 76-PE-1, as were bands at 156-157 and approximately 440 and 550 nucleotides. Northern blots probed with p76-c4 or p76-c7 did not reveal transcripts at the 2.0-2.1 kb size predicted by these larger primer extension products (Fig. 3-2). Start sites corresponding to the lower molecular weight primer extension products (nucleotides 980-981, 1018, 1062, and 1080) were judged most consistent with the 1.8 kb major transcript observed on Northern blots.

Reverse transcriptase-PCR analysis subsequently suggested that at least three classes of transcripts exist. Two classes are trans spliced
to the 22 nucleotide leader SL1 at nucleotides 1084 and 1102, and the third class appears to be colinear with the genomic DNA from at least nucleotide 620 of the genomic DNA to the end of the first exon. cDNA was made from total C. elegans RNA annealed to the primer 76-E1E2, which spans the boundary between exons 1 and 2 (7 nucleotides of homology with exon 1). A primer corresponding to the spliced leader SL1 was used for PCR amplification of the resulting cDNA, and from the prominent 620 nucleotide band, two PCR products were cloned and sequenced. These corresponded to the SL1 leader spliced to unc-76 at nucleotides 1084 and 1102, respectively, consistent with the prominent primer extension products of 57 and 75 nucleotides. These would yield transcripts of 1679 and 1661 nucleotides, respectively.

A series of primers corresponding to sites in the unc-76 5' end was used for PCR amplification with cDNA made with the primer 76-E1E2. Primers starting at nucleotides 620 (primer End-7), 743 (End-8), 936 (End-1), 955 (End-2), 979 (End-3), and 1018 (End-4) in the genomic sequence produced products of the size expected for a transcript colinear with the genomic DNA, while primer End-6 (nucleotides 377-397) did not give rise to an amplified unc-76 region product (data not shown). Because the downstream primer in all cases was 76-E1E2, which spans the exon1-exon 2 splice junction, it is unlikely that contaminating genomic DNA was amplified in these experiments. These results suggest that the highest molecular weight primer extension product arose from transcripts starting between nucleotides 377 and 620 of the genomic sequence. No PCR products indicating the use of the potential splice acceptor at nucleotide 960 were identified. These results, taken together, indicate that the Unc-76 protein is likely to
begin with the methionines encoded by nucleotides 1090-1092 (as indicated in Fig. 3-5) or nucleotides 1111-1113, because any potential transcripts longer than the SL1-spliced transcripts identified would not contain an in-frame methionine codon.

**Determination of unc-76 mutant allele sequences**

To demonstrate that the cloned DNA is the unc-76 gene, we analyzed the sequences of putative coding DNA and splice junctions from eight mutant unc-76 alleles. Six sets of PCR primers (described in Experimental Procedures) were used to amplify DNA from pools of six worms (B. Schrank and B. Waterston, personal communication), and the resulting PCR products were either subjected to asymmetric PCR and direct sequencing (for primer sets 1, 3 and 5) or they were cloned in the pCR II vector (Stratagene) and sequenced from single-stranded phage DNA. Mutations were confirmed by direct cycle sequencing of each mutant exon from PCR-amplified DNA from an independent pool of six worms.

Mutations were detected in seven of the eight alleles (Fig. 3-5), indicating that the cloned gene is unc-76. All of the identified unc-76 mutations are predicted to generate truncated protein products. Three were found to be frameshift mutations. The $^{32}$P-decay-induced mutation e911 had an 11 base pair deletion in exon 6 (nucleotides 6182-6192 of genomic sequence), shifting the reading frame and causing the termination of translation after 210 amino acids. The EMS-induced allele n2367 had lost a G/C base pair at nucleotide 5429. Another EMS-induced allele, ev424, was found to have a two base-pair insertion at nucleotide 4050, changing the sequence from TCTC to TCTCTC. The known
transposable elements in C. elegans cause duplications of (2-3) nucleotides at their insertion sites which often remain behind when excision occurs, but no transposon that leaves TC duplications has been reported. The predicted proteins terminate after 139 amino acids in ev424 and 165 amino acids in n2367.

Three EMS-induced alleles contained G/C > A/T changes that introduced premature stop codons. The alleles n2397 and n2399 introduced amber mutations at codons 105 (nucleotide 4016, TGG > TAG; Trp > amber) and 197 (nucleotide 5540, CAG > TAG; Gln > amber), respectively. The amber suppressor sup-7(st5), which inserts tryptophan at TAG codons (Bolten et al., 1984), suppressed the Unc phenotype of n2399 animals but had no effect on n2397. The allele n2398 carried a C/G > T/A change at nucleotide 6194, converting a CGA (Arg) to a TGA stop codon (opal) close to the site of the ey11 deletion.

The mutation found closest to the 5' end of the gene was the EMS-induced allele n2457, which was found to contain a G/C > A/T mutation at the splice acceptor site before exon 3, after amino acid 63 (nucleotide 2929), in which the splice acceptor TTTCA/GAC was changed to TTTCAA/GAC. RNase protection experiments with a probe covering the 120 nucleotide exon 3 entirely and spanning splice junctions at both ends showed that wild-type RNA protects a pair of products about one nucleotide apart in the 120 nucleotide range. RNA from n2457 animals protected only the smaller of these bands, consistent with the use of TTTCAAG/AC as an alternative splice site. This would create a frameshift, resulting in a truncated protein product of 76 amino acids.

unc-76 expression studies
Because the predicted unc-76 gene product showed no apparent similarity to any previously-identified protein, we sought clues to its function by determining the cells in which the protein is present, the subcellular localization of the protein, and the time during development in which the protein is present. The unc-76 expression pattern was determined with the use of three types of reagents: unc-76/lacZ fusions, epitope-tagged unc-76 derivatives, and anti-Unc-76 antibodies. All three techniques produced similar results.

**Expression of unc-76/lacZ fusions**

Translational fusions of the unc-76 promoter region and codons 1-12, 1-186, or 1-253 were constructed by insertion of fragments derived from the unc-76 rescuing genomic clone p76-16B into the lacZ-containing vector pPD16.43 (Fire et al., 1990a). These constructs contained 1 kb of genomic DNA 5' to the transcriptional start site of unc-76 and 1.7 kb from the 3' untranslated region. β-galactosidase activity in wild-type animals carrying any of these constructs on an extrachromosomal array with a plasmid encoding the rol-6(su1006) marker was detected throughout the nervous system but was largely absent from nonneuronal cells (Fig. 3-7). Staining of animals carrying p76-LJ11 with an anti-β-galactosidase monoclonal antibody showed that all neurons with cell bodies in the ventral nerve cord and on the lateral body wall expressed the fusion protein, as did most if not all of the neurons with cell bodies in the pharynx and the ganglia of the head and tail. A few non-neuronal cells, support cells of the amphid and labial sensilla in the head and the vm2
vulval muscles, also expressed the fusion protein. In addition, several
epithelial and muscle cells in the pharynx, all of which contain
elongated processes connecting the nucleus to the remainder of the cell,
showed β-galactosidase immunoreactivity. These nonneuronal cells were
not stained with anti-Unc-76 antisera in wild-type worms, however; see
below.

Expression of epitope-tagged unc-76 derivatives

In order to determine the localization of functional Unc-76
protein, sequences encoding peptide tags to which monoclonal antibodies
were available were added to unc-76 by site-directed mutagenesis. A
sequence from influenza hemagglutinin recognized by the monoclonal
antibody 12CA5 (Field et al., 1988) was inserted into a genomic rescuing
clone before the stop codon used by the p76-c7 splice form of unc-76,
and a c-myc sequence (recognized by the monoclonal antibody 9E10
(Kolodziej and Young, 1991) was added before the stop codon of the p76-
c4 variant. Both clones rescued unc-76(e911) mutants fully, suggesting
that the epitope tags did not interfere with gene function. No staining
of lines carrying the c-myc-tagged form was observed with 9E10, but
neuronal staining with 12CA5 was evident in lines carrying the 12CA5
epitope-tagged form. The staining pattern was similar to that observed
with unc-76/lacZ fusions stained with anti-β-galactosidase antibodies,
but 12CA5 staining was considerably weaker, either in animals carrying
extrachromosomal or integrated arrays of the tagged construct. Tagged
protein was observed throughout the nervous system in cell bodies and
axons but not in nuclei (data not shown). However, because experiments
with lacZ fusions suggested that overexpressed proteins can enter C. elegans axons nonspecifically (M. Rosoff, C. Li, M. Hamelin, and J. Culotti, personal communication; Chapter 4), the localization of epitope-tagged Unc-76 proteins was not considered a reliable indicator of the localization of endogenous Unc-76 protein.

Anti-Unc-76 antibodies recognize a set of proteins altered in unc-76 mutants

Polyclonal rabbit antisera against the p76-c7 form of the Unc-76 protein were raised as described in Experimental Procedures. Rabbits were injected with one or a series of bacterially-expressed fusion proteins containing either amino acids 13-385 or amino acids 48-385 of the predicted Unc-76 protein, and after the second boost, sera were affinity purified on nitrocellulose to which additional Unc-76 fusion proteins had been attached. Serum from rabbit 207, injected repeatedly with a Unc-76-maltose binding protein fusion, stained unc-76-overexpressing worms but not wild-type animals and was not analyzed further. Western blots of total worm protein with sera from each of three rabbits immunized with a series of different fusions (rabbits 273, 274, and 275) showed that all of these antibodies recognize a set of three or four bands not recognized by preimmune sera (Fig 3-8). Several lines of evidence indicate that these bands, approximately 54, 56, 57, 58 kDa in size, represent the unc-76 gene products. First, the bands were absent or reduced in abundance in protein extracts from all unc-76 mutants, and several mutants showed novel bands at lower molecular weights corresponding to the truncated forms predicted from the mutant.
DNA sequences (Fig. 3-9). Second, unc-76(e911) mutant animals transformed with the rescuing genomic clone p76-16B showed both the altered bands characteristic of e911 homozygotes and an abundant set of bands at the wild-type position. Third, wild-type animals transformed with an unc-76/lacZ fusion showed a novel high molecular weight band that was also recognized by an anti-β-galactosidase antibody.

The positions of the wild-type protein bands (54-58 kDa) suggest that the protein is larger than the predicted molecular weight of 42 kDa. Observations of bacterially-expressed Unc-76 fusion proteins (amino acids 12-385 with a His6 tag at the C terminus or amino acids 12-385 cleaved from a maltose binding protein fusion with factor Xa, both of which migrated close to the 49.5 kDa marker) suggested that the Unc-76 protein migrates more slowly on SDS-polyacrylamide gels than predicted from the sequence in the absence of C. elegans posttranslational modifications.

The source of multiple protein bands is not known. The two potential alternative C termini differ by 9 amino acids, consistent with the approximate difference of 1 kDa between bands. However, RNase protection experiments indicate that the smaller of the two forms is likely to be rare, while the protein forms appear to be roughly equal in abundance. Three of the protein bands are visible in mutants lacking the C terminal 180 amino acids (see below), suggesting that the source of the size variation lies in the N terminal 105 amino acids. Seven amino acids separate the initiator methionines predicted for the two SL1-spliced transcripts, again possibly accounting for a 1 kDa difference. Phosphorylation could also account for these size
differences; potential protein kinase C phosphorylation sites are present at amino acids 30, 137, 143, and 144.

The altered size variants of the protein in unc-76 mutants correspond with the nature of the mutations identified above. The mutant alleles e911 and n2398 are predicted to encode truncated proteins of 210 and 209 amino acids, respectively, compared to the wild-type 385 amino acids. The bands detected by anti-Unc-76 antibody 275 were at approximately 33-35 kDa in protein from both e911 and n2398, about 20 kDa smaller than the wild-type proteins, in agreement with the size predicted from the sequence (Fig. 3-9). Similarly, the mutation n2399 results in an amber codon at codon 197, generating a predicted protein 188 amino acids smaller than the wild-type Unc-76. A band corresponding to 33 kDa, a reduction of 29 kDa from wild-type, was observed. A pair of bands at 26-27 kDa was observed in protein from n2367 animals, which are predicted to produce a 165 amino acid protein.

Unc-76 protein is present in the axons of all neurons.

Immunofluorescence microscopy of wild-type worms stained with affinity-purified sera from each of three rabbits (sera 273, 274, and 275) showed a pattern of protein distribution similar to that observed with unc-76/lacZ fusions or epitope-tagged unc-76 derivatives. In adults, intense staining was observed in all of the major nerve bundles: the nerve ring, the dorsal nerve cord, and the left and right sides of the ventral nerve cord (Figs. 3-10, 3-11). In addition, all of the minor longitudinal and circumferential process tracts and bundles of sensory processes were stained with these antisera. Weaker staining
that varied with developmental stage was observed in neuronal cell bodies throughout the nervous system, as described in detail below. When visible, Unc-76 protein in cell bodies appeared to be excluded from the nucleus but distributed throughout the remainder of the cell.

Unc-76 protein appeared to be present in all neurons outside of the pharynx, consistent with the expression patterns observed for unc-76/lacZ fusions and epitope-tagged unc-76 derivatives. (Pharyngeal neurons expressed unc-76/lacZ fusions, but the fixation technique required for staining with Unc-76 antisera appeared to leave the pharynx poorly permeabilized. Staining of epitope-tagged Unc-76 protein was generally too weak to permit identification of cells in the head.) The absence of strong cell body staining in wild-type animals made the identification of specific Unc-76-expressing neurons difficult, particularly in ganglia of the head and tail. The strain MT6579, phenotypically wild-type animals homozygous for unc-76(e911), ces-2(n732ts), and nIs6, an array of the unc-76-rescuing plasmid p76-16B and the ces-2-rescuing cosmid C43F8 found fortuitously to be stable (M. Hengartner, personal communication), overexpressed the protein in cell bodies and thus facilitated identification of expressing cells (Fig. 3-11, 3-12). For all cells except those in the lateral, ventral, anterior, and ring ganglia in the head, observations made in MT6579 were subsequently confirmed in wild-type animals.

Unc-76 protein was observed in the cell bodies in all neurons of the ventral nerve cord in L1-L3 larvae (Fig. 3-13). Wild-type adults and, to a lesser extent, L4 larvae contained Unc-76 immunoreactive material in only a small subset of ventral cord cell bodies, the identities of which were not determined. Staining was clearly observed
in all neurons with cell bodies on the lateral body wall: ALMs, BDUs, CANs, HSNs, AVM, SDQs, PVDs, PDEs, and PVM. Except for the CAN and HSN neurons, these cells also showed reduced amounts of Unc-76 protein in their cell bodies in adults.

All neurons in the preanal, dorsorectal, and lumbar ganglia in the tail clearly stained with Unc-76 antisera in MT6579 animals (Fig. 3-14). Wild-type animals also appeared to have protein in all of these cells, and in contrast to the ventral cord neurons, a large subset of cells in the tail retained protein in adults. Similarly, all neurons in the retrovesicular ganglion and the deirids, located at the posterior part of the head, contained Unc-76 protein in MT6579 and wild-type animals (Fig. 3-12, 3-15). The close packing of cells in the ventral and ring ganglia made unambiguous identification of Unc-76 immunoreactive cells difficult in MT6579, but the positions of the small number of nonstaining cells in these ganglia were consistent with those of nonneuronal support cells (White et al., 1986). Wild-type animals showed widespread expression of Unc-76 protein in these ganglia in larvae and adults, but it was not possible to determine whether all neurons stained.

The Unc-76 protein appeared to be present exclusively in neurons. No staining was observed in cells that flank the ventral or dorsal nerve cords, such as body wall muscles or hypodermal cells. In the ventral nerve cords of young larvae, staining was observed in all neuronal cell bodies but not in the the Pn.p cells, precursors of the vulva and other hypodermal structures. Furthermore, nonneuronal support cells did not contain Unc-76 protein, while their associated neurons did. The postdeirids, ganglia containing four neurons and two support cells on
the left and two neurons and two support cells on the right, consistently showed Unc-76 staining in only four cells on the left and two cells on the right (Fig. 3-16). The positions of non-staining cells and the morphologies of stained axons indicated that the staining cells were neurons. Similarly, cells in the positions expected for phasmid sheath and socket cells in the tail and deirid and amphid sheath cells in the head consistently failed to stain in MT6579 and wild-type animals, while surrounding neurons did stain (Figs. 3-12, 3-15). While the identities of individual cells in the anterior ganglion of the head were not determined, non-staining cells were observed at the anterior margin of this ganglion, the positions expected for the nonneuronal support cells of the labial sensilla. The observed unc-76/lacZ staining of support cells in the head sensilla was not borne out by Unc-76 antibody staining in MT6579 or wild-type animals. Vulval muscle staining was observed in animals carrying unc-76/lacZ fusions stained with anti-β-galactosidase and in animals carrying an array of epitope-tagged unc-76 (nEx123) stained with anti-Unc-76 antibodies but not in wild-type animals and may represent an artifact arising from the short promoter region in these constructs.

In wild-type animals, Unc-76 protein was clearly present in all axonal tracts, including the longitudinal fascicles, the nerve ring, and the individual axons traversing the lateral body wall both longitudinally and in the dorsoventral axis. Although the morphologies of axons growing dorsally or ventrally on the lateral body wall are normal in unc-76 mutants (Desai et al., 1988; McIntire et al., 1992), Unc-76 protein was clearly visible in the dorsoventral segments of these axons. All motorneuron commissures extending from the ventral to the
dorsal nerve cord contained Unc-76 protein (Fig. 3-10), as did the
ventral-directed segments of the HSN, PDE, and PVD axons. Commissural
axons usually crossed longitudinal axons on the lateral body wall
without apparent changes of direction (Fig. 3-10).

No specific staining of Drosophila embryos (K. Lee, personal
communication) or adult ear norm ventral nerve cords (data not shown)
with affinity-purified anti-Unc-15 antiserum 275 was observed.

**Unc-76 protein is present throughout axon outgrowth and
adulthood.**

Unc-76 protein appeared in *C. elegans* neurons early in their
development. Protein could be detected with anti-Unc-76 antiserum in
MT6579 and wild-type embryos at the three-fold stage, although embryos
(particularly younger embryos) were generally poorly permeabilized by
the fixation procedure used. Expression of unc-76/lacZ fusions in a few
cells was observed in embryos shortly before the onset of morphogenesis,
when assembly of the embryonic nervous system occurs. During the first
larval stage, a wave of neurogenesis occurs in the ventral nerve cord,
and animals in which newly-derived neurons were present in the anterior
ventral cord but not in the posterior regions were observed to express
Unc-76 protein in the newly-born neurons (Fig. 3-17). It appears that
at least these neurons produce the Unc-76 protein at the time of axonal
outgrowth. The HSN neurons in MT6579 animals had visible Unc-76 protein
expression as early as the L2 stage, the time at which HSN axon
extension to the ventral nerve cord begins (Garriga et al., 1993).
Unc-76 protein clearly persisted through adulthood in wild-type and MT6579 animals. Adults showed strong staining in all axonal bundles and individual axons extending on the lateral body wall, and staining was variable and weaker but still detectable in cell bodies in the head and tail and in small numbers of ventral cord and lateral cell bodies (Figs. 3-13, 3-14, 3-15).

**Unc-76 protein is reduced in abundance in unc-76 mutant worms.**

Staining of unc-76 mutant animals with affinity-purified anti-Unc-76 antisera showed that all mutant alleles caused a reduced level of staining, and that two mutants, n2457 and n2367, had almost no detectible protein in whole-mount staining (Fig. 3-18). Very faint staining was occasionally observed in n2397, n2399, and rh116 animals, and somewhat stronger staining could be detected in ev424, e911, and n2398 animals. Preincubation of antisera with purified Unc-76 fusion proteins (maltose binding protein-Unc-76 or Unc-76-His6 fusions affinity-purified from *E. coli*) eliminated the residual staining from unc-76(e911) animals (data not shown), indicating that this residual immunoreactive material was Unc-76 protein.

When present in these mutant animals, Unc-76 immunoreactivity appeared in axons in the main neuronal bundles. This observation suggests that axonal-localizing sequences are retained in the truncated mutant proteins, indicating that these sequences are located within the N-terminal 105 amino acids. This is supported by the observation that
fusion of amino acids 12-186 to the *E. coli* lacZ gene product is sufficient to promote axonal localization of the fusion.

**Discussion**

unc-76 function is necessary for normal fascicle organization.

*C. elegans* animals carrying defective unc-76 genes have two types of axonal defects: axons in fascicles often do not reach their full lengths, and many axons that normally fasciculate tightly fail to do so. The same axons are morphologically normal in regions in which they are not associated with other axons in wild-type animals, suggesting that unc-76 is required specifically for axon-axon interactions. Experiments presented in Chapter 2 indicate that these defects result from a complete loss of unc-76 function. This was confirmed by the demonstration that seven of the eight mutant alleles encode truncated forms of the Unc-76 protein and that immunoblotting and whole-mount staining of these mutants showed that Unc-76 protein was reduced in abundance and/or altered in size.

unc-76 encodes a new class of axonal protein

The unc-76 gene was cloned by rescue of the uncoordinated phenotype of unc-76(e911) animals in germline transformation experiments. Evidence that the 10.7 kb rescuing clone contains the gene is provided by demonstration that mutant unc-76 strains contain
alterations in DNA sequence, mRNA levels, and size and abundance of the protein predicted from analysis of cDNA clones derived from this region. Two cDNA clones were identified by hybridization to the 10.5 kb rescuing clone, and DNA sequence analysis showed that the transcripts from which the cDNA clones were derived spanned the central 7.5 kb of the genomic DNA clone. These cDNA clones hybridized to a single major transcript in RNA from embryos, which was greatly reduced in abundance in unc-76(e911) mutants. DNA sequence analysis showed that seven of the eight unc-76 mutant alleles contained alterations within the coding region inferred from the cDNA sequences. Immunoblotting with antisera raised against fusion proteins derived from the putative unc-76 cDNA clones showed that all eight alleles caused alterations in protein size or abundance. Altered protein sizes corresponded with the sites of termination codons predicted by sequence analysis of the mutant alleles.

It is interesting to note that all of the seven mutations identified were predicted to cause truncations in the protein and that none were simple missense mutations. This could reflect either a high degree of functional redundancy within the Unc-76 protein (e.g., many residues are sufficient for function but few are necessary) or a very small region of the protein critical for its function, such that mutations affecting this site would be rare. Alternatively, the screen that produced most of the unc-76 alleles could have imposed a bias toward severe truncations. This possibility is supported by the observation that the allele e911, used in screens for new mutations, can provide partial function under certain circumstances. The genomic clone p76-10, which lacks exons 1-3 (amino acids 1-104) has little rescuing activity in n2397 homozygotes (which produce a truncated protein of
amino acids 1-105) but provides significant rescuing activity to e911 mutants (which encode amino acids 1-210). This observation suggests that either the N and C terminal halves of the Unc-76 protein provide independent functions or that intermolecular associations of the Unc-76 protein via sequences between amino acids 105 and 210 are important for protein function. The partial function remaining in e911 might have compensated for defects caused by many missense mutations and prevented their isolation in the screen used.

The predicted unc-76 gene product is a protein of 385 amino acids with no apparent similarity to proteins in the Swissprot and PIR databases. It is a hydrophilic protein, with several predicted protein kinase C phosphorylation sites, a concentration of acidic residues at its N terminus and no long hydrophobic domain that could serve as a signal sequence or transmembrane domain. It therefore appears to define a new class of axonal outgrown proteins.

Polyclonal antisera raised against unc-76 fusion proteins produced in E. coli recognized a group of four C. elegans proteins in the 54-58 kDa range in immunoblotting experiments. Immunofluorescence microscopy with each of the three sera showed staining in all C. elegans neurons, comparable to the pattern observed with unc-76/lacZ fusions and epitope-tagged unc-76 derivatives. No non-neuronal cells appeared to contain Unc-76 proteins. Although axons in developing C. elegans nerve cords are in close proximity to other cell types, including body muscles and the ridges of hypodermis that flank each cord, none of these cells expressed Unc-76 protein. Furthermore, neuronal expression was not restricted to a subset of ventral cord neurons, suggesting that the defects in ventral cord organization observed in unc-76 mutants did not
simply arise from early defects in the small set of axons that pioneer the ventral cord. The widespread expression of Unc-76 protein together with the widespread defects in axonal elongation and fasciculation suggest that the gene functions in most if not all of the neurons that make up the fascicles.

Unc-76 immunoreactivity was strongest in the fascicles of the ventral and dorsal nerve cords and the nerve ring but was evident along the lengths of individual axons as well. All axons outside of the major fascicles appeared to stain along their entire lengths, including motorneuron commissures, which extend circumferentially around the body wall. Circumferential growth of the VD, DD, and HSN motorneurons is normal in unc-76 mutants, and the presence of Unc-76 protein in portions of axons not affected by its absence suggests that its function is not required everywhere it is present. It is clear that the simple presence of Unc-76 protein in axons that contact one another is not sufficient to induce fasciculation, because single commissural and longitudinal axons containing the protein crossed at right angles without apparent alterations in their trajectories at the intersection (e.g., Fig. 3-10).

Unc-76 protein was observed in embryonic nerve cords and the nerve ring, and it persisted through adulthood. Immunoreactivity was visible in ventral cord motorneurons generated in late L1 larvae at a time when axonal outgrowth was occurring. The HSNs, which first extend axons in the L2 stage to the ventral nerve cord and then continue outgrowth in the ventral cord in the L4 stage, stained throughout this period in M76579.

Although present in cell bodies and axons in embryos and young larvae, the Unc-76 protein was never observed in nuclei. Cell body
staining in most ventral cord neurons gradually diminished during development, with only a few cell bodies retaining immunoreactive material in L4 larvae and adults. A number of cell bodies in the head and tail, and the CAN and HSN cell bodies on the lateral body wall, continued to express Unc-76 protein in adults. The significance of this difference is not clear. The outgrowth of HSN axons is not completed until the late L4 stage, and it is possible that late expression of Unc-76 protein in the somata of these cells reflects this late completion of development. Aside from overall elongation occurring as the animal grows, however, late morphological changes in other axons have not been described. To elongate during the animal's growth, cells with longer axons might be expected to synthesize more axonal components than do cells with shorter axons. The small proportion of ventral cord cell bodies with high levels of Unc-76 protein in adults, however, suggests that this cannot completely account for the variation in expression levels, because most of their axons are similar in length and yet only a few retain large amounts of Unc-76 protein.

**Possible functions of the Unc-76 protein**

Genetic analysis of unc-76 mutants suggests that this gene is required specifically for two aspects of axonal bundle formation in C. elegans, longitudinal extension of axons within fascicles and the lateral association of specific axons in these fascicles. The reagents currently available do not allow visualization of C. elegans neurons before they have completed outgrowth, and so the precise cause of the aberrant axon and fascicle morphologies observed in unc-76 mutants has
not been determined. The simplest explanation, based on the behavior of axons in the grasshopper and Japanese medaka fish central nervous systems deprived of their normal guidance cues (Raper et al., 1984; du Lac et al., 1986; Bastiani et al., 1986; Kuwada, 1986), is that axons in unc-76 mutants either grow slowly or stop growing in the ventral nerve cords. Alternatively, they could reach their normal length in embryonic or larval development but retract as the animal grows, as has been observed for the larval photoreceptor nerve in the Drosophila disconnected mutant (Steller et al., 1987). The observed defasciculation could arise at the time of axonal outgrowth, by a failure of follower axons to grow along the correct pathways, or by abnormal interposition of ingrowing axons between members of a fascicle. Methods for visualizing axons in early stages of outgrowth using the axonally-localized unc-76/lacZ fusions under the control of promoters with limited expression patterns are under development and should help to clarify this issue (Chapter 4; L. Bloom, D. Ginsberg, M. Koelle, and H.R. Horvitz, unpublished observations).

Fasciculation and extension of axons along axonal surfaces are believed to be mediated by cell-surface adhesion molecules. Some of the molecules identified in studies of vertebrates and invertebrates are calcium-dependent homophilic adhesion molecules of the cadherin family, while most are homophilic adhesion molecules of the immunoglobulin superfamily. Two insect cell surface molecules, fasciclin I in grasshopper and Drosophila and fasciclin IV in grasshopper, have structures that are not similar to those of previously-characterized adhesion molecules (Zinn et al., 1988; Kolodkin et al., 1992). Antibody perturbation experiments have shown that many of these molecules (L1,
NCAM, Fl1, TAG-1) function in both axonal elongation and bundling 
(Rathjen et al., 1987b; Chang et al., 1987; Thanos et al., 1984; Ruegg 
et al., 1989), and some, such as TAG-1 and fasciclins I, II, III, and 
IV, have spatially and temporally restricted expression patterns in the 
vertebrate central nervous system that suggest they could function in 
directing outgrowth of axons along specific pathways. (Dodd et al., 1988; 
Shiga and Oppenheim, 1991; Kolodkin et al., 1992; Patel et al., 1987; 
Bastiani et al., 1987). Drosophila mutants lacking fasciclin II 
function have aberrant fasciculation patterns (Goodman and Shatz, 1993; 
Grenningloh et al., 1991), while mutants in fasciclin I and fasciclin III 
have no clear defects (Elkins et al., 1990).

The unc-76 gene is unlikely to encode a cell-surface adhesion 
molecule or secreted protein because the predicted Unc-76 protein 
sequence contains neither a potential transmembrane domain nor a 
predicted signal sequence. This possibility cannot be entirely ruled 
out, however, because vertebrate growth factors such as bFGF are 
secreted without apparent use of the normal secretory pathway/without 
signal sequences. (Abraham et al., 1986) The apparently uniform 
distribution of Unc-76 protein along all axons of the nervous system 
contrasts with the fascicle-specific defects in mutants lacking Unc-76 
function and suggests that Unc-76 does not itself cause selective 
fasciculation but rather implements instructions from region-specific 
cues.

Two general roles can be proposed for an intracellular protein 
functioning downstream of extracellular cues. First, such a protein 
could have a structural role in the formation of fascicles, either by 
intracellular association with a cell-surface adhesion molecule or by
association with the cytoskeleton in response to region-specific cues. Second, it could act in the transduction of signals from cell-surface adhesion molecules to the intracellular signaling machinery that regulates axonal extension and adhesion. Studies in vertebrates provide evidence for the existence of molecules with either or perhaps both of these functions.

Several classes of vertebrate cell adhesion molecules implicated in axon-axon interactions are believed to be linked to the cytoskeleton. N-cadherin associates with three molecules, α, β, and γ-catenin, through which it is likely to associate with the actin cytoskeleton (Hirano et al., 1992). An isoform of N-CAM with a large cytoplasmic domain, N-CAM 180, shows reduced lateral mobility in the cell membrane compared to an isoform with a shorter cytoplasmic domain, N-CAM 140, and can associate with spectrin in vitro (Kadmon et al., 1990). In addition, N-CAM associates by unknown mechanisms with cell-cell contact sites (Pollerberg et al., 1987) and with the cell-adhesion molecule L1 (Thor et al., 1986). By analogy to the catenins and spectrin, the Unc-76 protein could act as an intracellular link between a cell-surface adhesion molecule and the cytoskeleton or other adhesion molecules.

The axonal cytoskeleton is not uniform in all regions of growing or mature axons. Specific phosphorylation patterns of the cytoskeletal proteins tau, MAP-1B, and high- and intermediate-molecular weight neurofilaments (NF-H and NF-M) have been observed in distal portions of axons (Papadosoumenos et al., 1987; Carden et al., 1985; Sato-Yoshitake et al., 1989). Recently, a phosphorylated derivative of NF-M has been shown to appear in subsets of chick motor axons at and distal to the points at which they make guidance decisions (Landmesser and Swain,
1992). While the functional significance of these local cytoskeletal modifications is not known, they suggest that cytoskeletal assembly and stability can be regulated in a region-specific fashion. Furthermore, disruption of filamentous actin by cytochalasin B treatment does not impair axonal elongation in the grasshopper limb bud or on certain adhesive substrates in vitro (Bentley and Toroian-Raymond, 1986; Marsh and Letourneau, 1984) (while it does disrupt elongation on other substrates, such as immobilized N-CAM; Abosch and Lagenaour, 1953), nor does depletion of the intermediate filament peripherin by antisense oligonucleotides inhibit neurite elongation on adhesive substrates (Troy et al., 1992). These observations suggest that, at least in vitro, certain extracellular conditions can make particular cytoskeletal structures appear unnecessary. Because axons in unc-76 mutants appear normal in their regions of contact with the lateral hypodermis and basement membrane, these observations suggest that a cytoskeletal role for Unc-76 could be masked by conditions of the hypodermal environment.

Recent pharmacological evidence suggests that several vertebrate cell adhesion molecules with unrelated cytoplasmic domains (L1, N-CAM, and N-cadherin) employ a common signalling mechanism to promote axonal extension and that this mechanism is different from that activated by extracellular matrix receptors (Troy et al., 1992; Williams et al., 1992; Saffell et al., 1992; Bixby, 1989; Bixby and Jhabvala, 1990). The outgrowth-promoting functions of these cell-adhesion molecules can be inhibited by a common set of treatments without affecting their adhesive function, and another set of experiments suggests that regulation of adhesion mediated by N-CAM, L1, and F11 is also controlled by a common signalling mechanism (Cervello et al., 1991). While the evidence for
use of specific signalling pathways is indirect and inferred largely through the use of relatively nonspecific pharmacological agents, it nonetheless suggests that intracellular interactions of cell surface molecules are likely to play an important role in stimulating axonal outgrowth. If common signalling mechanisms are indeed used by surface molecules with different cytoplasmic domains (or, in the case of glycosylphosphatidylinositol-linked P11, no cytoplasmic domain at all), intracellular adaptor proteins are likely to exist to couple receptors with regulatory proteins, by analogy with the SH2/SH3 domain adaptors that link growth factor receptors with several signalling systems (Pawson and Gish, 1992). Unc-76 is a candidate for such an adaptor.

This possibility is particularly attractive because both axonal elongation on axonal surfaces and axonal adhesion appear to be impaired in unc-76 mutants. Since these functions might be pharmacologically separable in vertebrate axons, a molecule that is required for both processes is likely to function at the intersection between receptors and signalling molecules. The functions of adhesion molecules associated with the T cell receptor provide an instructive model. Adhesion molecules (the T cell receptor/CD3 complex) bind T cells to their specific target antigen-presenting cells, but they also activate intracellular signalling pathways (possibly via associated tyrosine kinases), one function of which is to enhance the adhesive function of more generally-acting adhesive molecules on the T cell surface, integrin αLβ2 (Hynes, 1992). This, in turn, increases the strength of the originally-weak cell-cell adhesion. The growing evidence that in a variety of cell types, different adhesion molecules are often clustered and can affect the activity of the others through intracellular
signalling pathways suggests that intracellular links among axonal adhesion molecules, direct or indirect, are likely to play important roles in axonal development.

The Unc-76 protein is not absolutely required for fascicle formation, because considerable axonal extension and fasciculation takes place in animals lacking detectable Unc-76 protein. Genetic evidence suggests that at least two other genes, unc-34 and unc-71, the mutant phenotypes of which resemble that of unc-76, function independently in the process of fascicle formation (McIntire et al., 1992; G. Garriga, L. Bloom and H.R. Horvitz, unpublished observations). It is possible that the products of these genes work in signalling or structural processes parallel to that proposed for Unc-76 (e.g., in association with different cell-surface receptors or cytoskeletal elements). The observation that severe axon outgrowth defects are observed in Drosophila mutants lacking both fasciclin I and the abl tyrosine kinase while axons in mutant embryos lacking either protein alone are relatively normal suggests that redundant receptor-linked signalling mechanisms can exist in the same axons (Elkins et al., 1990).

Mutations in the gene unc-69 cause axonal outgrowth defects resembling those observed for unc-76 (G. Garriga, personal communication). In light of the possible role for Unc-76 as an adaptor protein, it is interesting to note that the predicted products of the unc-76 and unc-69 genes, which have similar mutant phenotypes, share a similar sequence near their N termini (DIPLASCDDDDD in Unc-76 and DIPLADDDDD in Unc-69) but are not otherwise similar (M. Hengartner, N. Tsung, and H.R. Horvitz, personal communication). While no function has been ascribed to this portion of either protein, it is tempting to
speculate that the two proteins could serve as adaptors for different
cell-surface molecules, with the common motif functioning in interaction
with a shared signal transducing molecule or cytoskeletal protein.

**Experimental Procedures**

**Isolation of unc-76 rescuing clones**

The overlapping cosmids C25D7, B0289, C56C4, T25A9, C08C1, C01G10,
T06H10, T01G5, C13G10, and C28G7 were isolated by Coulson *et al.* as part
of the *C. elegans* genome cloning project (Coulson *et al.*, 1986). Cosmid
DNA purified over Qiagen tip-20 or tip-100 columns (Diagen, Inc.,
Germany) was injected into the lumens of the distal gonad arms of unc-76
(e911) mutant hermaphrodites according to the procedure of Fire (1990b).
In initial experiments, pools of three overlapping cosmids (20 μg/ml
each) were coinjected with 40 μg/ml pRF4, a plasmid encoding the
dominant marker rol-6 (*su1006*; (Kramer *et al.*, 1990), into
hermaphrodites of the genotype *dpy-11(e224) unc-76(e911)/++. Rescue was
determined by scoring the broods of heterozygous F2 animals that
expressed the rol-6 marker for the presence of animals homozygous for
the *dpy-11(e224) unc-76(e911)* chromosome that were not uncoordinated.
In subsequent experiments, single cosmids were injected at 50 μg/ml into
unc-76(e911) hermaphrodites and the Uncoordinated phenotype was scored
directly in the F1 and F2 generations.

The minimal region necessary for rescue was determined by germline
transformation with fragments of the cosmid C56C4 subcloned into
pBluescript SK (+) (Stratagene, Inc.) or pIBI20 (IBI, Inc.). p76-6-18
was obtained by partial digestion of C56C4 with XbaI and self-ligation to produce a 19.5 kb insert in the cosmid vector pJB8, of which approximately 14 kb was contiguous DNA from the portion of the cosmid later found to contain the minimal rescuing fragment. p76-7 was constructed from a 6.4 kb ApaI-XhoI fragment of C56C4 ligated into pIBI20 digested with ApaI and SalI. p76-8 was constructed by ligation of an 8.6 kb ApaI-XbaI fragment of C56C4 into ApaI-XbaI-digested pIBI20, and p76-8ΔHind was derived from this plasmid by partial HindIII digestion and self-ligation. 7.0 kb SacII-BamHI and 7.5 kb BamHI fragments from p76-8 were ligated into the cognate sites in the polylinker of pBSK(+) to produce p76-10 and p76-11, respectively. p76-13 and p76-14 were constructed by partial digestion of p76-10 with XhoI or HindIII and self-ligation to leave inserts of 6.2 kb (SacII-XhoI) or 5.5 kb (SacII-HindIII). pKpn-1 was a derivative of C01G10 from which the 1.4 kb KpnI fragment was deleted by KpnI digestion and self-ligation. The smallest plasmids that gave complete rescuing activity were p76-16A and p76-16B, constructed from 10.7 kb XbaI fragments of p76-6-18 ligated in opposite orientations into the pBSK(+) XbaI site.

Additional plasmids used for sequencing unc-76 genomic DNA were constructed as follows: p76-15 contained the same 7.5 kb BamHI fragment used for p76-11 but in the opposite orientation in pBSK(+). p76-17 was constructed by ligation of a 3.5 kb XbaI-SacII fragment of p76-6-18 into the SpeI and SacII sites of pBSK(+). p76-21 was obtained by partial BamHI digestion and self-ligation of p76-16A to leave a 2.9 kb XbaI-BamHI insert.
Isolation of unc-76 cDNA clones and identification of unc-76 transcripts

The 8.6 kb ApaI-XbaI fragment from p76-6-18, identical to the insert in the partially-rescuing plasmid p76-8, was purified away from vector sequences by digestion with ApaI and XbaI and two rounds of electrophoresis in low-melting agarose (SeaPlaque) and was $^{32}$P-labelled by random oligonucleotide priming (Sambrook et al., 1989). 220,000 plaques from a mixed-stage C. elegans cDNA library in lambda gt10 (Kim and Horvitz, 1990) were transferred to nitrocellulose filters and hybridized to this probe for 16 hr in 6X SSPE, 5X Denhardt's solution at 65°C. DNA prepared from the seven positive clones identified after two rounds of plaque purification was radiolabeled and hybridized to blots of p76-8 DNA digested with EcoRI or a combination of ClaI and HindIII. Two clones, c4 and c7, hybridized to sequences throughout the region necessary for rescue, and their inserts were cut from the lambda vector with EcoRI and cloned in both orientations into pBSK(+) to generate p76-c4A, p76-c4B, p76-c7A, and p76-c7B.

Worms were grown in 1-l flasks in S Basal medium supplemented with E. coli (HB101 or C600 harvested from 1.5 mi cultures and frozen), and mixed-stage populations were harvested by centrifugation at 1000 rpm for 2 min in a Beckman J-6B centrifuge. Pure populations of embryos were obtained by treating these worms with a solution of 20% sodium hypochlorite in 0.5 M NaOH until all larvae and adults dissolved. Total RNA was obtained by freezing worm pellets at -80°C, crushing with a liquid nitrogen-cooled mortar and pestle, and sonication in 0.01 volumes $\beta$-mercaptoethanol, 2 volumes 1:1 phenol:chloroform, and 3 volumes lysis
buffer (2 % SDS, 10 mM EDTA, 50 mM Tris pH 7.5, 100 mM β-
mercaptoethanol). After five phenol-chloroform extractions, the RNA was
ethanol-precipitated, resuspended in loading buffer (0.5 M NaCl, 0.2 M
Tris pH 7.5, 10 mM EDTA, 1% SDS), and poly-A (+) RNA was purified on
oligo (dT) cellulose. RNA (10 µg per lane) was separated on a 1%
agarose-formaldehyde gel (Sambrook et al., 1989) and blotted to Nytran
filters (Schleicher and Schuell), which were baked for 3 hr under vacuum
and UV-crosslinked by brief exposure to a short-wave germicidal lamp.
EcoRI inserts from p76-c4A and p76-c7A were gel-purified away from
vector sequences and 32P-labeled by random oligonucleotide priming for
hybridization to Northern blots (6 X SSPE, 5X Denhardt's solution, 65°C;
with 1 wash in 1X SSPE, 0.1% SDS and 3 washes in 0.2X SSPE, 0.1% SDS at
65°C). Autoradiographs were made using Kodak XAR-5 film and Lightning
Plus or Quanta III intensifying screens (Picker).

**Primer extension analysis and reverse transcriptase-PCR**

Primers of sequence 5'-CGGTACTCGCATCTGCAGCTCCTCCATTGC-3' (76-PE-1)
and 5'-CGGATGACACACAAAAAGTGGAAGAGAAA-3' (76-PE-2) were end-labeled with
32P (Ausubel et al., 1991) and hybridized to 75 µg of total *C. elegans*
RNA or 17 µg of poly-A+ RNA in formamide/PIPES buffer (Ausubel et al.,
1991) overnight at 30°C. Primers were extended with AMV reverse
transcriptase (Promega) at 42°C for 90 min, and the products were
analyzed by electrophoresis on a sequencing gel and autoradiography.

Reverse transcriptase-PCR was performed using reagents supplied in
kit form (rTth kit, Perkin-Elmer Cetus). 2.25 µg of total *C. elegans*
RNA were annealed to primer 76-E1E2-1 (5'-CTCGTCTGATGAATGGTGCTC-3'),
which spans the boundary of exons 1 and 2. Reverse transcription was carried out at 66°C and PCR with each of eight primers (END-1 = 5'-CTGCATCTCTCCAACGTCGTC-3', END-2 = 5'-AGTTTGCTTTTTCCTCCTCT-3', END-3 = 5'-CTCCCAATTCTCCAATCCCCAAAAG-3', END-4 = 5'-CATTTCTTTTCTCTTCACAC-3', END-6 = 5'-ATGTCACCAGCATAATTCTTCCTTC-3', END-7 = 5'-ATGTACACTCATTTTGGTGATC-3', END-8 = 5'-TGTCTCTGCCGCTCTCTATCCG-3', SL1NHE = 5'-GGGGGCTAGCGTTAATTTACCAAAGTTTGAG-3') was performed according to the manufacturer's instructions. Control reactions were performed with each primer and RNA that had been treated with reverse transcriptase without addition of the 76-E1E2-1 primer. PCR products were analyzed on a 1.2% agarose gel and for sequence analysis were cloned using the pCRII vector (Stratagene).

Reverse transcriptase PCR analysis of the alternatively spliced region was conducted using the same protocol, with reverse transcription using two primers (c4B = 5'CACTAATCAATCAACACACAG-3' and c4D = 5'-GTAGTCTCTGCAGGCTGG-3') and PCR with these and primers c4A (5'-ATTGACAGCGACAATTCGT-3') and c4C (5'-TTTCTCTTCTCAATACCCCTTGAA-3').

RNase protection analysis

Radiolabeled probes were made by transcription in vitro with T7 RNA polymerase (New England Biolabs). Alternative splicing after exon 8 was investigated with a probe made from the genomic clone Δ40E, a clone derived from Exonuclease III deletion of p76-10. This plasmid was digested with EcoRI for production of a probe corresponding to genomic clone nucleotides 7713-8010 and a short vector sequence. Splicing in exon 3 in unc-76(n2457) was investigated with a probe made from the
clone Δ342B, a product of Exonuclease III deletion of p76-11. This plasmid was digested with BamHI for production of a probe corresponding to genomic nucleotides 2896-3141 and a short vector sequence.

Unincorporated nucleotides were removed from riboprobes by centrifugation in Millipore Ultrafree MC spin columns. Probes were annealed to 20 µg total RNA or yeast tRNA at 45°C or 60°C overnight as described by Ausubel et al. (1991). Hybrids were digested with a mixture of 40 µg/ml RNase A and 700 U/ml RNase T1 (Boehringer-Mannheim) for 60 min. at 37°C and analyzed on a sequencing gel.

**DNA sequence analysis**

For determination of the unc-76 cDNA sequences, p76-c4A and p76-c4B were digested with SalI and ApaI, and p76-c7A and p76-c7B were digested with SalI and KpnI. Nested deletions approximately 100 bp apart were generated by Exonuclease III and Nuclease SI or Exonuclease VII digestion (Sambrook et al., 1989; Ausubel et al., 1991). Recircularized deletion clones were transformed into the *E. coli* strain BB4, and single-stranded DNA production was induced by adding 5 x 10^9 pfu of the helper phage VCSM13 per 2 ml culture. One-third of the single-stranded DNA prepared from each culture was sequenced by the dideoxy chain termination method using Sequenase (United States Biochemical) with [α^35S]dATP and the -40 M13 sequencing primer.

Genomic DNA sequence from subclones of C56C4 was determined by the same method using nested deletions of the following pBSK (+)-based plasmids: p76-11 (digested with EcoRI/ApaI or SalI/ApaI), p76-14 (digested with SalI/ApaI, BglII/ApaI, or HindIII/ApaI), p76-15 (digested
with SmaI/ApaI, BglII/ApaI, or HindIII/ApaI), p76-17 (digested with SmaI/KpnI), and p76-21 (digested with SmaI/KpnI). Together this deletion series covered both strands of the 10.7 kb region cloned in p76-16A. Sequence was determined from multiple clones representing both strands. Regions not represented by deletion clones on both strands were sequenced with specific primers.

Sequence analysis was carried out using DNA Inspector, DNAsStar, GeneWorks and University of Wisconsin GCG sequence analysis packages. Database searches were carried out using BLAST and fasta algorithms.

Six pairs of PCR primers were designed to amplify all exons and splice junctions from unc-76: set 1 (primers A1, 5'-CACCTCATTGAGAGTGCGG-3' and A2, 5'-CAACTTGTGAGGGAGTTTGG-3'), set 2 (primers A3, 5'-CATATTGATTTGTCGTTGGAG-3', and A4, 5'-GGTGCTATTCTGAACAGGATTA-3'), set 3 (primers A5, 5'-CGAAATAGAGAAACGAGCA-3', and A6, 5'-CGCTCTCCACATAAGTAAGAG-3'), set 4 (primers A7, 5'-GTTTACTGATTTCCTAGGAT-3' and A8, 5'-CGATTGAAACAAAGATGTGAGA-3'), set 5 (primers A9, 5'-TACTTAACACTCTCACTGAC-3' and A10, 5'-GTCTTGATTTTCATGGAAATCC-3'), and set 6 (primers A11, 5'-CCGTATAATTTGTGCTAATTTGC-3' and A12, 5'-TGACACAATTTATGACACCGC-3'). Sequences of mutant unc-76 alleles were determined by PCR amplification of DNA from pools of six worms for each mutant strain (B. Schrank and B. Waterston, personal communication), followed (for sets 1, 3, and 5) by single-strand PCR amplification and dideoxy sequencing with Sequenase as described above or (for sets 2, 4, and 6) by cloning in the pCR II vector (Stratagene) and sequencing of single-stranded DNA with Sequenase. Sequencing primers internal to the primers used for PCR amplification were as follows: set 1, S1=5'-
GCATTCATTTAGTTTG-3'; set 2, S3=5'-TTCTATTCTGCCTATCC-3', S4=5'-
CGATTTATTGGAACCTTAAC-3'; set 3, S5= 5'-ATTAGTAGTAAACATATAA-3'; set 4,
S7=5'-CCTGCTTCTTCTCCCTAAA-3', S8=5'-CCTTTTCTGCTAAAGTC-3', S10=5'-
TTTTAGTTAATTTGGAATTTA-3'; set 5, S13=5'-GCCAGTAATAGTGAAT-3', S14=5'-
CATATTAAAAATCTGTATC-3'; and set 6, S16=5'-TGTAGAATAGAAGAGAAG-3'. The
sequences of all mutant exons were confirmed by direct 33P cycle
sequencing (fmol kit, Promega) of DNA amplified by PCR from independent
pools of worms.

Construction of unc-76 cDNA-based plasmids

Plasmids in which one of the two possible 3' ends of the unc-76
coding region had been eliminated were constructed. p76-22, in which
sequences from the 3' end of the p76-c7 cDNA replaced the equivalent
genomic sequence from exon 8 to the 3' untranslated region, eliminating
the alternative sequences present in the p76-c4 cDNA, was constructed by
the following steps: A 1.7 kb SpeI-XbaI fragment of unc-76 3' genomic
DNA from p76-8 was ligated into the SpeI site of pPD16.43, a lacZ fusion
vector (Fire et al., 1990a) to generate p76-L5. A 1.3 kb SpeI fragment
from p76-c7A (containing the entire cDNA coding sequence) was ligated
into the SpeI site of p76-L5, generating p76-L9B. The lacZ, linker
sequences, and most of the unc-76 cDNA sequences from this vector were
removed by XbaI/SacI digestion and replaced with a 7.6 kb XbaI-SacI
genomic DNA fragment (extending to the SacI site in exon 8) from the
unc-76 clone p76-16B, generating p76-22.

p76-24, in which 337 bp containing the splice acceptor, unique
coding sequences, and stop codon of p76-c7 were deleted (leaving only
the p76-c4 3' end, which retains sequences spliced out in p76-c7) was constructed as follows. The entire unc-76 rescuing region, a 10.7 kb XbaI fragment from p76-16B, was inserted into the XbaI site of p76-L5, generating p76-L8. This vector was completely digested with SstI, which cleaves at a site in a linker immediately 5' to the SpeI site in the unc-76 3' untranslated region, and was then subjected to partial digestion with KpnI and trimming with T4 DNA polymerase, and was self-ligated to generate p76-24. The net result was a 337 bp deletion from the unc-76 rescuing clone.

The plasmid p76-28, which contained the p76-c7 cDNA flanked by genomic 5' and 3' sequences, was constructed by digestion of p76-L9B with XbaI and BglII to replace lacZ and linker sequences with a 1.1 kb XbaI/BglII genomic fragment from p76-16A containing promoter sequences and part of the first exon of unc-76.

**Beta-galactosidase fusions**

Translational fusions of unc-76 to the *E. coli* lacZ gene were constructed by insertion of unc-76 genomic DNA sequences from both 5' and 3' ends of the gene into the pPD16.43 vector designed by A. Fire (1990a). The vector p76-L5 described above contained lacZ (including its stop codon) upstream of 1.7 kb of the unc-76 3' untranslated region, starting at a SpeI site 27 base pairs after the stop codon in p76-c7. All unc-76/lacZ fusions were constructed by deleting fragments from p76-L8, described above, which contained the entire 10.7 kb unc-76 genomic region cloned into the 5' polylinker of p76-L5. p76-L11 was derived from complete digestion of p76-L8 with SmaI followed by partial SacI
digestion and T4 polymerase trimming, separation on a low-melting-temperature agarose gel, and self-igation. p76-L13 was obtained by complete digestion of p76-L8 with BglII and SmaI, generation of blunt ends with Klenow fragment, and self-igation. p76-L15 was constructed similarly by complete digestion of p76-L8 (prepared from the dam E. coli strain GW5454, a gift of Dr. G. Walker) with SmaI and partial digestion with BclII, treatment with Klenow fragment, separation on a low-melting-temperature agarose gel, and self-igation. All of the above constructs contained a nuclear localization signal from SV40 T antigen flanked by KpnI sites. These were removed from p76-L11, p76-L13, and p76-L15 by KpnI digestion (partial, in the case of p76-L11) and self-igation to generate p76-L14, p76-L17, and p76-L16, respectively.

Cells expressing beta-galactosidase were visualized by acetone fixation followed by incubation with a solution of 5-bromo-4-chloro-indole-β-D-galactoside (X-gal; Fire et al., 1990a). Alternately, worms were fixed for 30 min. in 1% paraformaldehyde (Finney and Ruvkun, 1990) and stained with a 1:50 dilution of a monoclonal anti-beta-galactosidase antibody (Promega) at 4°C overnight, followed by a 2 hr incubation at 37°C with a 1:20 dilution of a fluorescein conjugated goat-anti-mouse IgG antibody (Jackson Immunoresearch). For enhanced sensitivity, this was sometimes followed by a 2 hr. incubation at 37°C with a fluorescein-conjugated rabbit-anti-goat IgG antibody (Jackson Immunoresearch). Following each antibody incubation, animals were washed several times in PBS pH 7.2 containing 0.5% Triton X-100 and 1% BSA for a total of 30-60 min. Stained animals were mounted for observation in 90% glycerol, 10% PBS pH 8.0 containing 1mg/ml p-phenylene diamine to reduce fading. For observation of nuclei, diamidinophenolindole (DAPI) was added to 1 μg/ml
in the final wash. Worms were observed on a Zeiss Axiophot microscope and photographed with Kodak Tri-X or Ektachrome 160T film.

Epitope-tagged constructions

Epitopes recognized by monoclonal antibodies C12A5 (peptide sequence YPYDVPDYA, from influenza hemagglutinin; (Field et al., 1988) and 9E10 (peptide sequence EEKLISEQDL, from c-myc; Kolodziej and Young, 1991) were inserted immediately upstream of the two possible unc-76 stop codons by site-directed mutagenesis (Amersham kit, cat. no. RPN1523). The 12CA5 epitope was inserted into a deletion clone of p76-15 (∆221B) at the p76-c7 stop codon (using oligonucleotide 76-C7-FLU, 5'-CCAAAAATATATATCGTGTGTTACCATACGACGTCCAGACTACGCTTAGCCATTGCCACC-3') to generate p76-25A, and the 9E10 epitope was inserted into ∆221B at the p76-c4 stop codon (using oligonucleotide 76-C4-MYC, 5'-CAGCTGTGTGTGTGATGAGCAAAAAACTATATACCGAAGAAGACTTGATTAGTGAGAAGAAG-3') to generate p76-26. The mutagenized portions of these clones were sequenced to demonstrate that the desired alterations had been made, and the corresponding regions of the wild-type genomic clone, p76-23 (10.5 kb XbaI fragment cloned into a pUC19 vector), were replaced with a 1.8 kb SacI-NcoI fragment of p76-25A or a 1.4 kb KpnI fragment of p76-26.

The mutant clones were named p76-tag7-flu-1 and p76-tag4-myc-1 to indicate the alternate cDNA form and epitope added.

Lines carrying extrachromosomal arrays of epitope-tagged clones were constructed by injection of either clone at a concentration of 50 μg/ml into unc-76(e911) hermaphrodites and isolation of non-Unc progeny of the injected animals. A line carrying an integrated array of p76-
Lag7-flu-1 was obtained by injection of this clone at 80 µg/ml and the same oligonucleotide used for mutagenesis at a concentration of 800 µg/ml (C. Mello, personal communication). Of 489 non-Unc progeny of injected unc-76(e911) animals, 22 gave rise to broods with non-Unc animals, and one of these broods contained animals that produced only non-Unc progeny. Genetic analysis demonstrated that these animals carried a dominant suppressor of unc-76 (presumably the rescuing DNA) linked to dpy-20(e1282ts)IV. The integrated array was named nIs4.

Localization of the epitope-tagged protein was determined by fixation of worms in 4% paraformaldehyde for 2 hours (Finney and Ruvkun, 1990), followed by incubation in 30 µg/ml 12CA5 or 9E10 (Harvard Cell Culture Facility) at 4°C overnight. Secondary and tertiary antibodies were used as described for the beta-galactosidase antibody.

Production of anti-unc-76 antibodies

Five female New Zealand White rabbits were immunized subcutaneously at four-week intervals with unc-76 fusion proteins described below. Rabbits 206 and 207 received four injections of 1 mg doses of a fusion of the E. coli maltose binding protein malE to amino acids 13-385 of unc-76 (using the p76-c7 C terminus). The fusion clone, p76-malc-2, was constructed by ligation of a 1.1 kb BglII (Klenow-filled)-SpeI fragment from p76-c7B into the StuI and XbaI sites of pMalC (New England Biolabs, Inc.). Expression in the E. coli strain BB4 was induced with IPTG, and protein was purified from a soluble protein fraction by affinity chromatography on an amylose column followed by SDS-PAGE on a 7.5% polyacrylamide gel. The gel slice containing the
fusion protein was excised and macerated for injection into the rabbits. Bleeds were taken two weeks after the second and third boosts. Sera were purified by absorption with column-purified MalE protein coupled to CNBr-activated Sepharose beads (Pharmacia) followed by affinity purification on a column of the malE-Unc-76 fusion coupled to CNBr-activated Sepharose (Harlow and Lane, 1988).

A second set of antisera was raised by a different procedure. For their first injections, rabbit 273 received 1 mg of the column-purified malE-Unc-76 fusion protein (approximately 90% pure) dialyzed against PBS pH 7.2, and rabbits 274 and 275 received 0.375 mg of the fusion protein purified by affinity chromatography and SDS-PAGE and electroeluted from a gel slice. All injections were in a total volume of 1 ml in Ribi adjuvant (Ribi ImmunoChem Inc). 1 mg of a glutathione-S-transferase-Unc-76 fusion was purified on a glutathione-Sepharose column (Pharmacia), dialyzed against PBS pH 7.2, and injected into each rabbit in Ribi adjuvant for a first boost. This fusion plasmid (p76-c7-GEX), expressed in DH5α, fused amino acids 48-385 of Unc-76 to the C-terminus of glutathione-S-transferase and was constructed by insertion of a 1.5 kb BamHI fragment of p76-c7B into the BamHI site of pGEX-2T. For a second boost, each rabbit received 1 mg of a fusion of amino acids 12-385 of unc-76 followed by six histidine residues. These were inserted by PCR amplification of p76-28 using the oligonucleotides 76-5'-Kpn (5'-CGGGTACCGATGCTTTTGGAGAG-3') and 76-His6 (5'-GGAAGATCTGTGATGTTGATGACACGATATATTATTATTTGGACAGAC-3'), which added a KpnI site at the 5' end of unc-76 and six His codons and a BamHI site at the 3' end. The PCR product was digested with BglII and BamHI, gel-purified, and ligated into the BamHI site of pQE6 (Diagen, Inc) to
produce p76-QE6-L. The protein was produced in the E. coli strain BB4, purified on a nickel-agarose column under denaturing conditions according to the manufacturer's instructions (Diagen, Inc.), and dialyzed against PBS pH 7.2. Rabbit 273 received 1 mg of the material eluted from the column, of which the desired fusion constituted approximately 25%. Rabbits 274 and 275 received 1 ml of macerated slices of gel-purified fusion protein (approximately 0.67 mg). Bleeds were taken at two-week intervals starting two weeks after the second boost. Sera were affinity-purified on nitrocellulose strips carrying either the Unc-76-His6 mixture or the Unc-76-TrpE fusion protein purified from bacterial inclusion bodies by electrophoresis and electroblotting. (The Unc-76-TrpE fusion plasmid p76-pATH-1A was expressed in the E. coli strain TG1. It was constructed by insertion of a 1.3 kb BglII-HindIII fragment of p76-c7B into the pATH10 vector digested with BamHI and HindIII.) Antibodies were eluted with 100 mM glycine, pH 2.5, neutralized with 1 M Tris pH 8.0, concentrated in a Centricon 100 spin column (Amicon), brought to 1 mg/ml BSA, and stored at -80°C.

A variety of fixation conditions was tested for each antiserum. Serum from rabbit 207 showed specific staining only on worms carrying high-copy arrays of the unc-76 gene and was not pursued further. The optimal fixation conditions for wild-type worms were determined for serum from rabbit 275, judged in initial experiments to give the best staining. Worms were fixed with 4% paraformaldehyde in PBS overnight at 4°C. Samples were divided into two 1.5 ml eppendorf tubes, one of which was subjected to two or three freeze/thaw cycles in liquid nitrogen alternated with a 37°C bath, and then the worms in each were partially
broken with several strokes of a Dounce homogenizer and washed free of fixative in PBS pH 7.2. They were incubated for 30 minutes at 37°C with 10% normal goat serum in PBS/0.5% Triton-X100 and then incubated at 4°C overnight with 10% affinity-purified serum 275 in PBS/0.5% Triton X-100/1% BSA, followed by a 2 hr incubation at 37°C with an-FITC conjugated goat-anti-rabbit antibody (Jackson ImmunoResearch). Washes were conducted as described for the beta-galactosidase antibody. Following the secondary antibody and washes, worms were mounted in Mowiol (Fluka) containing 1 mg/ml p-phenylenediamine (Sigma) and observed with epifluorescence optics on a Zeiss Axiophot microscope. In some preparations, horseradish peroxidase-conjugated goat anti-rabbit secondary antibodies were used, followed by treatment with diaminobenzidine and hydrogen peroxide to form a dark precipitate (all reagents from Vector Laboratories). These animals were mounted in Mowiol and observed with Nomarski optics.

Staining with antiserum from rabbit 275 was also observed in worms lightly fixed by the same procedure used for anti-β-galactosidase antibodies. Inclusion of glutaraldehyde above 0.2%, use of paraformaldehyde above 2%, or fixation for longer than 1 hr. reduced staining by this method. Because permeabilization with this technique was unreliable, however, it was not used routinely. Animals stained after being frozen between two slides in liquid nitrogen and subsequently fixed in -20°C methanol and acetone (Siddiqui et al., 1989; Krause et al., 1990) W. Li, personal communication) also showed strong staining in axonal bundles but poor cell body morphology. Substitution of 10 mg/ml ethylene glycol bissuccinimide (EGS; Pierce) for paraformaldehyde in the standard UNC-76 staining procedure described
above, a technique that has been used to expose masked epitopes
(Rabacchi et al., 1990), produced only weak staining and poor cell
morphology. Treatment of animals fixed by any of these techniques with
0.5% Triton X-100 and β-mercaptoethanol (10-50 mM) for longer than 2-3
hours increased background but did not improve staining. Treatment with
collagenase, as described for anti-serotonin staining (Desai et al.,
1988), eliminated all Unc-76 staining.

**Immunoblotting**

Proteins were separated on 10% SDS polyacrylamide gels and
transferred to nitrocellulose membranes (Schleicher and Schuell) by
electroblotting (Harlow and Lane, 1988). Filters were blocked with
either 1% BSA in TBST or 10% skim milk (used in liquid form, as
powdered milk left a residue on the filters) in PBS for 1-12 hours.
Primary antibodies were applied in TBST/BSA or PBS/milk for 1 hour at
room temperature or 12-16 hours at 4°C, and blots were washed in several
changes of TBST/BSA or PBS/milk on a rocker at room temperature. The
concentration of primary Unc-76 antibody used was approximately 3 μg/ml,
a 1:75 dilution of the dilution used to stain whole-mount worm
preparations. Proteins were detected by incubation with a horseradish
peroxidase-conjugated goat-anti-rabbit secondary antibody diluted
1:10,000 (Vector Laboratories), washing in TBST and PBS, and reaction
with the ECL chemiluminescent reagent kit (Amersham). Images were
recorded on Kodak XAR-5 film.

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References


Figure 3-1.

Physical map of the unc-76 region. A. Clones tested for unc-76 rescue by germline transformation are shown. + indicates a restoration of wild-type locomotion to unc-76(e911) homozygotes. B. Restriction map of the 10.7 kb unc-76 rescuing region, with an indication of structures of subclones used for identifying important genomic regions. +/- rescue indicates that young larvae had nearly wild-type locomotion, but older animals were quite uncoordinated.
Figure 3-1

A

\[
\begin{array}{cccc}
\text{Sac}^3 & \text{unc-76} & \text{V (R)} \\
\hline
\end{array}
\]

\[0.1 \text{ map units}\]

\text{unc-76 rescue:}

\[
\begin{array}{ccc}
\text{C28G7} & - \\
\text{T01G5} & - \\
\text{T06H10} & + \\
\text{C13G10} & + \\
\text{C01G10} & + \\
\text{C08C1} & + \\
\text{T25A9} & - \\
\text{C56C4} & - \\
\text{B0289} & - \\
p76-16A, B & + \\
\end{array}
\]

B

\text{unc-76 rescue:}

\[
\begin{array}{cccc}
\text{BamHI} & \text{ApaI} & \text{SacII} & \text{HindIII} \\
\hline
\text{XbaI} & \text{HindIII} & \text{BamHI} & \text{SacI} \\
\hline
\text{KpnI} & \text{XhoI} & \text{BamHI} \\
\hline
1 \text{ kb} & \text{XbaI} \\
\end{array}
\]

\[
\begin{array}{cccc}
\text{XbaI} & \text{ApaI} & \text{SacII} & \text{HindIII} \\
\hline
\text{BamHI} & \text{ApaI} & \text{SacII} & \text{HindIII} \\
\hline
\text{XhoI} & \text{BamHI} & \text{SacII} & \text{HindIII} \\
\hline
\text{p76-16A, B} & \text{p76-8} & \text{p76-11, p76-15} & \text{p76-10} \\
\text{p76-13} & \text{p76-14} & \text{p76-8\text{A}Hind} & \text{p76-7} \\
\text{p76-7} & \text{p76-7} & \text{p76-7} & \\
\end{array}
\]
Figure 3-2.

Northern blots of polyA+ RNA probed with the inserts from p76-c4 and p76-c7. A single major transcript of 1.8 kb is visible, while a long exposure (right) reveals a minor band at about 2.5 kb. 10 µg of embryonic RNA were loaded in lanes 1 (N2), lanes 2 (unc-76(e911)), and lanes 3 (smg-1(e1228)him-2(e1065);unc-76(e911)). Lane 4 contains wild-type RNA from a mixed-stage population.
Figure 3-3.

Genomic sequence of *unc-76*

Coding regions from p76-c4 and p76-c7 are indicated. The alternative C terminus of p76-c4 is indicated by parentheses. Dashed line at nucleotides 950-960, potential 3' splice acceptor. Labeled underlines, primers used for primer extension (76-PE1 and 76-PE2) or PCR to verify alternative splicing (c4A, c4B, c4C, c4D). SL1 at nucleotides 1084 and 1112 indicates the sites at which the 22 nucleotide leader sequence SL1 was found to be spliced to *unc-76* sequences by reverse transcriptase-PCR analysis. Small arrow at nucleotide 989, start of p76-c4 cDNA. Open arrowhead at nucleotide 1107, start of p76-c7 (following poly-A). Underlined ATAAA sequence, potential polyadenylation signal.
Figure 3-4

Genomic organization of the *unc-76* locus. Top: restriction map of the 10.7 kb rescuing insert in p76-16A and p76-16B. Bottom: The exons of p76-c4 and p76-c7 are indicated by dark lines. The two clones differ by the inclusion or exclusion of intron 8.
Predicted Unc-76 proteins. A. Protein sequences deduced from p76-c4 and p76-c7. The sequences are identical through amino acid 354, and the alternative p76-c4 C terminus is shown beginning at amino acid 355. The positions in the proteins affected by unc-76 mutations are indicated. B. Kyte-Doolittle hydrophobicity plot for the p76-c7 form of the Unc-76 protein. The protein is hydrophilic overall and lacks clearcut membrane-spanning hydrophobic regions.
Figure 3-5  Predicted Unc-76 protein

A. Predicted Unc-76 protein sequences and sites of mutations

1  MRFGEVAMEA ADLRPVDIPL ASCDDDDDIDS NKNLSNHS SD EKHHNCNSNSD 50
   n2457 (splice acceptor)
51  EERLHDEFGS SLDELQVNF SDKIAACLKDH EVTTADIPV QIRTQEEVMN 100
   n2397 (amber)  ev424 (2 bp insertion)
101  ESQTVWTLTG NFRNIIQYLDF GTSSICKKMA AALDSDSLKD DASTRSMTN 150
   n2367 (1 bp deletion)  n2399 (amber)
151  SDDDELLRQG MDVHQMIGHH HGSTDYGGT PPQTADQVIE EIDEMLASC 200
   e911 (1 bp deletion)  n2398 (opai)
201  FTGPPLEKRT MESVDSMHSS MRSPFPSSIQ SSDADIKLRS AQALVSNPIN 250
251  LQELSYSKLV TLCAEMEQLI RYINESLVE LAHRDELVYE KEMKNSFISL 300
301  LLAIQNKRKV YANDRKRKVG KASDASQLPQ YLTATIPYNQ HQIDNASIA 350
351  SLIKILRAIH DNTTVPTLL TDYILTHVCP KNISC 385  p76-c7 C terminus
       ....SEKMR DINKYLIDKE YQLCVD 376  p76-c4 C terminus

B. Ryte-Doolittle hydrophobicity plot for p76-c7 form of unc-76
Figure 3-6

Primer extension analysis of the unc-76 5' end.

Primers 76-PE1 and 76-PE2 were end-labeled with $^{32}$P, hybridized to total C. elegans RNA or poly A+ RNA, and extended with AMV reverse transcriptase. Lanes 1 and 2 are the labeled primers 76-PE1 and 76-PE2 in the absence of RNA. Lane M is a sequencing ladder used to ascertain the molecular weights of primer extension products. Lanes 3 and 4, 76-PE1 extension of total and poly A+ RNA, respectively. Bands are visible at 57, 75, 119, 156, 440, and 550 nucleotides. Lanes 5 and 6, extension of 76-PE2 using total and poly A+ RNA.
unc-76/lacZ fusion expression. Wild-type animals carrying an extrachromosomal array of p76-L11 were stained with X-gal. Note intense staining in the ventral nerve cord and nerve ring of the L4 animal shown in A (left lateral view), and also the absence of staining in the lateral hypodermis. The cell bodies of the neurons HSNL and CANL are visible near the center of the animal on the lateral surface. Staining in individual cell bodies in head and tail ganglia can be seen. Faint staining of four nonneuronal support cells is visible in the head (wide fan-like cells extending halfway from the tip of the nose to the nerve ring). B. unc-76/lacZ expression in a late embryo partially removed from its eggshell. Staining of cell bodies and axons can be seen in the ventral cord, head, and tail ganglia. Note the absence of stain from nuclei. Scale: A: 1 cm = 23 μm; B: 1 cm = 9.5 μm.
Three antisera raised against Unc-76 fusion proteins recognize the same set of proteins. Antisera from rabbits 273, 274, and 275 were affinity-purified on Unc-76-His6 fusion proteins immobilized on nitrocellulose. Western blots of total protein from mixed-stage populations N2 (right lanes in each set) and unc-76(e911) (left lanes) were stained with horseradish peroxidase-conjugated secondary antibodies and reacted with the chemiluminescent reagent ECL (Amersham). A set of bands is visible in the 54-58 kDa range in each N2 lane (3 bands visible with serum 273, 4 bands visible with the others) and is absent in unc-76 lanes, replaced by a set of bands in the 33-35 kDa range.
Figure 3-9

Unc-76 protein expression in unc-76 and unc-34 mutants. Immunoblot of protein from mixed-stage populations of animals using serum 275 affinity purified with an Unc-76/TrpE fusion indicates that Unc-76 protein is altered or reduced in abundance in all unc-76 mutants. Note the reduction in staining in the 54-58 kDa range in all eight unc-76 mutant strains and smaller allele-specific bands appearing in e911, n2367, n2398, and n2399 lanes. Three unc-34 strains (e315, e566, and e951) were included at right but appeared to contain normal levels of protein. Left, pairs of lanes of protein from N2 and from a strain expressing the unc-76-lacZ fusion p76-L11. The left lanes were incubated with an anti β-galactosidase polyclonal rabbit antiserum, and the right lanes were incubated with antiserum 275. A set of bands is visible in the fusion-bearing strain with either antibody. Lane 3, main panel (*e911;unc-76(+)*)", protein from MT6579, which carries an array of the rescuing plasmid p76-16B in a homozygous unc-76(e911) background. Note that both heavy wild-type bands and truncated e911 bands are visible.
Figure 3-10

N2 animals stained with purified anti-Unc-76 antiserum 275 and an FITC-conjugated secondary antibody. A. Left lateral view of a wild-type adult. Anterior is at left. The nerve ring, dorsal cord, ventral cord, and several longitudinal processes can be seen to stain in this immunofluorescence micrograph. Cell bodies of the HSN and CAN neurons are visible. B. Both longitudinal and dorsoventral axons contain Unc-76 protein. The dorsal cord (thick horizontal band), two dorsal sublateral cords (thinner horizontal bands), and three commissural axons (vertical lines) are visible in this N2 adult. Scale: A: 1 cm = 34 μm; B: 1 cm = 16 μm.
Fluorescence micrographs of animals stained with antiserum 275. A. A late-stage embryo and L2 larva of the Unc-76 overexpressing strain MT6579 show bright staining in the ventral cord and nerve ring (bright semicircle at upper right in L2 larva, bright band in lower right of embryo). B, C. Wild-type adults stained with antiserum 275. B shows staining in the nerve ring (bright vertical band at left), the left and right sides of the ventral cord (brightest horizontal stripe, bottom), ventral and dorsal sublateral cords (narrow horizontal stripes flanking each major cord), and the dorsal cord (wide horizontal stripe, top). C shows staining of the right HSN cell body (arrow) and axon (faint vertical stripe) extending into the ventral cord, as well as the PLM axon, which extends laterally from the posterior (left) and appears to contact the HSN cell body. Scale: A: 1 cm = 15 µm; B, C: 1 cm ≈ 23 µm.
Figure 3-12

Four focal planes of the head of an Unc-76 overexpressing (MT6579) adult stained with antiserum 275 and viewed from the dorsal side. Anterior is at left, and the nerve ring is roughly at the center of each image. The outline of the pharyngeal bulbs can be seen in E. The four pairs of anti-Unc-76 (A,C,E,G) and DAPI (B,D,F,H)-stained images progress from ventral (A,B) to dorsal (G,H). Most of the cells in the head can be observed to stain with anti-Unc-76 antibodies. A,B. Complete staining of the retrovesicular ganglion, indicated with an arrow. C,D. Lateral ganglia of the head (arrowheads), just posterior to the nerve ring, stain brightly. E,F. The remainder of the lateral ganglia are visible. Note a small five-cell ganglion to the right of the arrowhead. All five cells in this plane stain, but a sixth in the more dorsal plane (G,H) does not stain with anti-Unc-76 (arrows). This cell is likely to be the ADEsh cell, a nonneuronal support cell. Scale: 1 cm = 18.5 µm.
Figure 3-13.

Ventral nerve cord cell body expression of Unc-76 protein varies with developmental stage. A. Most neuronal nuclei in this fragment from a wild-type L2 ventral nerve cord stain with anti-Unc-76 (serum 275). Stain is visible in each cell body in a ring surrounding the nucleus, which does not stain. Anterior is at left, and the ventral side is down. B. Nuclei from the animal shown in A visualized with DAPI. C. Portion of a ventral nerve cord stained with anti-Unc-76 antiserum 275. Only a subset of adult ventral cord cell bodies stain brightly, while axons stain brightly and a few cell bodies (arrow) show faint Unc-76 immunoreactivity. Anterior is at left. The right ventral cord is the thick stained neuronal bundle below the indicated cell bodies, and the thin bundle above these cell bodies is the left ventral cord. D. Nuclei from the animal in C visualized with DAPI. Scale: A,B: 1 cm = 11.5 μm; C, D: 1 cm = 15 μm.
Wild-type adults stained with antiserum 275. A. In the posterior half of this adult, staining of the HSNR cell body (arrow) is visible, as well as staining of CANR and the two neurons of the right postdeirid (cell bodies in the center of this micrograph, adjacent to the CANR process). Ventral and dorsal cord staining are also visible. Anterior is at right. B, C. Anti-Unc-76 and DAPI staining of the tail region of the worm shown in A. The three cells of the dorsorectal ganglion (arrow) clearly stain with anti-Unc-76 (B). D, E. Ventral views of the head of a wild-type adult stained with anti-Unc-76 antibodies (D) and DAPI (E), showing the intense anti-Unc-76 staining of cell bodies just posterior to the nerve ring (large arrow). Anterior is at left. Only one of the six cells in the ganglion visible at lower right (small arrow) fails to stain with anti-Unc-76, similar to the pattern observed in Unc-76 overexpressing animals (see Fig. 3-12, G-H). Scale: A: 1 cm = 24 μm; B, C: 1 cm = 14.5 μm; D, E: 1 cm = 15 μm.
Figure 3-15

Two focal planes of a lateral view of the head of a wild-type adult stained with anti-Unc-76 antibodies (antiserum 275; A,C) and DAPI (B, D). Anterior is at left. The outline of the pharyngeal isthmus and posterior bulb can be seen in C. Note the intense staining of nerve ring axons in A and C (bright vertical band at left) and the weaker staining of head ganglion cell bodies flanking the nerve ring. Comparison with DAPI staining (B,D) indicates that most if not all cells in the ganglion contain Unc-76 protein. Scale: 1 cm = 16.5 μm.
Figure 3-16.

Postdeirid neurons but not nonneuronal support cells express Unc-76 protein in Unc-76 overexpressing (MT6579) animals. A. Dorsal view (anterior is to the right) of an MT6579 animal stained with antiserum 275. Two cells on the right side (side with arrow) and four on the left stain, while two cells on each side do not stain (arrow). B. Nuclei in the animal shown in A stained with DAPI. The two posteriormost cells in the cluster on the right side (arrow) do not stain with anti-Unc-76 antibodies. The numbers and positions of cells are consistent with the failure of nonneuronal support cells to stain. Scale: 1 cm = 12 μm.
Figure 3-17.

The onset of Unc-76 expression begins early neuronal development. This L1 animal has begun to generate postembryonic motorneurons in the anterior ventral cord, while the posterior still retains the embryonic pattern. Newly-derived anterior neurons (at the bottom of this micrograph) in this unc-76-overexpressing animal stain darkly with antiserum 207.
Figure 3-18

Unc-76 protein is reduced in abundance in unc-76 mutant animals. Only traces of stain (antibody 275 visualized with an FITC-conjugated secondary antibody) are evident in e911 (A), n2397 (B), n2399 (C), rh116 (D), and n2457 (E) animals. These images represent some of the most intense staining observed in these strains. Arrows indicate the nerve ring (A, B, D) or the ventral cord (C, E), where staining is most intense in wild-type animals. Note that the weak staining is still localized in axons. Scale: 1 cm = 15 μm.
Chapter 4. New techniques for the study of *C. elegans*

neuronal development
Summary

To expand the set of techniques available for the study of axonal outgrowth in *C. elegans*, methods for labeling axons and culturing embryonic neurons were developed. Vital fluorescent dyes that could be activated in specific cells, a photoactivated fluorescein derivative and a compound that releases fluorescein when cleaved by β-galactosidase, failed to produce specific staining. Fusions of Unc-76 protein sequences to β-galactosidase were localized to axons in transgenic worms, and specific cells were labeled by placing unc-76/lacZ fusions under the control of the *unc-86* promoter. Cells expressing these fusions could also be observed in the electron microscope, although the label was not well localized. Techniques for the short-term primary culture of embryonic cells were explored, and media, substrates, and methods of cell preparation that produced neurons capable of extending long neurites were determined.
Introduction

The small size of *C. elegans* and the ease with which mutations affecting nervous system development can be obtained and analyzed have allowed extensive genetic studies of axonal outgrowth. The worms' small size, however, prevents the use of some of the most powerful techniques available in vertebrates and larger invertebrates, such as direct surgical manipulation, labelling of individual neurons by intracellular dye filling, and electrophysiological recording. In addition, the available reagents for visualizing specific axons do not permit labeling of axons until outgrowth is complete, making interpretation of mutant phenotypes difficult. Finally, techniques for the culture of *C. elegans* neurons *in vitro* have not been developed, making the study of neuronal behavior on defined substrates impossible. The two parts of this chapter will focus on three lines of experimentation designed to expand the set of tools available for the study of *C. elegans* neurobiology. In Part I of this chapter, a technique for labeling specific subsets of axons during development using an *unc-76/lacZ* fusion will be described, and its application to electron microscopy will be presented. In Part II, a technique for the short-term culture of primary embryonic cells, in which extensive axonal outgrowth is observed, will be discussed.

I. Development of axonal markers

Introduction
Several stains allow the visualization of small numbers of C. elegans axons, e.g., vital dyes such as DiO and FITC and antibodies against serotonin, GABA, FMRPamide, acetylated tubulin, and horseradish peroxidase \( \text{(Desai et al., 1988; McIntire et al., 1992; Schinkmann and Li, 1992; Siddiqui, 1990; Siddiqui and Culotti, 1991). All of these, however, suffer from an important limitation: they can be used only to visualize neurons that have already completed development. The HSN axons in unc-34 and unc-76 mutant adults are shorter than normal, but because the cells do not express serotonin (and therefore cannot be visualized) until adulthood, the events leading to this abnormal axonal morphology are unknown. In the simplest scenario, the HSN axons grow at their normal time and rate and simply stop growing at the point observed in adults. However, precedent from the Drosophila disco gene suggests that failure to attach to a target reached early in development can lead to detachment as the animal grows, causing the appearance of abnormal axonal trajectories in older animals \( \text{(Steller et al., 1987). In addition, abnormal timing or rate of outgrowth could lead to the observed HSN morphology. These issues could be resolved if a method by which developing axons could be observed were available.}

Several methods for studying axons prior to their terminal differentiation and expression of antigens such as serotonin are available. Reconstruction of the wild-type nervous system from serial electron micrographs showed that HSN axons were not present in the nerve ring of an L4 larva but were present in adults, implying that outgrowth occurs in the late L4 stage \( \text{(White et al., 1986), but reconstructions are too laborious to be used to assay defects in axon outgrowth. Several alleles of the gene clr-1 cause animals shifted to 25°C to}
become filled with fluid in a way that allows fine neuronal processes and muscle arms to be seen in Nomarski optics (Hedgecock et al., 1990). Axons in the lateral regions of the animal can easily be seen because there are few other structures nearby, whereas axons in the nerve cords cannot be distinguished from one another. The HSNs have been observed in clr-1 mutants (Garriga et al., 1993) as they grow ventrally along the lateral hypodermis to the ventral nerve cord in the L2 stage. HSN outgrowth in the ventral cord cannot be observed in clr-1 animals.

An ideal marker would allow any single axon to be observed in a living animal over the course of the animal's life. In larger animals, this can be approximated by injecting specific cell bodies with a dyes such as lucifer yellow or di0 or diI. The small size of C. elegans and tough cuticle make accurate injections into living animals prohibitively difficult. An alternative technique involves the use of caged fluorescent molecules that can be activated by a focused beam of light of the appropriate wavelength. O'Farrell and colleagues activated a caged fluorescein derivative covalently linked to a dextran of approximately 70,000 daltons which was also coupled to a short peptide containing a nuclear localization sequence (Vincent and O'Farrell, 1992). This technique provided an intensely fluorescent signal in the nuclei of specific cells.

P. Candido et al. (personal communication) have recently reported success with a similar technique that involves transgenic worms carrying the lacZ gene under the control of a heat shock promoter. The desired cell is heated by means of an attenuated laser microbeam, and at the desired time, worms are fixed and treated with X-gal to visualize the
induced β-galactosidase. Candido and colleagues reported that axons can be visualized in this manner.

The approaches described above require the manipulation of individual animals and risk damage to the neuron under observation. A second kind of approach to the problem of labeling axons during development involves the use of axonally-localized β-galactosidase fusion proteins that are expressed under the control of promoters that are active in a small number of neurons as they develop. It allows the study of axons in a population of animals without the manipulation of every individual to be studied. While visualization of β-galactosidase-containing cells presently requires fixation of the animals, the successful use of vital fluorescent β-galactosidase substrates such as FDG in intact zebrafish embryos suggests that this may eventually be possible in living C. elegans animals as well (C. Fulwiler, personal communication).

A successful axonal marker based on β-galactosidase requires two elements: a protein sequence that, when fused to β-galactosidase, will target the fusion to the axon, and a promoter that is active in the desired neuron but not in neighboring cells. Fusions of Drosophila kinesin sequences to β-galactosidase for axonal targeting in Drosophila enhancer trap screens have been reported to label axons (E. Giniger, personal communication). A comparable kinesin/β-galactosidase fusion in C. elegans, however, gave only small spots of β-galactosidase immunoreactive material in the vicinity of the nerve ring, suggesting that the kinesin motor may have transported the fusion protein to the nerve terminal without leaving any along the axon (G. Ruvkun, personal communication). The difference may result from expression levels in the
different experiments; overexpressed fusion proteins in the Drosophila case may have saturated all of the available sites on the microtubules, leaving some protein along the axons. The experiments described below involve the use of part of the unc-76 coding region to target β-galactosidase to axons. While β-galactosidase alone expressed from an extrachromosomal array appears to enter axons, unc-76 sequences appear to prevent entry of the fusion protein into the nucleus and to enhance the proportion of protein located in the axon. An unc-76/lacZ fusion that could be placed downstream of any promoter could facilitate the study of any set of desired neurons.

In order for a promoter to be useful specifically in the study of HSN outgrowth, it would have to be active in the HSNs during larval development, and it could not be active in other cells with axons near the HSN axons in the ventral nerve cord, or it would be impossible to distinguish them in the light microscope. Only one cloned C. elegans gene, unc-86, is known to fulfill both of these criteria (Finney and Ruvkun, 1990). The unc-86 gene is required for the differentiation of many neuronal types, and it is expressed in 57 neurons, including the HSNs, beginning early in their development. The only unc-86-expressing cells with processes in the anterior half of the ventral nerve cord are HSNR, PVR, PVM, and AVM in the right ventral cord and HSNL in the left ventral cord bundle. PVR, PVM, and AVM run together in the ventralmost portion of the ventral nerve cord, while HSNR runs at the dorsal edge of the cord, quite far from the other unc-86-expressing cells (White et al., 1986). An unc-76/lacZ fusion under the control of the unc-86 promoter would allow visualization of the left HSN axon, and possibly the right HSN axon as well, throughout their development without
interference from neighboring processes. Ruvkun and Finney (personal communication) subsequently demonstrated that a truncated form of the unc-86 promoter activated β-galactosidase fusion expression in only a subset of the normal unc-86-expressing cells, including the HSNs but not any of the other cells with processes in the ventral cord. This appeared to be close to the ideal promoter for the study of HSN outgrowth.

The attempts to apply specifically-expressed lacZ fusions to electron microscopy discussed in this chapter were motivated by limitations in the study of axonal outgrowth at the level of resolution available in the light microscope. The interaction of neighboring axons is critical in normal pathfinding and bundle formation, but the precise positioning of neighboring axons can be ascertained only by electron microscopy. In addition, electron microscopic studies of fasciculating axons in the grasshopper central nervous system have provided strong evidence for the preferential association of filopodia and growth cone lamellae with specific axons known to be essential for guidance (Bastiani et al., 1984; Bastiani and Goodman, 1986; du Lac et al., 1986). Equivalent studies of the interactions of the HSN growth cones with the axons they normally follow (PVP and PVQ; Garriga et al., 1993) would be helpful in interpreting the phenotypes of mutants discussed in Chapter 2. In addition, because defasciculation of neighboring axons is difficult to detect at the light microscope level unless axons are spread far apart, electron microscopic studies of neighbor-neighbor interactions would be useful for interpretation of defects in fasciculation mutants.
Few C. elegans axons can be identified in electron micrographs without reconstruction from serial sections, which is prohibitively tedious for most studies. A marker for specific axons that could be used in the electron microscope would overcome this difficulty because in many cases examination of a few sections would be sufficient to ascertain the positions of marked axons. In larger animals, injection of reagents that give rise to electron-dense products (combinations of diaminobenzidine with horseradish peroxidase, lucifer yellow, or diI) is used for axonal labeling (e.g., Bastiani et al., 1984; Bastiani and Goodman, 1986; du Lac et al., 1986; Chitnis et al., 1992), but accurate injections into living C. elegans animals are not possible.

β-galactosidase fusions are potentially useful as electron microscope-level markers because the blue reaction product of X-gal with the enzyme, a 5-bromo-4-chloro-3-indolyl precipitate, is electron-dense and can remain close to the site of the enzyme (Bonnerot et al., 1987). X-gal histochemistry combined with electron microscopy has been used to locate cells in mouse cerebella infected with a β-galactosidase-expressing retrovirus (Snyder et al., 1992). Efforts to apply the unc-76/lacZ fusion vectors developed in this chapter to electron microscopy are discussed below.

Experimental Procedures

Injection of caged fluorescein compounds

Fluorescein di-β-D-galactopyranoside, or FDG (Molecular Probes) was dissolved in DMSO and brought to a concentration of 8 mM in worm
injection buffer/10% DMSO (Fire 1986). Animals were injected by the same technique used for DNA transformation: animals were placed on an agarose pad and the lumen of the syncytial portion of the adult gonad was filled with injection solution by microinjection. The animals were rehydrated in recovery buffer, floated off of the agarose pad with M9, and allowed to lay eggs on a seeded agar plate overnight. Embryos and young larvae on the plate were placed on a standard Nomarski agarose pad (Wood, 1988) and viewed under epifluorescence optics.

Caged fluorescein-linked dextran (MW 10 kDa; Molecular Probes) was brought to 10 mg/ml in PBS, diluted to 1 mg/ml in worm injection buffer, and injected by the technique described above. Activation of the fluorescein requires wavelengths of ultraviolet light not transmitted well with glass optics, and so a microscope with a minimal number of lenses and mirrors between the ultraviolet light source and the specimen was used. A Zeiss Universal microscope with direct fluorescence optics (a 100 W mercury vapor lamp focused on the specimen from below through the condenser) was arranged to illuminate a small portion of the field of view. With only the visible lamp on, the field diaphragm was closed to its minimum aperture and covered with a piece of aluminum foil pierced with a pin, and the resulting spot of light, still relatively large, was centered in the field visible with a 100x Nomarski objective lens. The specimen was placed onto the stage and the spot of light was focused on the desired region. For fluorescein activation, the light path from the objective lens to the eyepieces was closed, and the light path from the mercury lamp to the specimen was opened for periods of 20 sec.-2 min. Worms or test spots of the caged fluorescein-dextran
solution were observed with a Zeiss Axiophot microscope equipped with epifluorescence illumination and the standard fluorescein filter set.

Construction of unc-76/lacZ fusion plasmids

The construction of plasmids in which lacZ was placed under the control of the unc-76 promoter region are discussed in Chapter 3.

The vector p76-L18, containing a polycloning site at the 5' end of a portion of the unc-76 cDNA (codons 1-197) and followed by the lacZ gene and the 3' untranslated region from the C. elegans unc-54 gene, was constructed as follows (Fig. 4-4). The PCR primers 76-5'-Kpn (5'-CGGGGTACCAGCTTTTCCGAGG-3') and 76-mid-2 (5'-CGGGGTACCTGTAACATTTTACATTCTTC-3'), which include KpnI sites at their 5' ends, were used to amplify a 590 base-pair fragment of unc-76 cDNA from p76-28 (construction described in Chapter 3). This fragment was digested with KpnI and ligated into the KpnI site of pPD21.28LA, a lacZ vector modified from pPD21.28, in place of the nuclear localization signal sequences in the parent vector (Fire et al., 1990a; T. Burglin, B. Reinhardt, and G. Ruvkun, personal communication).

Three sets of unc-76/lacZ fusions were placed under the control of the unc-86 promoter. All included at least the first exon and first intron of unc-86 because the first intron is required for maintenance of expression (M. Finney and G. Ruvkun, personal communication). In all fusions, the unc-86 reading frame was maintained. The first fusion, p86/76-1, contained approximately 5 kb of unc-86 upstream sequences and transcribed DNA to the middle of exon 3, unc-76 genomic DNA sequences from codons 13-187, lacZ (including a termination codon), and 1.8 kb of
the unc-76 3' untranslated region. It was constructed by digestion of
the unc-76/lacZ clone p76-L16 (Chapter 3) with BglII, creation of blunt
ends with Klenow fragment, and digestion with EagI, followed by ligation
into the unc-86/lacZ vector SA2 digested with StyI, blunted with
nuclease S1, and digested with EagI. SA2 (kindly provided by G. Ruvkun)
was constructed by insertion of an 8 kb SpeI fragment of unc-86-region
DNA into the XbaI site of pPD22.04 (Fire et al., 1990a). A fusion
similar to p86/76-1, called p86-L1, contained the same unc-86 promoter
and coding regions fused directly to the lacZ gene, with all unc-76
sequences removed and the unc-54 3' untranslated region downstream of
lacZ. It was constructed by digestion of SA2 with KpnI, removal of the
3' overhang with T4 DNA polymerase, digestion with StyI, filling of the
5' overhang with Klenow fragment, and self-ligation.

The second set of fusions used the unc-54 3' untranslated region
in place of unc-76 3' sequences, and unc-86 coding sequences after the
second codon of the second exon were eliminated. DNA from the SA2
vector was amplified by PCR with the primers 86-Hind (5'-
GTCAAGTTTCAATGGATTTCTAC-3'), which corresponds to a position in the
first intron 5' to a HindIII site, and 86-poly (5'-
CGGGGATCCATCCAGGTTGAACTGAAAATTCAGAATCTT-3'), which adds StyI (CCTTGG)
and KpnI sites after the second codon of exon 2 of unc-86. This
fragment was digested with HindIII and KpnI and ligated into the HindIII
and KpnI sites of the lacZ vector pPD21.28, generating p86-L2A. This
plasmid, in turn, was digested with HindIII and SpeI, and inserted into
SA2 digested with SpeI and partially digested with HindIII, generating
p86-L3A. This served as a control vector, because it contained the unc-
86 promoter and modified third exon linked directly to lacZ without unc-
76 sequences. The unc-76-containing vector p86-L5A was constructed by insertion of the KpnI-digested unc-76 cDNA fragment described above (amplified by PCR primers 76-5'-Kpn and 76-5'-mid2) into the KpnI site of p86-L3A.

The third set of fusions used the clone C2, which contains a truncated unc-86 promoter active in only a subset of unc-86-expressing cells (M. Finney and G. Ruvkun, personal communication). C2 was constructed from a 5.7 kb EcoRI fragment of unc-86 DNA in pBluescribe, into which an XbaI-SpeI fragment of the lacZ vector pPD22.04ΔI (T. Burglin, B. Reinhardt, and G. Ruvkun, personal communication) was inserted at the unc-86 SpeI site. p86/76-4A was constructed by insertion of the KpnI-digested unc-76 cDNA PCR fragment used above into SA2 digested partially with KpnI. The unc-76 sequence was inserted at the 3' end of the nuclear localization signal cassette from this vector, which was not removed by partial KpnI digestion. p86/76-4A was digested with EcoRV to delete the nuclear localization cassette and a potential unc-86 nuclear localization signal near the SpeI site, generating p86/76-6. This construct lacked the unc-86 homeodomain but contained the POU domain. A final construct, p86/76-7A, was built by digestion of p76-L18 (described above) with StyI, filling with Klenow fragment, digestion with SpeI, and ligation into C2 prepared by the same enzymatic treatment. This construct contained unc-76 sequences inserted at the same position in unc-86 as they were in p86/76-1 and lacked the unc-86 POU domain.

p86/76-1, p86/76-L1, p86/76-L3A, and p86/76-L5A were injected into wild-type worms at 10-50 μg/ml together with 20 μg/ml pRF4 (encoding rol-6(su1006); Kramer et al., 1990). Strains were maintained at 20°C.
p86/76-4A, p86/76-6, and p86/76-7A were injected at 20 μg/ml with 30 μg/ml pMH86 (encoding the wild-type dpy-20 gene; Han and Sternberg, 1991) into dpy-20(e1282ts) hermaphrodites. Strains were maintained at 25°C, where the dpy-20 phenotype is most clear.

Antibody and X-gal staining

Anti β-galactosidase antibody staining was done by the method of Finney and Ruvkun (1990). Animals were washed from plates into a 1.5 ml eppendorf tube, washed in M9, and flash-frozen in a fixative solution (1% paraformaldehyde in 25% methanol, 80 mM KCl, 20 mM NaCl, 10 mM EGTA, 5 mM spermidine HCl, and 15 mM PIPES pH 7.4). Samples could be stored at -80°C for several days. Frozen samples were thawed for 30 min on ice, washed twice in a 100 mM Tris pH 7.5/1% Triton X-100/1 mM EDTA buffer, and incubated on a rotator for 2 hours at 37°C in 100 mM Tris pH 7.5/1% Triton X-100/1 mM EDTA/1% β-mercaptoethanol. Worms were then washed once in 25 mM borate buffer, pH 9.5, incubated on a rotator for 15 min. at 37°C in borate buffer/10 mM dithiothreitol, washed once in borate buffer, and incubated on a rocker for 15 min at room temperature in borate buffer/0.3% hydrogen peroxide. Worms were washed once in borate buffer and once in 0.5% Triton X-100/1% BSA in PBS pH 7.2, and were stored in the same Triton/BSA/PBS buffer at 4°C.

Animals were stained with a 1:50 dilution of a monoclonal anti-β-galactosidase antibody (Promega) in Triton/BSA/PBS at 4°C overnight, washed three times at room temperature in Triton/BSA/PBS, and incubated with a FITC-conjugated goat-anti-mouse secondary antibody (Jackson ImmunoResearch) for 2 hr at 37°C, followed by three washes in
Triton/BSA/PBS. Stained animals were mounted in Mowiol (Fluka) containing 1 mg/ml p-phenylene diamine and observed on a Zeiss Axiophot microscope equipped with epifluorescence illumination.

For electron microscopy, worms were fixed as described in the text. The staining solution used for worms to be processed for electron microscopy was composed of 200 mM sodium phosphate pH 7.5, 1 mM MgCl₂, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, and X-gal in dimethylformamide. After staining, worms were processed for electron microscopy as described in Chapter 2.

X-gal stained animals were removed from the staining solution with a glass micropipette, rinsed through several changes of cacodylate buffer, and pooled for fixation for electron microscopy.

Results

Labeling of living cells with caged fluorescein derivatives

Vincent and O'Farrell (1992) injected Drosophila embryos with a caged fluorescein derivative covalently linked to a 70 kDa dextran and a peptide encoding a nuclear localization signal. This compound was not fluorescent unless activated with 350 nm light. Illuminated cells retained the fluorescent label in their nuclei. Efforts in another laboratory to achieve similar results in C. elegans without the use of a nuclear localization signal, necessary for the visualization of axons, have produced weak diffuse signals but never a localized signal of
sufficient intensity to be useful for visualizing axons (J. Maloof and C. Kenyon, personal communication).

Attempts to improve on these results were not successful. Spots of caged fluorescein placed onto a microscope slide could be activated by illumination with unfiltered light from a 100 W mercury lamp attached to a Zeiss Universal microscope (see experimental procedures). These were the best optics available that would allow transmission of wavelengths of light that could activate the compound; quartz optics are needed for optimal activation. Nevertheless, exposures as short as 20 seconds were sufficient to cause the exposed region to fluoresce brightly when observed under epifluorescence optics. When the same dye was injected into adult *C. elegans*, however, fluorescence could not be activated with exposures of up to several minutes either in young progeny of the injected animals or the injected adults themselves.

A second caged fluorescein derivative tested for axonal labeling was fluorescein di-β-D-galactopyranoside, or FDG. This compound is a β-galactosidase substrate that releases a fluorescein moiety when cleaved by the enzyme (Nolan et al., 1988). It can be loaded into living cells by osmotic shock and observed by fluorescence microscopy without apparently harming the cells (Nolan et al., 1988). Whole dechorionated zebrafish embryos can be stained with FDG if soaked briefly in an FDG solution (C. Fulwiler, personal communication). Because growing *C. elegans* axons expressing β-galactosidase fusions might be labeled by this compound, it was injected into worms carrying the unc-76/lacZ fusion p76-L11 (for construction, see Chapter 3) on an extrachromosomal array.
FDG was injected into the gonads of adult hermaphrodites, and they and their progeny were examined approximately 16 hours later by fluorescence microscopy. Specific neuronal staining was not observed, but bright fluorescence was observed in the intestine of embryos. Staining had largely disappeared by the L1 stage. Injected adults showed similar intestinal staining, which was not observed in uninjected adults or their progeny. It is likely that FDG, like other low molecular weight compounds, is transported to the intestine during embryogenesis and is therefore not retained in neurons.

**Design of unc-86/unc-76/lacZ vectors**

**unc-76 sequences required for axonal localization**

Several fusions of the unc-76 promoter and coding sequences to lacZ were tested to determine the unc-76 coding regions needed for localization of the fusion protein to axons (Fig. 4-1). Animals carrying arrays of p76-L16 and p76-L15, which contained unc-76 genomic DNA up to codons 186 and 253, respectively, fused directly to β-galactosidase, both contained β-galactosidase immunoreactive material in their axons and cell bodies but not in nuclei (Fig. 4-2). Animals carrying plasmids with an SV40 T antigen nuclear localization sequence (NLS) between unc-76 and lacZ sequences also showed β-galactosidase immunoreactivity in axons and perinuclear regions of neuronal cell bodies, indicating that either the NLS did not function in these positions within the fusion protein or axonal localization functions present in unc-76 coding sequences were able to override nuclear
localization functions of the NLS. However, a similar Unc-76/$\beta$-galactosidase fusion protein containing only the first 12 amino acids of unc-76 (expressed from an array of p76-L13) was localized to the nucleus when the NLS was present. When the NLS was removed from this construct (in p76-L17), the resulting fusion protein expressed from an extrachromosomal array was still found in axons (Fig. 4-2). However, axons in these animals stained less intensely with anti $\beta$-galactosidase antibodies relative to the cell bodies than did axons in animals carrying arrays of p76-L11, p76-L15, or p76-L16. Furthermore, the fusion protein from p76-L17 appeared to be present in neuronal nuclei, in contrast with the other unc-76/lacZ fusion proteins. Therefore, sequences between amino acids 13 and 186 of the Unc-76 protein were judged likely to be involved in exclusion of the fusion protein from the nucleus and preferential localization in the axons. This conclusion is supported by the observation that unc-76 mutants encoding proteins truncated after 105, 165, and 197 amino acids occasionally show faint Unc-76 immunoreactivity in their axonal bundles, suggesting that the N terminal 105 amino acids are sufficient to localize these protein fragments (Chapter 3).

To produce unc-76/lacZ fusion proteins that would be present in the HSNs but few other ventral cord axons, fusions were constructed with the unc-86 promoter (Finney and Ruvkun, 1990). The first unc-86 promoter-unc-76/lacZ fusion constructed was p86/76-1 (Fig. 4-3), consisting of 5 kb of unc-86 upstream sequences and unc-86 coding sequences up to codon 83 fused to p76-L16 sequences starting at codon 13 of unc-76. Animals carrying this plasmid on an extrachromosomal array showed $\beta$-galactosidase activity and immunoreactive material in the axons
and cell bodies, but not nuclei, of a subset of neurons approximately that expected to express unc-86 (Fig. 4-5). The axonal localization of both this fusion protein, which included amino acids 13-187 of unc-76, and the protein expressed from p76-L17, which included amino acids 1-12 of Unc-76, suggested two possible interpretations of the β-galactosidase localization. First, two distinct Unc-76 regions, one in amino acids 1-12 and the other in amino acids 13-187, are capable of directing axonal localization. Alternatively, β-galactosidase expressed at high levels in neurons enters axons without a specific localization signal (e.g., when expressed from p76-L17), but the protein is excluded from the nucleus and the distribution of protein is shifted toward the axons when unc-76 sequences provide a specific localization signal (e.g., in amino acids 13-187). The second possibility appears to be more likely, because several other β-galactosidase fusion proteins have been reported to enter axons in the absence of any additional coding sequences (M. Rosoff, C. Li, M. Hamelin, and J. Culotti, personal communication). Furthermore, animals carrying an array of p86-L1, a plasmid identical to p86/76-1 except that the unc-76 sequences (coding and 3' untranslated region) were removed and the unc-54 3' untranslated region inserted after lacZ, showed staining similar to that observed with p76-L17 (strong staining in cell bodies and nuclei, weaker staining in axons; Fig. 4-2). Thus the presence of β-galactosidase fusion proteins in the axons does not depend on unc-76 sequences, and so β-galactosidase fusions do not provide a sensitive assay for axonal localization signals. Nonetheless, they do allow the marking of axons for developmental studies.
Expression of unc-86/unc-76/lacZ fusions

Animals carrying extrachromosomal arrays of either p86/76-1 or p86-L1 showed β-galactosidase immunoreactivity in a relatively small subset of neurons, approximately the set shown to express unc-86 (Finney and Ruvkun, 1990; Fig. 4-5, 4-6). Axons in the left and right sides of the ventral cord could be clearly distinguished, and in L2 animals, HSN cell bodies extending short processes with growth cone-like structures at their tips were observed. However, two problems were also observed in these animals. First, more cells with processes in the ventral nerve cord were observed than were predicted from the unc-86 expression pattern. In particular, at least one process extended the length of the animal in the left ventral cord, while the left HSN, the only unc-86-expressing cell with a process in the left side of the ventral cord, has an axon only in the anterior half of the ventral cord. This made observation of outgrowth of the left HSN impossible, since the fusion no longer provided a unique label to this axon. The second problem was that several neurons, including the HSNs and the mechanosensory neurons, appeared to function abnormally. Animals were often insensitive to light touch with an eyelash, indicating that the six mechanosensory neurons, all of which express unc-86 and require its activity for their differentiation (Finney and Ruvkun, 1990), were not functioning.

Animals were also frequently egg-laying defective (Egl), a trait of animals in which the HSNs are abnormal. Observation of the HSNs in two lines of p87/76-1-bearing animals by anti-serotonin antibodies showed that 54-60% (n=24, 10) of the HSN cell bodies were in the tail rather than in their normal position near the vulva. This position indicated a
defect in the embryonic migration of the HSNs from their birthplace in
the tail, which is known to cause an egg-laying defect (Desai et al.,
1988). HSN migration defects resulting from the p86/76-1 plasmid were
unexpected, since HSN migration defects have not been associated with
unc-86/lacZ fusions, unc-76/lacZ fusions, or loss-of-function mutations
in either unc-86 or unc-76. The expression of Unc-86 protein is first
observed in the HSNs of wild-type animals during migration (Finney and
Ruvkun, 1990) and persists through adulthood, raising the possibility
that the fusion protein is produced during migration and interferes in
some way with this process.

The HSN migration and mechanosensory function defects do not
depend on the presence of unc-76 sequences in the fusion protein. unc-
76/lacZ fusions under the control of the unc-76 promoter do not cause
such defects. In addition, animals carrying an extrachromosomal array
of p86-L1, the coding region of which is equivalent to p86/76-1 without
unc-76 sequences, are Egl and Mec. This observation raised the
possibility that unc-86 sequences were responsible for the observed
neuronal defects. Simple transcriptional fusions to the unc-86
promoter, which would eliminate unc-86 protein sequences, are not
possible because the first intron of unc-86 is required for unc-86/lacZ
expression (G. Ruvkun and M. Finney, personal communication).
Therefore, a KpnI site was added after the second codon of the second
unc-86 exon to allow the insertion of unc-76 sequences after a minimum
of unc-86 coding sequence (20 codons). However, animals carrying
extrachromosomal arrays of this modified plasmid, p86-L3A, were often
Egl and Mec, as were animals carrying arrays of the same plasmid into
which the amino acids 13-187 of unc-76 had been inserted, p86-L5A.
Neurons expressing these fusion proteins often had abnormal appearances, with thickened or abnormally-located processes (similar to the right HSN seen in Fig. 4-5B). Similarly, animals carrying arrays of p86/76-6 and p86/76-7A, unc-86/unc-76/lacZ fusions using a truncated form of the unc-86 promoter expressed only in the HSNs and a small number of other cells (Figs. 4-5, 4-6), were also Egl, and HSNs in these animals visualized with an anti-β-galactosidase antibody often had defects in migration or axonal outgrowth (either thickened or misplaced processes). These observations suggested that either the first 20 amino acids of the Unc-86 protein or the truncated unc-86 promoter itself caused the defects. The concentration of injected DNA and the frequency with which extrachromosomal arrays were transmitted to the next generation (both of which are often correlated with the copy number of the injected plasmid-REF) were roughly correlated with the severity of the Egl and Mec phenotypes, consistent with either model. However, a nuclear-localized unc-86/unc-76/lacZ fusion (p86/76-4A) caused egg-laying defects, while nuclear-localized unc-86/lacZ fusions did not (G. Ruvkun, personal communication). Some retention of eggs in the parental dpy-20(e1282ts) strain used for p86/76-4A injection (along with a plasmid carrying wild-type dpy-20 sequences), and it is possible that genetic background effects could contribute to the egg-laying defect.

Axonal defects caused by unc-86-based fusion proteins have precluded their use for observation of HSN outgrowth. Even lines of fusion-bearing animals with comparatively few defects cannot be treated as wild-type, given the potential for subtle defects caused by low levels of toxic proteins. To improve these fusions in the future, the site-directed mutagenesis of unc-86/unc-76/lacZ constructs to eliminate
four ATG codons in the unc-86 coding region upstream of the site of unc-76/lacZ insertion may eliminate toxic regions of the fusion protein while preserving an intact regulatory region.

However, this caveat in mind, it is possible to note the time at which HSN process extension in the ventral nerve cord begins in animals carrying arrays of p86/76-6 or p86/76-7A, fusions under the control of the truncated unc-86 promoter. Preliminary observations showed that normally-positioned HSN cell bodies in L1, L2, L3, and early L4 animals carrying these fusions extended a short process, which was often directed ventrally and slightly anteriorly. The earliest stage at which HSN processes were observed to extend anteriorly in the ventral nerve cord was the late L4 stage, at the point at which vulval morphogenesis had reached the "Christmas tree" stage. While the possibility exists that even normal-looking HSNs in these animals were altered in the timing of their development by the fusion construct, the conclusion that HSN axon extension in the ventral nerve cord occurs during the late L4 stage confirms the inference from electron microscopic reconstructions of L4 and adult ventral nerve cords (White et al., 1986).

**General unc-76/lacZ-based axonal markers**

Because the axonal defects induced by unc-86/unc-76/lacZ fusions appeared not to be caused by unc-76 sequences, the unc-76/lacZ-based fusions still appeared to be potentially useful axonal markers. A segment of unc-76 cDNA sufficient for axonal localization, codons 1-197, was cloned in the general lacZ fusion vector pPD21.28LA (Fire et al., 1990a; Burglin et al., personal communication) in place of the nuclear
localization signal. This plasmid, p76-L18 (Fig. 4-4), is suitable for
the construction of translational or transcriptional fusions, as it
contains the initiator methionine codon from unc-76. It was used
successfully for the construction of unc-30/unc-76/lacZ transcriptional
fusions (Y. Jin, personal communication) without any apparent disruption
of the function of the VD and DD motorneurons, in which the fusion was
expressed. Mutations in unc-30 prevent the normal differentiation of
the GABAergic VD and DD motorneurons, and unc30/lacZ fusions are
expressed transiently in these cells as well as several others in the
head and tail. The unc-30/unc-76/lacZ fusion protein was detected in
the axons of these cells. unc-30 mutants have a characteristic
locomotory defect resulting from the lack of VD and DD function, but
animals bearing the unc-30/unc-76/lacZ fusions showed no signs of this
locomotory defect, suggesting that the fusion caused few or no problems
in the development of the cells in which it was expressed.

Conclusions:

The successful use of unc-30/unc-76/lacZ fusions, together with
the observation that unc-76/lacZ fusions under the control of the unc-76
promoter do not cause obvious neuronal defects, suggests that unc-
76/lacZ-based axon markers show promise as powerful tools. Not only can
they be used in the identification of neurons that express a particular
cloned gene, but they may also be used to study axons for which no other
marker is available. Detailed examination of neuronal morphology in
animals carrying unc-30/unc-76/lacZ fusions is necessary to ascertain
whether any morphological abnormalities in the labeled axons exist.
Visualization of axons by X-gal or antibody staining is sufficiently rapid and sensitive to allow screens for mutants with abnormal outgrowth of these axons, even when these mutants have no behavioral defects.

The use of unc-76/lacZ fusions for marking axons for electron microscopic observation

The product of the reaction of X-gal with β-galactosidase is electron-dense. In order to use the unc-76/lacZ fusions described above to label specific axons at the electron microscope level, a series of experiments was conducted to determine staining conditions that would allow formation of the X-gal reaction product while preserving axonal membrane ultrastructure. The results of preliminary experiments are described below. All processing of stained worms for electron microscopy and subsequent sectioning and microscopic observation were conducted by Erika Hartwig.

Technical considerations

The labelling of specific axons with X-gal requires the optimization of two parameters, the amount of blue product deposited specifically in axons and the fixation of axonal membranes to allow axons to be distinguished from one another in the electron microscope. Methods that improve one parameter tend to worsen the other. β-galactosidase catalyzes the cleavage of X-gal to yield a soluble intermediate, two molecules of which subsequently react to form an electron-dense blue precipitate (Bonnerot et al., 1987). The intensity
of staining can be improved by elevating the amount of β-galactosidase, the concentration of X-gal, the temperature at which the X-gal reaction occurs, and the duration of the X-gal reaction. Long incubations and high temperatures also accelerate tissue damage that occurs in lightly-fixed animals, however, and they promote the diffusion of soluble intermediates in the reaction, diminishing the localization of the blue product. One set of experiments was designed to produce staining of the highest possible specificity and intensity at the light microscope level. A second set of experiments was designed to determine whether specimens prepared under optimal conditions for light microscopy could be used for electron microscopy.

Optimizing staining intensity

In worms stained with the standard X-gal staining procedure for light microscopy (Fire, 1986), single axons can sometimes be observed as solid blue stripes similar to those seen in Fig. 4-7. More often, however, only bundles of neurons are solid stripes of stain, while single axons appear as rows of distinct blue particles, which are often separated by several micrometers. Thin sections for electron microscopy are usually less than 100 nm thick, so a gap of five µm between blue particles would mean that a labelled axon would not be detected in about 50 adjacent sections. We therefore sought to develop a staining technique that consistently produced solid staining in single axons on the assumption that this would provide label in every thin section.

The rate and extent of the β-galactosidase-catalyzed reaction depends on the concentrations of enzyme and substrate and the reaction
temperature. The concentration of enzyme was maximized by using a strain carrying the unc-86/unc-76/lacZ fusion plasmid p86/76-1 on an extrachromosomal array with the plasmid pRF4 (rol-6(su1006)) and stains rapidly under standard X-gal staining conditions.

Substrate concentration can be controlled by adjusting the concentration of X-gal in the staining solution and by controlling the degree to which the staining solution can diffuse into the fixed worms. Previous experiments using X-gal in the electron microscope (Bonnerot et al., 1987; Snyder et al., 1992) used cultured cells or tissue slices, in which penetration of reagents presents a less serious obstacle than does the C. elegans cuticle. Staining of intact worms for light microscopy is normally done in the presence of detergents such as SDS that enhance the penetration of X-gal into internal tissues. However, detergents damage membranes and are therefore unacceptable for electron microscopy. Several different fixation conditions and methods of exposing internal tissues to X-gal were tested to determine which would allow strong staining in the absence of detergents.

For all experiments, the standard X-gal staining mixture with detergent omitted was used (see Experimental Procedures). Worms were fixed either in 1% glutaraldehyde in 40 mM NaPO₄ pH 7.5/4 mM MgCl₂ for two hours at room temperature (Koga and Oshima, personal communication) or in a mixture of 2% paraformaldehyde and 2.5% glutaraldehyde in 0.2 M PIPES pH 7.4/2 mM MgCl₂/2 mM EGTA for three hours at room temperature. Both conditions appeared to allow equal staining intensity. X-gal is soluble in N,N-dimethylformamide (DMF) and dimethyl sulfoxide (DMSO; Bonnerot et al., 1987; Snyder et al., 1992); good staining was observed with both solvents. Tissues were exposed to the X-gal mixture by
placing fixed worms in a drop of staining solution in a glass depression slide and cutting them with a scalpel blade. Transverse cuts were made posterior to the vulva, posterior to the head, or anterior to the anus to allow observation of neurons in these regions. Animals were then transferred to eppendorf tubes or microtiter plates to prevent drying. Staining was generally most intense near the site of the cut, particularly when the incubation time in staining solution was short, but after overnight incubations, cells distal to the cut site in adults often stained intensely. When uncut worms were left in staining solution for several days, staining was observed in a small proportion.

The optimal X-gal concentration and reaction temperature were determined by incubating fixed, cut worms at 4°C, 20°C, 37°C, and 42°C in staining solutions containing X-gal at concentrations ranging from 0.1 to 4 mg/ml, close to the limit of solubility. Staining was observed at all temperatures, with strong staining appearing within 2-4 hours at 37°C and 42°C and 16 hours at 4°C. However, the localization of the blue precipitate was strongly temperature-dependent: samples incubated at 37°C and 42°C often had large blue crystals near nerve cords and single processes, while staining was better confined to specific cells in samples incubated at 4°C. Since distinguishing between specific stain and stray crystals in the electron microscope was judged likely to be a serious problem, 4°C incubations were used to determine the optimal X-gal concentration. Staining could be detected after overnight incubation at 4°C in 0.1 mg/ml X-gal in either DMF or DMSO, and it increased in intensity with increasing X-gal concentration up to 4 mg/ml. At concentrations above 1 mg/ml, however, crystals of X-gal appeared in the reaction solution and stray blue precipitate crystals
were evident in stained animals. Therefore, 0.4-0.6 mg/ml was
determined to be the optimal range of X-gal concentrations. Animals
left in X-gal solutions at this concentration at 4°C for several weeks
developed intense but well-localized staining with little stray stain
and still retained good cell morphology at the light microscope level.

Nitroblue tetrazolium (NBT) is sometimes included with reactions
that release the bromochloroindole moiety to produce a dark purple
stain. (e.g., Grenningloh et al., 1991). In one experiment, 0.34 mg/ml
NBT was added to 0.175 mg/ml X-gal in a buffer compatible with NBT (100
mM NaCl, 5 mM MgCl₂, 100 mM Tris pH 9.5). After overnight staining at
37°C, NBT/X-gal-treated worms appeared generally dark, in contrast to
worms stained with X-gal, which were dark only in areas of specific
stain. Nerve cords were distinctly darker than the surrounding tissue
in NBT/X-gal-stained worms, but these conditions did not appear to be
generally useful for light microscopy.

Electron microscopy of stained worms:

Worms were fixed for electron microscopy as soon as they developed
dark staining. After dehydration and embedding in resin, 1 μm thick
sections were taken in a plane transverse to the long axis of the worm.
If blue stain was observed in these sections, thin sections were taken
and viewed in the electron microscope. To ensure that the most highly
electron-dense material was the X-gal precipitate, thin sections were
not stained with lead citrate or uranyl acetate before viewing. The
osmium tetroxide fixation provided sufficient staining for the
visualization of membranes and did not form large electron-dense deposits.

The blue stain formed under optimal conditions for light microscopy, at 4°C for 48 hours, appeared to be washed out of the samples during the dehydration steps. Stain formed at 37°C, however, did remain in the samples and was visible in worms embedded in resin. Thick sections showed that blue stain was usually visible only in neurons, as expected from light microscopic observation of stained worms carrying p86/76-1 (Fig. 4-7). In thin sections viewed in the electron microscope, electron-dense stain was concentrated primarily in neurons, but other cell types in some preparations also had small patches of electron-dense material similar in appearance to those observed in neurons (fig. 4-8A). Muscles adjacent to the ventral nerve cord or lateral seam cells adjacent to ganglia in the head were the most frequent sites of "stray" stain. In general, however, staining was confined to neurons.

At high magnification, it was clear in most preparations that staining was not restricted to a small number of axons in the ventral cord (Fig 4-8B). As mentioned above, examination of p86/76-1-bearing animals stained with anti-β-galactosidase antibodies showed that a small number of processes in the left and right sides of the ventral nerve cord contained the fusion protein. However, in the electron microscope, electron-dense precipitate was visible throughout the ventral nerve cord and did not appear to be concentrated over any specific axons. Furthermore, axonal membranes were often difficult to distinguish. The dark stain often appeared as patches 50-150 nm in diameter consisting of a dark core inside a light area surrounded by a dark rim, but this dark
rim appeared even in patches of stain inside muscle cells and was therefore unlikely to represent axonal membrane. Preparations in which blue crystals outside of the nerve cords were observed in the light microscope tended to have larger patches of stain visible in the electron microscope. These patches were often oblong with straight sides, suggesting that crystals had been cut obliquely. Preparations in which nitroblue tetrazolium was used along with X-gal, which were generally darkly stained at the light microscope level, had much finer-grained electron-dense material in the nerve cords, but this too was not concentrated over specific axons, and it was often difficult to distinguish this fine material from other osmium-tetroxide-stained structures.

While axons were not made distinct by X-gal deposition, some neuronal cell bodies had patches of stain surrounding the nucleus. This pattern is similar to that observed by others using constructs containing a specific nuclear-localization signal (Bonnerot et al., 1987; Snyder et al., 1992). While the SV40 T antigen nuclear-localization signal was not present in the p86/76-1 construct, Unc-86 is a nuclear protein (Finney and Ruvkun, 1990), and it is possible that sequences at the N terminus of the Unc-86 protein are sufficient to produce this localization. The appearance of stain adjacent to nuclei (Fig. 4-8B) suggests that X-gal staining could be a useful way of labeling specific nuclei for use as landmarks in electron microscopy.

Conclusions
The appearance of X-gal-stained worms in the electron microscope indicated that further refinements of this technique are necessary before it can be used to label specific axons. Low temperatures during X-gal staining produced well-localized dark stain at the light microscope level, but this stain was apparently not stable in the dehydration and embedding conditions used. The prolonged incubations necessary for X-gal staining at 4°C also lead to poor membrane ultrastructure. The large size and wide distribution of X-gal crystals formed at higher temperatures also were incompatible with specific axon labeling.

Among C. elegans subcellular structures, axonal membranes are one of the most sensitive to fixation conditions (E. Hartwig, personal communication). Successful labeling requires that conditions that disrupt these membranes (e.g., long incubations in staining solution or high temperatures) be avoided. Although the strain used in these experiments had high levels of β-galactosidase expression, as judged by the rate of X-gal staining, even higher expression levels may have been necessary to allow shorter staining periods at low temperature. Also, worms used for X-gal staining were fixed relatively lightly before the staining period, and heavier fixation prior to staining might preserve membranes better. Finally, it might be possible to alter dehydration and embedding conditions so that they do not dislodge the smaller precipitate particles formed in low-temperature staining.

Despite the fact that immunoelectron microscopy of C. elegans in a way that maintains axonal structure has not been achieved, β-galactosidase fusions might be good markers for use by this technique. Multiple anti-β-galactosidase antibodies are available, and conditions
for immunoelectron microscopy of cultured cells expressing β-galactosidase fusions have been developed (Bonnerot et al., 1987). Conditions that allow immunoelectron microscopy with a β-galactosidase antibody could be used for any fusion construct, creating a technique for labeling any cell in which a cloned promoter is active.

II. Development of techniques for primary culture of C. elegans embryonic neurons

Introduction

One of the major limitations of the study of axonal outgrowth in C. elegans is that direct manipulation of the environments of specific neurons is not possible, either in partially-dissected preparations or in culture. In experimental systems in which culture is available, detailed studies of the interactions of growing axons with their substrates are possible, including antibody perturbation studies of cell-surface molecules, direct observation of growth cone behavior, and pharmacological manipulation of growing axons' electrical activity, second messengers, or cytoskeleton. In Drosophila, primary culture of neurons from mutant strains has enabled study of membrane cycling in shibire mutants and the electrophysiological defects in nap mutants (Wu et al., 1983). The availability of a technique for the culture of C. elegans neurons would provide a new method for the analysis of mutants defective in neuronal development and function. In particular, several genes required for normal axonal outgrowth are believed to encode molecules that affect the cytoskeleton, membrane structure, interaction
with the extracellular matrix, and signal transduction (Leung-Hagesteijn et al., 1992; T. Otsuka et al., in preparation; Oshima, R. Steven, A. Ruiz, J. Mancillas, and J. Culotti, personal communication). A technique for studying these processes in mutant cells lacking specific molecules in a defined environment might provide information applicable to the study of axonal outgrowth in a variety of species.

No technique for culturing *C. elegans* neurons has been published. Hedgecock et al. (1987) cited unpublished observations that embryonic cells plated on an adhesive substratum send out single, unbranched processes, but gave no indication of the conditions used. Conditions that allow normal cell division to occur after embryos are permeabilized or partially dissociated and reassembled have been reported. L. Edgar (personal communication) has developed a technique for removing the eggshells of embryos as young as the 1-cell stage by a combination of enzymatic digestion and pipetting through a narrow aperture. These embryos, which remain surrounded by a membrane, continue to divide to produce up to 500 cells, and normal differentiation of the major lineages occurs (as judged by markers for gut, muscle, and germline). Occasional neuronal processes have been observed in these permeabilized embryos (L. Edgar, personal communication). Blastomeres that are separated, reassociated, and cultured under these conditions continue to divide and differentiate normally (Goldstein, 1992).

The experiments described below were designed to extend these techniques to the growth of large numbers of dissociated embryonic cells. The goal was to define media, substrates, and cell isolation techniques that would permit neuronal differentiation and axonal outgrowth in short-term cultures. Because long-term culture was not
anticipated, efforts to maintain sterility were made only in preparation of media. Bacterial and fungal contamination was sometimes evident in three-day cultures, but not before this. Cells were kept on ice or at room temperature during initial experiments with cell isolation procedures. As this appeared to make little difference in the health of the cells, experiments with different substrata and media were conducted with cells kept at room temperature throughout the procedure.

**Experimental Procedures**

Details of the procedures used are discussed in the text.

Cultures were observed under Nomarski optics using a 100x Planapo objective lens on a Zeiss Axiovert 10 inverted microscope or a Zeiss Axiophot microscope. For antibody staining, cultures were fixed for 30 min at room temperature in 4% paraformaldehyde in PBS, followed by three washes in PBS pH 7.2 containing 1% Triton X-100 and 1% BSA. Cultures were blocked for 30 min. with 10% BSA in PBS, followed by overnight incubation in primary antibody at 4°C, three room-temperature washes in PBS, and a 1 hr incubation in secondary antibody at 37°C. Following three washes in PBS, the cultures were mounted in Mowiol containing 1 mg/ml p-phenylenediamine and observed.

Ascites fluid from monoclonal antibody 611B1 (G. Pipierno) was used at a 1:10 dilution, the anti-tubulin monoclonal antibody YL1/2 was used at a 1:50 dilution. Rhodamine-conjugated goat-anti-mouse (Cappell) and fluorescein-conjugated goat-anti-rat secondary antibodies (Jackson ImmunoResearch) were used at a 1:400 dilution. All antibodies were diluted in PBS.
Results

Dissociation methods

Dissociation of cells for primary culture from other organisms is usually achieved by a combination of dissection of neurons away from other tissue, mild proteolytic digestion of basement membranes and other connective tissues, and physical separation of cells. The small size of C. elegans embryos makes dissection impossible, and so an additional necessary step is the removal of the eggshell. Krasnow et al. (1991) dissociated whole Drosophila embryos simply by gentle Dounce homogenization. Edgar's technique for removal of the eggshell from single embryos involved a brief digestion of intact embryos in a mixture of chitinase and chymotrypsin to begin breakdown of the eggshell followed by passage of the embryo through a drawn-out micropipet with a diameter slightly smaller than that of an embryo.

Both Dounce homogenization and enzymatic digestion were used to free C. elegans embryonic cells from the eggshell. In all cases, populations of mixed-stage embryos were obtained from mixed-stage C. elegans populations by washing in M9 followed by treatment with 20% sodium hypochlorite solution in 0.5 M NaOH until all larvae and adults were dissolved. Embryos were washed several times in M9 to remove hypochlorite and then resuspended in egg buffer for chitinase/chymotrypsin digestion or Ca/Mg-free medium for Dounce homogenization (8 g/l NaCl, 200 mg/l KCl, 50 mg/l Na H₂PO₄·H₂O, 1 g/l NaHCO₃, 1 g/l glucose; Wu et al., 1983). Dounce homogenization was
performed on a 1 ml cell suspension in a 15 ml glass homogenizer. A drop of the supernatant was inspected under the dissecting microscope every 10-20 strokes, and homogenization was stopped when a large number of individual cells were visible (60-100) strokes. The cells and embryos were then incubated in a cocktail of collagenase type IA, IV, and VII (Sigma; 0.1 mg/ml each in Ca/Mg-free medium) for 60 min at room temperature. In some preparations, collagenase-digested embryos were sucked up and down repeatedly (triturated) in a drawn-out pasteur pipet to separate the cells mechanically. Yields from the Dounce procedure were usually low, regardless of the number of Dounce strokes, the collagenase mixture used, and the inclusion of a trituration step.

Embryos to be dissociated by enzymatic digestion were prepared by hypochlorite treatment as above. They were then incubated at room temperature in a mixture of 5-10 mg/ml each chitinase (Sigma) and α-chymotrypsin (ICN) with gentle agitation until the embryos in a sample observed under the dissecting microscope began to round up and the outlines of individual cells at the edges of the embryos began to become more distinct (usually 5-6 minutes). The reaction was stopped by several washes in culture medium (see below) containing fetal bovine serum, which contains protease inhibitors. Treatment with two washes of soybean trypsin inhibitor before the serum washes did not improve the apparent health of the cells. Cells were then mechanically dissociated by trituration in a pasteur pipet with a slightly drawn-out tip, followed by a period of several minutes in which whole embryos and large clumps of cells were allowed to settle out of the suspension. The mechanical dissociation and settling steps were repeated with material that settled out of suspension until few intact embryos remained. High
yields of cells could be obtained with this technique if the
dissociation was sufficiently gentle, a condition aided by keeping the
tip of the pipet only slightly smaller than the normal pasteur pipet tip
and by keeping the amount of pipetting to a minimum. In addition,
overdigestion with the chitinase/chymotrypsin appeared detrimental to
the health of the cells.

Following dissociation, cell suspensions were filtered through two
layers of fine nylon mesh stretched over the end of a 3 ml plastic
syringe. This effectively removed all of the whole embryos and most of
the L1 larvae that were released from their eggshells during the
dissociation procedure, but it allowed large clumps of cells to pass
through. Filtrates were then subjected to two rounds of low-speed
centrifugation (750 x g) to separate intact cells from particulate
material produced during dissociation. Cells were resuspended in a
volume of medium equivalent to 50 μl per sample to be plated
(approximately three samples per 9 cm plate of worms). This yielded a
drop of cells that was confluent in the center but allowed observation
of individual cells at the edges.

Substrates

Dissociated cells from a variety of species generally attach to
glass or tissue culture plastic coated with nonspecific charged
molecules such as poly-L-lysine or polyornithine, relatively nonspecific
adhesive proteins such as the lectin concanavalin A (Chiquet and Acklin,
1986), or species-specific extracellular matrix molecules such as
laminin, fibronectin, or collagen (Banker and Goslin, 1991). Because
nearly all neurons are reported to show some adhesion and axonal outgrowth on polylysine, initial experiments were done with glass coated with 0.01-1 mg/ml polylysine. *C. elegans* embryonic cells from some preparations adhered well to PLL-coated glass cover slips, but often they failed to remain adhered, or when they sent out axons, the axons seemed very loosely attached and appeared to float in the medium. Because a more adhesive substrate appeared to be necessary, the silane derivative TESPA (3-aminopropyl-triethoxysilane; Sigma), often used for attaching tissue sections to slides, was tested for its ability to support *C. elegans* cell attachment and axonal outgrowth. Initial experiments (using cells prepared by chitinase/collagenase treatment and trituration and grown in modified Edgar's medium; see below) showed that TESPA-coated cover slips allowed more extensive axonal outgrowth than did PLL-coated cover slips, but this, too, was variable. Reactive aldehyde groups can be added to TESPA by brief treatment with paraformaldehyde; cover slips covered with 1% TESPA and activated with 4% paraformaldehyde produced the most consistent axon outgrowth (Fig. 4-9). Cover slips prepared less than two days before use appeared to be more reliable than older cover slips. Cells grown on uncoated clean glass failed to adhere.

Poly-L-lysine applied to activated TESPA-coated cover slips appeared to be no better than activated TESPA alone. Initial experiments with the vertebrate extracellular matrix proteins laminin, fibronectin, thrombospondin, collagen (types I, III, and IV) applied to PLL-coated cover slips showed no obvious improvement in cell attachment or axonal outgrowth over that observed with PLL alone.
The apparent advantage of paraformaldehyde-activated TESPA over other, less adhesive, substrates suggested that cells prepared by chitinase/chymotrypsin and trituration were not particularly adhesive. This might be caused by loss of cell surface adhesion molecules through excessive protease treatment. The adhesivity of the culture substratum has been shown in other organisms to affect the amount of neurite outgrowth and cell spreading (Bray and Chapman, 1985). It is possible that less adhesive substrata would have promoted different behavior of C. elegans cells, such as cell division rather than differentiation.

Media

Cell culture media typically contain salts, a buffering agent, vitamins, precursors for amino acid and nucleic acid biosynthesis, antibiotics, and a source of growth factors. While some invertebrate cell culture systems use invertebrate tissues as a source of growth factors (e.g., Aplysia or Helisoma hemolymph), many invertebrate cell types have been successfully cultured in fetal bovine serum (Beadle et al., 1988). Because invertebrate hemolymph is not commercially available, fetal bovine serum was used to develop media for C. elegans cell culture. (Coelomic fluid isolated from earthworms (Arlington Bait and Tackle, Arlington, MA) showed considerable toxicity to C. elegans cells in an initial experiment and was not tested further.)

Most invertebrate cells are cultured in air incubators rather than in the environment of CO2 in air used for most mammalian cells. Several media designed for use in air incubators were tested for their ability to support C. elegans cell adhesion, differentiation, and survival. A
medium similar to that used by Wu and co-workers for the culture of
*Drosophila* larval neurons (Wu et al., 1983) was tested in initial
experiments with cells isolated by Dounce homogenization and plated on
PLL-coated cover slips, and subsequent experiments were conducted with
modifications of the medium designed by Edgar for use with permeabilized
*C. elegans* embryos. In all experiments, a drop of dissociated cells
(approximately 50 μl) was placed in the center of a 22 x 22 mm or 24 x
50 mm glass coverslip recently coated with PLL or TESPA. In some
experiments, the drop was held in place with by a thick line made with a
grease pencil, but this could be omitted without serious difficulty.
Cover slips were placed cell-side up onto parafilm-covered microscope
slides in a moisture chamber made from a plastic box lined with water-
soaked Whatman filter paper. The chambers were covered in aluminum foil
to protect the cells from light and placed in the same 20°C incubator
used for growing worms. Cells were viewed with Nomarski optics and a
100x oil-immersion objective lens. 24 x 50 mm coverslips could be
placed directly onto the stage of an inverted microscope for observation
without disturbing the drop of medium. Smaller coverslips were viewed
with a conventional microscope after being inverted onto viewing
chambers made from microscope slides to which two coverslips had been
glued, separated by a 5 mm gap. This configuration allowed the cells to
remain in culture medium during observation without being squashed, but
was prone to drying. Cultures that were to be observed multiple times
were kept on larger coverslips.

The first medium tested was similar to that used by Wu and co-
workers (1983), which contained 25% L-15 medium (Gibco), 66% modified
Schneider saline, and 9% fetal calf serum, heat-treated to inactivate
complement. After preparation by Dounce homogenization, *C. elegans*
cells were washed in this medium and plated on freshly-prepared PLL-
coated cover slips. In some experiments, some cells extended short
processes (4-5 cell diameters in length) after an overnight incubation,
but the proportion was generally low. A few rare cultures contained
many cells with processes as long as 10 cell diameters. These processes
appeared well attached to the surface, and processes arising from
neighboring cell bodies were sometimes bundled together. Well-attached
cells with processes were observed in one- and two-day cultures, while
three-day cultures usually contained a high proportion of cells that
appeared to have detached from the surface. However, most of the
neurons in the intact worm nervous system complete their outgrowth
within 24 hours of fertilization, and the observation that neurites
appeared to grow from some cells in some cultures and remained healthy
for at least this long suggested that successful primary *C. elegans* cell
culture was possible.

Alternative substrates and methods of cell dissociation were
tested in conjunction with a modified version of the medium designed by
Edgar for use with permeabilized embryos. Because adhesion and axonal
outgrowth were dramatically improved by the use of TESPA and modified
Edgar's medium, Wu's medium and PLL were not used in further
experiments. Edgar's medium is similar to Wu's medium, except that
inulin and polyvinylpyrrolidone (PVP) are included to raise the osmotic
pressure of the medium, and chicken egg yolk is added as a source of
cholesterol. The salts and buffer in Edgar's optimal medium are
slightly different from those in the modified Schneider saline used by
Wu and co-workers but are similar to those in several commercially-
available media. Edgar (personal communication) indicated that commercial L-15 medium was nearly as good for permeabilized embryos as her optimized salt/buffer solution, so bulk embryonic cell culture experiments were done with prepared media obtained commercially. The modified Edgar's medium used for initial studies consisted of 61% L-15, 20% fetal bovine serum, 15% inulin solution (7.5 mg/ml in egg salts; see protocol below), 1% cell culture base mix, 0.25% egg yolk, 5 mg/ml polyvinylpyrrolidone, and 1% penicillin-streptomycin mix. The egg yolk was omitted after the initial experiments without apparent ill effect.

Cells prepared either by Dounce homogenization or by chitinase/chymotrypsin treatment and trituration were tested with this medium on PLL-coated coverslips. Because attachment was poor in an initial experiment, cells were allowed to attach to the coverslips for two hours in a serum-free version of this medium (in which serum was replaced by an equal volume of egg buffer) and then placed in serum-containing medium. This led to a clear improvement in cell attachment, probably because serum proteins can compete with the cells for attachment to adhesive sites on the coverslip, and the period without serum probably allowed the relatively poorly adhesive cells to resynthesize surface and extracellular matrix adhesion molecules lost during proteolytic dissociation. This improvement was not due simply to the absence of serum, as cells kept in serum-free medium were considerably less healthy than cells returned to serum-containing medium.

Several other commercially-available media, many of which are used for the culture of other invertebrate neurons, were compared with L-15 in modified Edgar's medium. Cells were initially dissociated and plated
on activated TESPA in L-15-based media and then switched to serum-containing media based on Schneider's Drosophila medium, Medium 199, Grace's insect cell culture medium, or CO₂-independent medium (Gibco; see protocol below). Most media were identical to the L-15 based medium except for the different commercial medium component. Grace's insect cell culture medium has a higher osmolality than the other media, and so the inulin and PVP were reduced by 10% in this medium. A qualitative assessment of the survival and differentiation of cells in these media suggested that some media promoted rapid neurite outgrowth but poor survival while others promoted survival at the expense of neurite outgrowth. For example, cells grown in Medium 199 exhibited striking neurite outgrowth after 20 hours, with some processes as long as 20 cell diameters, but a high proportion of cells appeared to be degenerating by 48 hours. Cultures that had had cells with long processes at 20 hours now had cells with thick stumps about a cell diameter in length. By contrast, cells in Grace's medium showed much shorter neurites after 20 hours but also much less detachment and degeneration after three days in culture. CO₂-independent medium gave an intermediate level of cell survival and neurite outgrowth in 20-hour cultures, but had a high proportion of long, well-attached neurites after three days. L-15 medium was generally similar to Medium 199, with somewhat shorter neurites, and Schneider's Drosophila medium produced both intermediate short-term neurite outgrowth and intermediate longer-term survival. By four days in culture, cells in all media were detaching and degenerating.

The use of L-15-based medium for initial cell preparation and plating appeared to be important. Cells prepared and plated in Medium-
199-based media and then shifted to serum-containing Medium-199-based medium consistently showed poorer adhesion and neurite outgrowth in overnight cultures than did cells prepared and plated in L-15-based media and then switched to Medium-199-based serum-containing medium. Cells cultured in CO₂-independent medium similarly showed better adhesion and neurite outgrowth if they were prepared and plated in L-15-based media than they were if prepared in plated in in Medium-199-based media. The reason for the importance of the initial plating medium is not clear.

Nature of the cultured cells

Cells observed two hours after plating were usually flattened and round. About 25 percent had extended short processes, usually less than one cell diameter long. Almost none extended longer processes, suggesting that the long processes observed after 16-20 hours in culture had grown after plating.

Cultured cells with long processes after overnight growth resembled cultured neurons from other organisms. They usually had relatively flat, small cell bodies and a single major process, which often spread out at the tip into a flattened, multiply-branched growth cone-like structure (Fig. 4-9). This major process occasionally branched, but most did not. Some cells were bipolar, with long neurites roughly equal in caliber extending from opposite ends of the ovoid cell body. Neurites often appeared to extend to another cell body and terminate there. They rarely seemed to interact with one another, crossing without changing trajectory when they intersected rather than
forming fascicles. Neurites arising from nearby cells in a group, however, often grew out in fascicles, staying together for most of their length but occasionally splitting into individual processes.

Evidence that the cells extending processes in culture were neurons was provided by staining with a neuron-specific antibody. The monoclonal antibody 611Bl was prepared against sea urchin axonemal microtubules (Siddiqui et al., 1989) and stains only neurons in whole-mount C. elegans preparations. The most intensely-staining cells are the mechanosensory cells ALM, PLM, AVM, and PVM, but all neuronal processes are reported to stain to some degree. Cultured cells were fixed, permeabilized, and stained with 611Bl or a control anti-tubulin monoclonal antibody, YL 1/2, that stains all C. elegans microtubules (Hyman, 1989). Most cells appeared to stain with the control tubulin antibody (Fig. 4-10). Cells without processes had a ring of stain in the cytoplasm but none in the nucleus. Cells with processes showed intense staining along the length of the processes. By contrast, cells without processes were not stained with 611Bl, and a subset of the cells with long neurites were stained (Fig. 4-10). This suggests that at least some of the cells with long processes were neurons.

Optimized protocol

The optimized procedure for culturing cells determined by the experiments described above is listed below. This procedure gave reasonably reproducible cell survival and axonal outgrowth but the variables have been by no means exhaustively explored.
L-15 Culture medium (L-15/CM)
24 ml L-15 (Gibco cat. 320-1415A; includes glutamine)
6 ml inulin (ICN cat. 102055; 7.5 mg/ml in egg salts, autoclaved)
8 ml fetal bovine serum (heat inactivated; Gibco cat. 230-6140AG)
0.4 ml base mix
200 mg PVP
0.4 ml penicillin-streptomycin mix (Gibco cat. 600-5140AG)
Mix, let sit on ice 1-2 hr, filter-sterilize (0.22 μm).

egg salts: 11.8 ml 1 M NaCl
4.8 ml 1 M KCl
83.6 ml H₂O
base mix: 100 ml H₂O
100 mg adenine
10 mg ATP
3 mg guanine
3 mg hypoxanthine
3 mg thymine
3 mg xanthine
3 mg uridine
5 mg ribose
5 mg deoxyribose
autoclave

PVP: polyvinylpyrrolidone MW 40,000
Dialyze against H₂O 1-2 days and lyophilize; store frozen.

L-15 Serum-free medium (L-15/SFM):
24 ml L-15
6 ml inulin
0.4 ml base mix
10 ml egg buffer
0.4 ml penicillin-streptomycin
100 mg PVP
Filter through 0.2 μm filter and keep refrigerated.

Medium 199 Culture medium (M199/CM)
24 ml Medium 199 (Gibco cat. 320-1151AG; includes glutamine)
6 ml inulin (ICN cat. 102055; 7.5 mg/ml in egg salts, autoclaved)
8 ml fetal bovine serum (heat inactivated; Gibco cat. 230-6140AG)
0.4 ml base mix
200 mg PVP
0.4 ml penicillin-streptomycin mix (Gibco cat. 600-5140AG)
Mix, let sit on ice 1-2 hr, filter-sterilize (0.22 μm).

Egg buffer:
11.8 ml 1 M NaCl
4.8 ml 1 M KCl
0.34 ml 1 M CaCl₂
0.34 ml 1 M MgCl₂
2.0 ml 0.25 M HEPES (pH 7.4)
82.5 ml H₂O

Filter-sterilize or autoclave before adding sterile HEPES.

Chitinase/Chymotrypsin:

5 mg chitinase (Sigma C-6137)
5 mg α-chymotrypsin (ICN 152272)
1 ml egg buffer

Dissolve; place on ice 30 min.; add 10 µl penicillin-streptomycin mix. Spin if cloudy. Better after a day or so on ice. Can be frozen.

Alternative culture media:
In the recipe for M199/CM above, substitute an equal volume of CO₂-independent medium (Gibco cat. 320-8045AG), Grace's insect cell culture medium (Gibco cat. 350-1590AG), or Schneider's Drosophila medium (Gibco 350-1720AG). CO₂-independent medium does not come with glutamine, so add L-glutamine to a final concentration of 4 mM. Reduce the amounts of inulin and PVP by 15% when using Grace's insect cell culture medium.

Culture Procedure:

1. Grow N2 hermaphrodites on 9 cm plates until the plates are crowded with gravid adults (but not starved). Plates can be left at 15°C overnight before use. The yield of cells from the procedure below is often not very high, so plan to use one plate per 2-3 culture samples.

2. Prepare culture media a day in advance.

3. Prepare cover slips with TESPA:

   --Wash cover slips in 70% ethanol, 1% HCl.
   --Rinse in several changes of H₂O over an hour or so.
   --Air dry.
   --Dip cover slips in 0.5%-4% TESPA (3-aminopropyl-triethoxysilane; Sigma) in acetone for 5 min. at room temperature.
   --Wash in 2 changes of acetone.
   --Air dry.

To add reactive aldehyde groups:
   --Soak in 4% paraformaldehyde in PBS 30 min. at room temperature.
   --Wash in several changes of H₂O.
   --Air dry.

Use within 1-3 days.

4. Pour M9 onto plates and scrape worms and eggs off with a rubber policeman. Transfer to a 15 ml tube.
5. Pellet worms in clinical centrifuge and resuspend in M9. Repeat until supernatant is clear (usually 2-3 washes).

6. Add 5 ml hypochlorite mixture and mix gently at room temperature until almost all worms are dissolved (about 5 min.).

   Hypochlorite mixture:
   1 ml sodium hypochlorite (should be fairly fresh)
   2.5 ml 1 M NaOH
   1.5 ml H2O

7. Wash 3 times with M9.
8. Wash once with Egg Buffer.
9. Add 0.5 ml chitinase/chymotrypsin mixture. Mix gently at room temperature until embryos round up, single cells start appearing, and the outlines of individual cells at the edges of embryos are more distinct than the eggshell (about 5 min.) It helps to monitor a small drop under the dissecting microscope. Too much digestion almost certainly damages the cells.

10. Add 5 ml L-15/CM and spin for 1 min at 2000 rpm in clinical centrifuge.

11. Wash twice in 2 ml L15/CM.
12. Resuspend cells in 1 ml L-15/SFM.
13. Dissociate cells by sucking up and down about 30 times in a pasteur pipet (slightly drawn out to narrow tip). Gentle dissociation is critical for good attachment and survival, so the pipet tip should not be too narrow or the pipetting too vigorous. Allow whole embryos and large clumps of cells to settle 3-5 min.
14. Remove supernatant to a sterile eppendorf tube. Add 1 ml L-15/SFM to pellet and repeat dissociation, again allowing 3-5 min for settling. Repeat once or twice more, until there are no cells in the supernatant following the settling step. If anything settles out of the supernatants once they have been removed from the first tube, this material can added back to the first tube for further dissociation.
15. Filter supernatants through 2 layers of fine nylon mesh (as small as possible--test to be sure that embryos don't pass through). A 1 cm square can be attached to the end of a plastic syringe (3cc) by cutting the tip off the cap that comes with the syringe and screwing the proximal half of the cap over the nylon.
16. Spin 10 min at 750 rpm (clinical centrifuge) or 1100 rpm (swinging bucket microcentrifuge). Resuspend in 1-2 ml L-15/SFM and repeat.
17. Resuspend cells in L-15/SFM, assuming 25-30 μl per cover slip. Plate 25-30 μl on each cover slip and incubate in a moist chamber at 20°C in the dark.
18. After 2 hr, remove most of the drop of medium with a drawn-out pipet and replace with the same volume of M199/CM for best neurite growth. Repeat. Incubate in a moist chamber in the dark at 20°C (up to 3 days).

Conclusions
Although the techniques for cell culture developed in this section are not fully reproducible, the observation that axons can be grown from cultured *C. elegans* neurons is encouraging and suggests that further optimization should be attempted. Techniques developed for mass culture of all embryonic cells could form the basis for culture of specific neuronal types. Experiments in *Drosophila* suggest that the isolation and culture of specific cell types from a population of dissociated embryonic cells might be possible. Krasnow and colleagues (1991) used a β-galactosidase substrate, FDG, that can be introduced into living cells and releases a fluorescein molecule when cleaved. They dissociated populations of transgenic *Drosophila* embryos carrying *lacZ* fusions, including a neuron-specific enhancer trap line, stained with FDG, and isolated a population of *lacZ*-expressing cells by fluorescence-activated cell sorting. Neurons isolated by this technique survived and sent out axons. This technique could probably be used in *C. elegans* in conjunction with neuron-specific *lacZ* fusions to isolate specific neuronal populations for culture. If neurons can grow at low density (perhaps with conditioning factors released into the medium by cells in denser cultures), the interactions of particular cells might be studied both in culture and intact worms. In particular, *unc-30/lacZ* fusions might be used to isolate DD neurons, which in embryos send out processes that change direction or stop when they contact the cell body of the adjacent DB or process of the adjacent DD neuron (Durbin, 1987).

Another potential use for *C. elegans* cell culture is in the study of the electrical properties of neurons and muscle cells. The electrophysiology of cultured *Drosophila* neurons, which are similar in size to the *C. elegans* cultured cells described above, has been studied
by intracellular and patch recording techniques (Wu et al., 1983). An attempt to establish patch clamps with C. elegans cells was unsuccessful (D. Raizen, E. Jorgensen, B. Sawin, and L. Bloom, unpublished results), due largely to the poor adhesion of the cells to the coverslip. Improvements in the culture technique could permit electrophysiological analysis of a variety of mutants with defects in the regulation of muscle contraction (Levin and Horvitz, 1992), calcium channels (L. Lobel, personal communication), anaesthetic sensitivity (P. Morgan, personal communication), etc.

Finally, vertebrate cell culture techniques have been used to quantify the elongation of axons on other axonal substrates (for example, see Chang et al., 1987), and it is possible that the C. elegans culture techniques can be applied to the study of fasciculation. In particular, it would be interesting to determine whether axons from mutants defective in fasciculation in vivo—unc-34, unc-71, and unc-76—are unusual in their fasciculation behavior in vitro. Genetic mosaic experiments for the study of fasciculation are difficult in vivo because neighboring neurons are often closely related by lineage, but an in vitro fasciculation assay would offer an opportunity to test the cell autonomy of the fasciculation defects observed in these mutants.

Discussion

Although none of the techniques discussed in this chapter has been brought to the point at which it can be used reliably, the results of both the labeling studies and the culture experiments are encouraging. Fusions of Unc-76 protein to β-galactosidase label axons strongly and
can be used both for detailed observations of cell morphology (by anti-
β-galactosidase antibody staining) and for relatively rapid screening
for axon morphology mutants (by X-gal staining). Examination of axons
in animals carrying fusions of unc-76/lacZ to the promoters of several
genes (unc-30, Y. Jin, personal communication; unc-4, D. Miller, personal
communication; and unc-36, L. Lobel, personal communication) should
demonstrate whether the axonal outgrowth problems encountered with unc-
86/unc-76/lacZ fusions are peculiar to the unc-86 promoter. Further
modification of the unc-86 promoter or use of other promoters likely to
be HSN-specific based on the specificity of mutant defects (egl-43, ham-
1; G. Garriga, personal communication) might generate the HSN markers
that were the origial goal of these experiments.

Another possible approach to HSN labeling involves injection of
the lipophilic dyes diI or diO into fixed worms near the HSN cell
bodies. While the cuticle of living animals is too tough to permit
injections of sufficient accuracy, the cuticle of fixed animals is more
easily pierced, and it is likely that a fine needle can be positioned
close enough to the HSN cell bodies in L4s or adults to permit dye
labeling without labeling of the rest of the ventral nerve cord. The
use of dye filling in fixed tissue followed by photoconversion to an
electron-dense product can be used for electron microscopic examination
of growth cones (for example, see Chitnis and Kuwada, 1992), and so an
investigation of this technique seems worthwhile.

While the important parameters for successful C. elegans cell
culture are not well understood, it is encouraging that cells can be
isolated and cultured in the short term with a reasonable degree of
reproducibility. The observation of neurons growing in defined
conditions required further refinement of these techniques to allow lower-density cell culture (perhaps with the addition of conditioned medium from C. elegans cultures). The requirement for a TESPA substrate suggests that the isolated cells were poorly adhesive, and investigations of fasciculation or of the effects of specific substrates on axonal growth (e.g., the laminin-related protein Unc-6 (Ishii et al., 1992) require isolation techniques that yield healthier cells. Nevertheless, the initial success of these culture experiments suggest that further investigation can yield a useful technique.

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References


Figure 4-1.

Structures of unc-76/β-galactosidase fusions. Construction of these three clones, and the equivalent clones containing nuclear localization signals just upstream of β-galactosidase, is described in Experimental Procedures. Black areas indicate exons, white areas indicate introns or other untranslated sequence, and hatching indicates the β-galactosidase gene. A "+" under axonal localization indicates strong β-galactosidase immunoreactivity in axons relative to cell bodies, while "+/-" indicates weak axonal immunoreactivity relative to cell body staining. A "-" under nuclear exclusion indicates that cell body immunoreactivity was observed in a ring surrounding the nucleus but not in the nucleus itself, while a "--" indicates that the entire cell body appeared to stain uniformly. Clones p76-L14 and p76-L16 produce fusion proteins that are localized to axons and excluded from nuclei, while the protein encoded by p76-L17 is more weakly expressed in axons but stains nuclei strongly. The equivalent clones containing an SV40 T antigen nuclear localization signal (p76-L11, p76-L15, and p76-L13 are the counterparts of p76-L14, p76-L16, and p76-L17, respectively) include this sequence immediately preceding the β-galactosidase sequence. Restriction sites indicated represent sites from the original unc-76 genomic clone and may not be retained intact in the β-galactosidase fusion clones.
Fig. 4-1  unc-76/lacZ fusion structures

unc-76 genomic clone

axonal localization  nuclear exclusion

p76-L14

p76-L16

p76-L17
Axonal localization of lacZ fusion proteins occurs in the absence of unc-76 sequences but is enhanced by their presence. A. Animals carrying the unc-76/lacZ construct p76-L11, which encodes two-thirds of the Unc-76 protein, show axonal staining but relatively little in nuclei (despite the presence of an SV40 nuclear localization signal), while (B) animals carrying p76-L17, which encodes only 12 amino acids of Unc-76 show more intense nuclear staining and weaker axonal labeling. Similarly, (C) animals carrying p86/76-L1 show more axonal staining and less nuclear staining than do animals carrying p86-L1, the comparable plasmid lacking unc-76 sequences (D). Scale: A,B: 1 cm = 15 μm; C,D: 1 cm = 23 μm.
Figure 4-3.

Structures of unc-86/unc-76/lacZ clones. Construction of these clones is described in Experimental Procedures. The first four clones (p86/76-1, p86-L1, p86-L5A, and p86-L3A) contain the large 5 kb upstream unc-86 region that promotes expression in the full set of Unc-86-expressing cells. The remaining clones (p86/76-4A, p86/76-6, and p86/76-7A) contain a 500 bp promoter fragment from unc-86 that produces expression only in the HSNs, NSMs, and a small number of other cells. p86/76-6 and p86/76-7A are identical except for the amount of unc-86 coding sequence they contain: p86/76-6 contains amino acids 1-243 (including the POU domain but not the homeodomain), while p86/76-7A contains amino acids 1-83 of Unc-86 (and does not include the POU domain or homeodomain). Stippled regions represent unc-86 upstream or intron sequences, black regions represent C. elegans exons, diagonal striped regions represent lacZ, crosshatched regions represent the unc-54 3' untranslated region (including a polyadenylation signal), and open areas in p86/76-1 represent unc-76 introns. Restriction sites indicated represent those from the original genomic clone or PCR amplified product from unc-76 or unc-86 and are not necessarily retained intact in the fusion clones.
Figure 4-4.

Structure of the unc-76/lacZ fusion vector p76-L18. Construction of this vector, designed for general use with any neuronally-active promoter, is described in Experimental Procedures. It is based on the lacZ vector pPD21.28LA (Fire et al., 1990a; T. Burglin, B. Reinhardt, and G. Ruvkun, personal communication) and includes codons 1-197 of the Unc-76 protein to enhance axonal localization of the fusion protein. A. Cloning sites in the 5' region of the vector are indicated by solid lines. Unique cloning sites are shown without parentheses, while sites represented twice in the vector are shown in parentheses. The unc-76 cDNA sequence is indicated in italics. The translational fusion p86/76-1, in which unc-86 sequences are inserted at the BglII site of the unc-76 cDNA, produces an axonally-localized fusion protein, and so any site 5' to this BglII site is probably appropriate for construction of translational fusions. B. Structure of the entire p76-L18 vector, with unique restriction sites indicated. Transcription occurs in a clockwise direction from the polycloning site at nucleotides 18-85, through 600 nucleotides of unc-76 cDNA, 3 kb of lacZ sequence (including a stop codon), and 794 bp of the unc-54 3' untranslated region, including a polyadenylation site.
Fig. 4-4 Structure of the unc-76/lacZ fusion vector p76-L18

A. Cloning sites in p76-L18 5' region

HindIII  (SphI)  (PstI) Sall  XbaI  (BamHI)
ATG ACC ATG ATT ACG CCA AGC TGG CAT GCC TGC AGG TCG ACT CTA GAG GAT CCC
SmaI  BglII  (PvuII)
CGG GAT TGG CCA AAG GAC CCA AAG GTATGTTTCGAATTGACGCTCGAGCTGAGCTCAGTG
synthetic intron

(PvuII)  (BstII)  NsiI  EspI  XhoI  (BglII) PmII
CGAATGACTAACATAACATAACACTTTTCAG GAG GAC CCT TGG AGG GCA CCC ATG CTT
StyI  (KpnI) cDNA

(PstI) (BglII)  TTT GGA GAG GTG GCA ATG GAG GCT GTC GCA GAT CTG CGA GAA GAC ATT CTC
GCC TCG TGT GAT GAT GAT ATC GAT ATC GAT AGT AAT AAG AAT TTG AGC AAC CAT TCA
(Tth111 I)  GAG TCC TCT GCA TCC CCT GAG GCT TGT GGC AAC TTT GAC GAA AAA ATT GCG
GCA TCC CGT AAG GAC CAC GAG GTG ACG ACA GCG GAT ATT GCA CCT GTG CAG AGA
SnaBI  ... (287 bp) ... GAA GAA ATT GAT GAA ATG TTA CAG GTA CCG GTG GTT
(AgeI)

B. Structure and unique restriction sites of p76-L18
Figure 4-5.

Fluorescence micrographs of animals carrying unc-86/unc-76/lacZ fusions stained with anti-β-galactosidase antibodies. A. The head of an adult expressing p86/76-1 shows strong labeling of a subset of sensory processes, axons in the nerve ring, and cell bodies. B. HSN defects in an animal expressing p86/76-6. This ventral view (anterior at upper left) shows the left HSN extending normally to the nerve ring (arrow) while the right HSN fails to enter the ventral cord and instead loops back posteriorly in a short thickened process. Scale: A: 1 cm = 12 μm; B: 1 cm = 25 μm.
Figure 4-6.

Fluorescence micrographs of animals expressing p86/76-7A stained with an anti-β-galactosidase antibody. Animals expressed this fusion in a small number of cells. A. shows the NSM motorneurons in the head of an adult worm (anterior is at left). B-E. Although the HSNs frequently migrated and extended axons abnormally, HSNs with cell bodies in the normal position appeared to send out processes in the ventral nerve cord in the late L4 stage. B. β-galactosidase immunoreactivity in the left HSN of a relatively young L4, based on the position of the vulval nuclei (stained with DAPI, C). This HSN has only a short process. The older L4 shown in D and E has an HSN that extends anteriorly in the ventral nerve cord (arrows). Staining became faint toward the head, but it was not clear whether this was a reflection of the end of the axon or of antibody penetration. Scale: 1 cm = 15 μm.
Figure 4-7.

X-gal staining of animals expressing unc-86/76/lacZ fusions. A. Both HSN axons are clearly visible in this animal carrying p86/76-7A. Anterior is at left. HSN cell bodies are out of the plane of focus. An additional unidentified cell in the head also contains X-gal stain. B. The head of an animal carrying p86/76-1 stained for several weeks at 4°C. Note the fine detail in individual processes. C. Cross section through a p86/76-1-expressing worm stained similarly to the animal shown in B and processed for electron microscopy. The ventral nerve cord is the blue region at the bottom of the micrograph. Electron micrographs of sections from this animal are shown in Fig. 4-8. Scale: A,B: 1 cm = 23 μm; C: 1 cm = 15 μm.
Figure 4-8.

Electron micrographs of X-gal-stained animals expressing p86/76-1. A. Low-magnification image of the ventral portion of the same animal seen in Figure 4-7C shows electron-dense material largely restricted to the region of the ventral nerve cord (a roughly triangular region in the center of the lower part of this micrograph). B. A high magnification image of the ventral cord of the same animal shows that stain is not restricted to the small number of axons expected to contain the fusion protein. Note the perinuclear staining in the neuronal cell body (arrows). Scale: A: 1 cm = 1.55 μm; B: 1 cm = 710 nm.
Figure 4-9.

Cultured N2 neurons on a substrate of paraformaldehyde-activated TESPA. Cells grown in culture overnight often extend neurites similar to those seen here, sometimes with a pronounced growth cone (A,B), but often with a fine tip (C). Both monopolar (C) and bipolar cells (A,B) are observed. Scale: 1 cm = 8 μm.
Figure 4-10.
Cultured N2 neurons stained with monoclonal antibodies against tubulin. Cells in A (Nomarski image in B) were stained with the anti-tubulin antibody YL 1/2, which stains all C. elegans tubulin. The axon in C (Nomarski image in D) is stained with the neuron-specific acetylated tubulin antibody 611B1. Scale: 1 cm = 7.5 μm.
Perspectives on genes affecting HSN axon outgrowth

Genetic analysis of uncoordinated mutants *C. elegans* has led to the identification of at least 13 genes that affect the longitudinal extension of the HSN axons (Fig. 5-1; Desai et al., 1988; McIntire et al., 1992). These can be subdivided on the basis of the environments in which HSN outgrowth is affected in mutants and whether HSN cell body migration is affected as well as axonal outgrowth. Some of the groups that emerge from this subdivision appear to reflect related molecular functions as well as mutant phenotypes, and so consideration of these groups might prove instructive.

As discussed in Chapters 1 and 2, two groups of genes affect axonal outgrowth in specific environments, the lateral hypodermis and nerve bundles. The likely functions of *unc-6* and *unc-5* as extracellular matrix component and receptor based on sequence analysis are consistent with the anatomical focus of defects in *unc-6* and *unc-5* mutants (Ishii et al., 1992; Leung-Hagesteijn et al., 1992; Hedgcock et al., 1990). Roles of the fascicle-specific genes have been addressed in Chapters 2 and 3 and will be discussed further below. Genes required for outgrowth in both fascicles and the lateral hypodermis appear to have regulatory or structural roles in axonal outgrowth processes common to both environments. Functions inferred from sequence analysis or ultrastructural examination of mutants include cytoskeletal assembly (*unc-33*, *unc-44*, and *unc-73*) and membrane trafficking (*unc-14* and *unc-51*). Mutations in *mig-2* affect the migration of several cells and cause
uncoordination but have not been extensively characterized (Hedgecock et al., 1987).

Genes involved in HSN cell body migration and axonal outgrowth

One grouping of these genes that has not received much attention is the set of genes required both for normal axonal extension and for normal HSN cell body migration. At least four of these genes exist, two required for axonal extension only in fascicles (unc-34 and unc-71) and two required for axonal extension both in fascicles and along the lateral hypodermis (unc-73 and mig-2). Mutations in each of these genes cause defects in the embryonic migration of the HSN cell bodies from the tail to their normal positions near the vulva (Desai et al., 1988; this work). The migration defects are relatively weak, as the cell bodies migrate about 75-80 percent of their normal distance.

Recent observations suggest the involvement of two members of this group in functions common to the movements of growth cones and motile cells. First, as discussed in Chapter 1, sequence analysis of unc-73 suggests that it might be involved in regulation of actin filament organization. The Unc-73 protein is similar to the yeast CDC24 protein, which is believed to act as a guanine nucleotide exchange factor for the small ras-like GTPase CDC42 (J. Culotti, personal communication). Together these genes in yeast regulate the localized deposition of actin during budding (Johnson and Pringle, 1990; Adams and Pringle, 1984), and a comparable role in the motility of growth cones and migrating cells appears likely. Other ras-like GTPases have been shown to affect
leading-edge behavior of fibroblasts (Ridley and Hall, 1992; Ridley et al., 1992). Because limited characterization of the effects of mig-2 on HSN axon outgrowth and cell body migration shows traits in common with those of unc-73, this gene could serve a comparable function. The isolation of a CDC42 homolog from C. elegans has recently been reported (Run, J-Q., Hung, M-S., Wang, A., Way, J., Steven, R., and Culotti, J., personal communication), as well as the identification of dominant extragenic suppressors of unc-73. These suppressors, which are reported to confer no striking phenotype in the absence of unc-73 mutations, map to chromosome II, while mig-2 is located on the X chromosome (Hedgecock et al., 1987).

A second recent observation of interest suggests a role for unc-71 in migration of a different cell type, the sex myoblasts (SMs). These cells migrate anteriorly from the tail to positions flanking the vulva during the L2 stage, and their position is determined by a combination of signals, of which one emanates from the developing gonad and one is probably associated with the migration route along the lateral basement membrane (Thomas et al., 1990). A gene with roles both in this migration process and in signal transduction during vulval development, sem-5, has recently been reported to encode a protein consisting primarily of one SH2 and two SH3 domains, and genetic evidence suggests that in its vulval development function, it serves as an adaptor between a cell-surface receptor and a ras-like protein (Clark et al., 1992). It has recently been observed that at least two unc-71 mutant alleles enhance the sex myoblast migration defects caused by sem-5 mutations (M.J. Stern, personal communication). The connection between unc-71 and sem-5 is not clear, but this interaction suggests a striking parallel
between the kinds of molecules implicated in vulval development and those involved in cell migration and axonal outgrowth. The Sem-5 protein appears by genetic criteria to affect ras function, but its target in sex myoblast migration is unknown. The function of a ras-like protein, a rho homolog, in HSN cell body and axon migration is suggested by the unc-73 sequence. Because unc-71 shows genetic similarity to other genes likely to function in receiving or processing signals in fasciculating axons (unc-34, unc-69, and unc-76; see below), it could act at an intermediate step of a signal transduction pathway in a similar manner to sem-5. The function of sem-5 and unc-71 in processes involving different ras-like molecules, together with the effects of mutations in these two genes on the migration of the same cell, suggests a possible convergence of regulatory machinery. The effects of unc-34, unc-69, and unc-76 mutations on sem-5 are currently being tested. Connections among sem-5, unc-71, unc-73, the CDC42 homolog, and the unc-73 extragenic suppressors bear further investigation.

Genes affecting axonal outgrowth in fascicles

What do unc-34, unc-69, unc-71, and unc-76 do? This question has been discussed at length in Chapters 2 and 3, but the principal observations that must be considered will be reviewed briefly here. First, unc-34, unc-69, unc-71, and unc-76 appear to affect both the extension of axons along other axons and the formation or maintenance of fascicles. Second, these genes appear to be required only for axonal function in fascicles and not in regions in which axons grow along the hypodermis and basement membrane. Third, mutations in these genes
affect a large proportion of the cells in the nervous system. Fourth, double mutant experiments suggest that unc-34, unc-71, and unc-76 provide independent contributions to the process of axonal elongation. Fifth, the predicted Unc-76 and Unc-69 proteins are probably intracellular and, aside from a nine-amino acid motif common to the N termini of these proteins, show no sequence similarity to each other or to other proteins in the available databases (M. Hengartner and N. Tsung, personal communication). Sixth, the Unc-76 protein is found in the axons of all or nearly all neurons from the time of onset of axonal outgrowth through adulthood.

A model for the function of these genes is based primarily on in vitro studies of vertebrate cell-cell adhesion molecules thought to be involved in fasciculation. Though speculative, it provides a framework for thinking about the relationships among the genes in this group and suggests experiments with which to explore them.

As discussed in Chapter 3, the likelihood that the Unc-76 protein is intracellular places it at an interesting position in the axonal outgrowth process. Pharmacological evidence suggests that multiple vertebrate cell adhesion molecules could use the same intracellular signals to promote axonal outgrowth, and this signalling function can be inhibited independently of the adhesive function of these molecules (Williams et al., 1992; Bixby and Jhabvala, 1990). A common phosphorylation-dependent mechanism of regulating the activity of multiple receptors has also been suggested (Cervello et al., 1991). If structurally-diverse cell surface proteins do in fact interact with common signalling or cytoskeletal molecules, intracellular adaptor molecules would be likely to serve as protein linkages between receptors
and effectors (signalling molecules or the cytoskeleton). While no
direct evidence for such a role for the Unc-76 protein exists, the
restriction of unc-76 mutant defects to fascicles, the axonal
localization of Unc-76 protein throughout development, and unc-76 mutant
defects suggestive of both impaired axonal elongation and adhesion
indicate that the Unc-76 protein functions at a point at which axon-axon
interactions, axonal elongation, and axonal adhesion converge. A role
as an intracellular adaptor therefore appears plausible. Likewise, the
apparent similarity of unc-76 and unc-69 mutant defects and a predicted
Unc-69 protein sequence indicative of intracellular localization (M.
Hengartner and N. Tsung, personal communication) suggest a similar role
for the Unc-69 protein. The involvement of cell-adhesion molecules in
intracellular signalling as well as in adhesion has been well documented
for integrins and the T cell receptor/CD3 complex (Hynes, 1992; Rudd,
1990), and emerging evidence from vertebrate fasiculation molecules such
as L1 and N-CAM suggests that these molecules are similarly bifunctional
(Saffell et al., 1992; Williams et al., 1992). The possibility that C.
elegans genetics has led to the identification of intracellular
molecules that participate in linking the two functions is appealing and
worth investigating further.

Molecular characterization of unc-34 (see Appendix) and unc-71
should indicate whether the phenotypic similarity of these two genes to
unc-76 and unc-69 arises from similar molecular structures. These two
genes share features that distinguish them from unc-76 and unc-69,
however, which could suggest a slightly different role. First, unc-34
mutants are somewhat less Unc and unc-71 mutants are considerably less
Unc than unc-76 and unc-69 mutants (although the null phenotypes of unc-
71 and unc-69 have yet to be determined). This could suggest a more
restricted set of cells in which the functions of unc-34 and unc-71 are
required. Second, unc-34 and unc-71 mutations affect HSN cell body
migration while unc-76 and unc-69 mutations do not. The enhancement of
sex myoblast migration by unc-71 and the potential parallels this
suggests with other receptor-adaptor-ras signalling pathways could
indicate a signalling role for unc-71 and unc-34 as well. A function as
cell-adhesion molecules involved in cell migrations and axonal outgrowth
cannot be ruled out, although the cellular environments in which these
processes take place are quite different (basement membrane vs.
fascicle) and axonal outgrowth is affected by these genes only in
fascicles.

The apparent independent function of unc-34, unc-71, and unc-76
based on the increased severity of axonal outgrowth defects in double
mutants is difficult to interpret without the certainty that the mutants
tested are null. In the context of the receptor-adaptor model,
independent functions might suggest that each gene represents an
independent receptor-adaptor pair. However, the sharing of the adaptor
Sem-5 between at least two distinct signalling pathways, in vulval
development and sex myoblast migration, suggests that adaptors need not
be restricted to a single receptor or effector, and so adaptor mutants
could affect multiple pathways and in genetic experiments show some
independence from the receptors with which they interact.

Future prospects
How can the functions of unc-34, unc-69, unc-71, and unc-76 be better defined? Analogy with the SH2/SH3 family of adaptor proteins suggests that at least a fraction of the proposed adaptor molecules should be associated with the cytoplasmic domain of the appropriate cell-surface receptor (Pawson and Gish, 1992). Demonstration of a membrane association of Unc-76 protein through cell fractionation and immunoblotting is a first step. Mutations eliminating the receptor are likely to alter the localization and possibly the levels of the intracellular adaptor (as observed in cells expressing cadherins unable to bind catenins; Kintner, 1992), and therefore if Unc-76 protein is membrane-associated, a test of its localization in unc-34, unc-71, and unc-69 mutants could be informative. Similarly, immunoprecipitation of Unc-76 protein from wild-type and mutant strains could identify interacting proteins.

It is of particular interest to test the function of the DIPLA(SC)DDDD sequence common to the Unc-76 and Unc-69 proteins, since adaptor proteins in the same signalling pathway might share common protein docking motifs. Peptides corresponding to this motif could interfere with the binding of these proteins to their target in vitro, and this can be tested by inclusion of the appropriate peptide in anti-Unc-76 immunoprecipitation experiments. Overexpression of proteins with such a motif in C. elegans neurons might be expected to mimic unc-76 or unc-69 loss of function in a dominant negative fashion, and in this regard it is surprising that overexpression of the wild-type unc-76 or unc-69 genes or lacZ fusion constructs containing fragments of these genes do not appear to cause axonal outgrowth defects (but see discussion of unc-86/unc-76/lacZ fusions, Chapter 4). This could
suggest that these proteins can exist in active and inactive forms such that the mere presence of protein is insufficient to produce binding. Finally, the ability of a genomic clone lacking codons 1-104 (including the DPLASCDDD sequence) to rescue an unc-76 mutant encoding amino acids 1-105, albeit extremely weakly, suggests that this sequence might not be essential for unc-76 function.

Should the DPLA(SC)DDD sequence be found to affect binding to another protein, a screen for additional genes with this motif using PCR primers corresponding to this sequence and the C. elegans spliced leader sequences could identify other genes with similar functions. Isolation and sequence analysis of unc-76 and unc-69 homologs from other species could be informative in this regard. In addition, site-directed mutagenesis of this sequence might reveal residues essential for function. If this is the case, the isolation of extragenic suppressors of engineered unc-76 or unc-69 mutations could be a powerful method of identifying physically-interacting proteins.

A major impediment to the understanding of the functions of all fascicle-specific axonal outgrowth genes is the lack of techniques for observing axons during their outgrowth. The strength of the work of Goodman and colleagues in documenting selective fasciculation in the grasshopper central nervous system lies in the high degree of detail—down to individual filopodia—with which normal and perturbed growth cone-axon interactions can be described. Until the outgrowth of the HSN axons can be studied in this way, the effects of loss of gene function on specific cell behavior will remain difficult to assess. Perhaps the development of markers such as those described in Chapter 4 will allow the necessary fine-structure analysis.
At a minimum, it is reasonable to expect that for light microscopy, the unc-76/ lacZ fusions under the control of the unc-86 promoter discussed in Chapter 4 can be modified to eliminate the axonal outgrowth problems believed to be caused by the first constructs. A potential alternative for studying wild-type and mutant axonal elongation at the light microscope level, although inconvenient for mutant screens, is the anterograde labeling of HSN axons in fixed worms by microinjection of di0 or diI. Though not yet tested in C. elegans, this technique works in fixed tissue (e.g., Chitnis et al., 1992), and fixation reduces the resilience of the C. elegans cuticle, the primary obstacle to accurate injection in living animals. If successful diI or di0 labeling can be achieved, photoconversion with diaminobenzidine to an electron-dense product (e.g., Chitnis et al., 1992; Bastiani et al., 1984) might allow electron microscopic observation as well. Electron microscope-level resolution would allow observation of the interaction of HSN growth cones with the PVP, PVQ, and other axons in wild-type and mutant animals and might reveal defects in growth cone-axon recognition. Light microscopic observation of these axons should provide information concerning the rates of axonal elongation and the time at which defects in HSN morphology are first evident.

How will molecules mediating specific axon-axon interactions in C. elegans be found?

Multiple axons are abnormal in each of the axonal outgrowth mutants discussed in this section. While it is unlikely that every axon-axon interaction will be mediated by a unique molecule, the
widespread effects of *unc-34*, *unc-69*, *unc-71*, and *unc-76* mutations on axonal outgrowth suggest that these genes function in too many cells to specify details of fascicle organization. Since a principal goal of these studies has been to understand the mechanisms by which selective fasciculation occurs, approaches for identifying molecules involved in more specific cell-cell interactions will be considered briefly.

Two lines of evidence suggest that mutations affecting specific axon-axon interactions might not produce obvious behavioral defects. First, elimination of specific *C. elegans* neurons by laser ablation can produce significant alterations in axonal bundle organization without causing significant behavioral alterations. The AVG axon pioneers the right side of the ventral nerve cord, and ablation of this neuron causes the ventral cord to break up into several smaller fascicles, some of which run on the wrong side of the ventral hypodermal ridge; yet uncoordinated movement of animals in which AVG has been ablated is extremely subtle (Durbin, 1987). This probably results from the ability of *C. elegans* muscles to send out processes that can find the appropriate motorneurons even in disorganized ventral cords. The second observation suggesting that molecules mediating selective fasciculation might be difficult to isolate by screening *unc* mutants is that *Drosophila* mutants lacking fasciclin I or fasciclin III have fairly normal behavior and nervous system organization, even at the level of single neurons (Chiba et al., 1992), although adult *fasI* mutants do show uncoordinated walking (Elkins et al., 1990). These observations suggest that *C. elegans* genes involved in small numbers of specific axon-axon interactions, if they exist, might not have strong mutant phenotypes.
Given the existence of multiple cellular and molecular cues by which axons navigate in many organisms, perhaps more attention should be paid to mutants with subtle or undetectable behavioral defects, such as those caused by ablation of AVG. The use of laser ablations to test the effects of specific cells on the guidance of other axons might be useful in this regard. Ablation of PVPR and PVQL was found to cause the left HSN axon to cross to the right side of the ventral cord, and while this did not result in a behavioral alteration, it suggested that loss of a guiding cell should have a morphological consequence (Garriga et al., 1993). Examination of HSN axons in mutants identified from other screens has identified three genes that can cause the expected mutant defect (unc-30, unc-42, and enu-1), suggesting that direct screening for mutants with morphological defects predicted from laser studies could yield genes with more specific functions. Direct screening with antibodies or other histochemical stains is labor-intensive, but it avoids a reliance on behavioral defects. Such screens have produced a few mutants (pvp-1 from a PVP-specific antibody screen; C. Norris, H. Bhatt, and E. Hedgecock, personal communication; and enu-1 from a phasmid axon dye-filling screen; J. Culotti, personal communication). Antibodies against serotonin or cell-specific axonal markers discussed in Chapter 4 are also potential reagents on which to base a screen. Given the subtlety of fasI and fasIII mutant defects, however, it is possible that screens for altered morphology alone could miss important molecules.

While the existence of functionally redundant processes can make isolation of single recessive mutants defective in the process difficult, two approaches to overcome this are possible. Dominant gain-
of-function mutations or synthetic mutations, the phenotypic effects of which are observable only in double mutant combinations, have both revealed defects caused by Drosophila fasciclin mutations that are not readily apparent in single mutants (Elkins et al., 1990; Chiba et al., 1992; Lin et al., 1992). While these defects were observed in strains built from known mutants or by ectopic expression of a known molecule, this approach could, in principle, be used in conjunction with direct morphological screening in C. elegans. A screen for synthetic mutations affecting HSN outgrowth, for example, might be carried out in the background of enu-1 mutants, which cause the left HSN to cross to the right side in about 50% of mutant animals but do not cause premature termination (Chapter 2). Alternatively, cell-specific suppression of general outgrowth mutants (e.g., suppression of unc-76 HSN, PHA, and PHB defects by tax-2) might prove a generally useful technique. Since PHA and PHB can be examined in living animals and mutations in at least one gene are expected to be obtained, this screen is particularly attractive.

Extragenic suppressors, in principle, provide a powerful genetic tool with which to explore the relationships among these genes. Although suppression of unc-76(e911) did not produce mutations involved in nervous system development, unc-34 suppressors were easily obtained (Chapter 2). Their general lack of visible phenotypes has been an impediment to genetic analysis, but further efforts are warranted given the likelihood of redundant functions in adhesion and signalling. The observation that one suppressor of multiple unc-34 alleles, n1953, confers a weak egg-laying defect when separated from unc-34(e566) is particularly interesting in light of the recently-observed connection
between the axonal outgrowth gene unc-71 and sex myoblast migration, defects in which also cause variable defects in egg laying.

References


Figure 5-1  Genes required for different phases of HSN outgrowth

unc-69  unc-34  unc-73  unc-33  unc-5
unc-76  unc-71  mig-2  unc-44  unc-6
unc-14  unc-14  unc-51  unc-40

growth in fascicles  migration of HSN cell body  growth on hypodermis
Appendix: Physical mapping of unc-34
Summary

Experiments to locate the unc-34 gene on the physical map by RFLP mapping defined a 400 kb interval in which the gene was likely to be located. Although germline transformation experiments failed to identify rescuing clones, independent PCR-based deficiency mapping (K. McKim, personal communication) and successful rescue of unc-34 with a new YAC clone in this 400 kb interval (D. Ginsberg, personal communication) indicated that these mapping experiments were correct.

Introduction

Genetic studies of unc-34 indicate that it plays a role in axonal outgrowth similar but not identical to that played by unc-76 and that the two genes probably function in independent pathways. It was therefore of considerable interest to clone the unc-34 gene in order to understand the structure of the unc-34 gene product and to generate molecular tools with which to study its function. This appendix summarizes the experiments conducted to identify clones containing the gene.

The approach taken relied on physical map data and DNA clones developed as part of the C. elegans genome cloning project conducted by John Sulston and Alan Coulson at the MRC Laboratory of Molecular Biology, Cambridge, England (Coulson et al., 1986). Cosmid clones shown by the Cambridge group to be located at the end of the left arm of linkage group V, where unc-34 maps genetically, were used to identify restriction fragment length polymorphisms (RFLPs) between wild-type
strains. RFLPs were then mapped genetically with respect to unc-34, and clones close to the gene were tested for their ability to rescue unc-34 mutants in germline transformation experiments. Although clones were localized to within approximately 400 kb of the gene, no rescuing clones were identified. Subsequent experiments with YAC clones not available at the time of these experiments showed that a YAC clone within this interval does rescue the unc-34(e566) mutant phenotype (D. Ginsberg, personal communication).

**Experimental Procedures**

Preparation of DNA and radiolabeled probes and Southern blotting were done by standard techniques (Coulson et al., 1986; Sambrook et al., 1989; Ausubel et al., 1991). YAC DNA was prepared by lysis of yeast cells in agarose, two rounds of pulsed-field gel electrophoresis, and phenol extraction and isopropanol purification of YAC DNA.

RFLPs were identified by preparing Southern blots of genomic DNA from the strains N2 and EM1002 digested with EcoRI, HindIII, and XbaI or BamHI and hybridization to cosmids probes radiolabeled by random oligonucleotide priming.

RFLP mapping was performed by crossing unc-34(e566)unc-60(e677)/+ males with EM1002 hermaphrodites, growing individual progeny on single plates, and inspection of the broods of animals containing unc-34(e566)unc-60(e677) animals for Unc-34 non-Unc-60 recombinants. Seven progeny of these animals were grown on individual plates, and clones that were homozygous for the recombinant unc-34(e566) chromosome were maintained for DNA preparation.
Germline transformation experiments were conducted by injection of cosmid DNA along with the rol-6(su1006) plasmid pRF4 (Kramer et al., 1990) into unc-34(e566) or unc-34(e566)/+ hermaphrodites (Fire et al., 1990) and establishing lines of Rol animals. Rescue was scored on the basis of coordination of the characteristic rolling motion of rol-6 animals.

Attempts to construct let-447(s1654) or let-448(s1363) unc-34 double mutants were made as follows: males of the genotype dpy-18(e364)/et1;let-447 or 448 unc-46(e177)/et1 let(s2165) were crossed to unc-34(e566) hermaphrodites. Broods of non-Unc progeny were examined for the presence of Dpy animals. From these broods, Unc-34 animals were cloned, and 8-10 of their progeny were examined individually for sterility.

Results

Mapping of restriction fragment length polymorphisms linked to unc-34:

In preliminary experiments, cosmids were used to probe DNA from animals heterozygous for unc-34 region deficiencies and balancer chromosomes. Experiments with the clones pCes263, C11E5, C44B1, C56B12, C26D7, ZK1005, F33E11, and C39F7, contained in contigs mapped to the left end of linkage group V, failed to identify the breakpoints of the deficiencies sDf32, sDf28, sDf34, sDf39, or sDf53. Quantitative Southern blotting to determine whether these cosmids identified DNA reduced in concentration by half in deficiency heterozygotes was judged unreliable. However, the cosmid C26D7 was shown to be absent from sDf34/sDf39 trans-heterozygotes. sDf39 deletes genes to the left of
unc-34 but not unc-34 itself, and sDf34 deletes genes both right and left of unc-34. The heterozygote sDf34/sDf39 is viable, and the lack of hybridization of the cosmid C26D7 to DNA from this heterozygote suggested that this cosmid lies to the left of unc-34.

A panel of cosmids near the ends of contigs mapped to the left end of linkage group V was tested by Southern hybridization to identify RFLPs between N2 and the Bergerac strain EM1002. At the start of these experiments, two contigs without genetically-mapped clones had been mapped by in situ hybridization to the distal part of chromosome V, but the orientation of these contigs with respect to the chromosome was not known. The cosmids K08B11, R09A1, C44B1, F59C5, and F33B11 detected RFLPs. In order to map the identified RFLPs with respect to unc-34, males of the genotype unc-34(e566) unc-60(e677)/++ were crossed to EM1002 hermaphrodites, generating hermaphrodite progeny heterozygous for the N2 and EM1002 variants of each identified RFLP. These heterozygotes produced occasional self progeny in which a recombination event had occurred in the 1.1 map unit interval between unc-34 and unc-60. Animals homozygous for unc-34 were easily distinguished from the unc-34 unc-60 homozygotes, but the double mutant was very similar in appearance to unc-60 single mutants, and so only unc-34-non-unc-60 animals were isolated. Clones of progeny from these animals in which the recombinant chromosome was homozygous were established, and DNA from 24 of these lines was analyzed by probing Southern blots with the cosmids that identified RFLPs.

Three cosmids were positioned with respect to unc-34 by this technique (Fig. A-1). F59C5, a cosmid at one end of the largest contig in the region, identified a HindIII RFLP. Of 12 unc-34-non-unc-60
recombinants, 7 retained the N2 band pattern, suggesting that this cosmid was between the two genes, about 0.5 map units to the right of unc-34. These data are also consistent with the segregation expected for a marker unlinked to either gene, however, but a cosmid closer to the center of the same contig, R09A1, showed clear linkage to unc-34. A HindIII RFLP identified by R09A1 showed the N2 band pattern in 15/17 unc-34-non-unc-60 recombinants, placing this marker approximately 0.13 map units to the right of unc-34.

The cosmid K08B11 identified a HindIII RFLP that cosegregated with unc-34 in 23/23 recombinants analyzed. This result suggested that K08B11 was either located to the left of unc-34 or within 0.05 map units of the gene on the right (a maximum of 1/24 of the 1.1 map unit distance between unc-34 and unc-60). K08B11 was used as a probe of DNA from several unc-34 mutants generated by mutagens likely to cause DNA rearrangements in an effort to identify RFLPs associated with specific mutant alleles. The gamma-ray-induced alleles s138 and n1890 and the deficiency sDf39 did not appear to be altered in the region represented in K08B11, but the allele e951 appeared to have an 0.5 kb EcoRI band and an 0.4 kb XbaI band shifted to slightly smaller sizes. However, this could not be interpreted as an indication that the unc-34 mutation was located in this region, because e951 also carried an allele-specific HindIII RFLP identified by R09A1, which maps 0.13 map units to the right of unc-34. Bands identified by the same cosmid were altered identically in all alleles isolated by a non-complementation screen, suggesting that the strain used for mutagenesis contained an alteration in this region that did not cause an unc-34 mutation. No allele-specific RFLPs were identified by T03D5 or W08D7.
Recombination between unc-34 and a marker on the left would have clarified the map position of K08B11, but the only genes known to be left of unc-34 had lethal or sterile mutant phenotypes. Attempts to build double mutant combinations between unc-34 and either of two of these sterile mutations, \textit{let-447}(s1654) and \textit{let-448}(s1363) were unsuccessful. These genes were positioned to the left of unc-34 by deficiency mapping, and the map distance between them and unc-34 is unknown (Johnsen and Baillie, 1988, 1991). It is possible that insufficient numbers of animals were examined in the search for recombinant chromosomes (160 broods for each lethal gene). However, \textit{let-447}(s1654) was isolated by formaldehyde mutagenesis, which induces small deficiencies (Johnsen and Baillie, 1991); deficiencies in this region of linkage group V have been observed to inhibit recombination to the right (Rosenbluth et al., 1990). The mutation \textit{let-448}(s1363) does not appear to reduce recombination (Rosenbluth et al., 1990). The apparent tight linkage of K08B11 to unc-34, however, suggested that germline transformation with clones near K08B11 might be a more efficient method of locating the gene.

The approximate positions of K08B11 and R09A1 were confirmed independently by Kim McKim (personal communication), who tested the positions of a number of cosmids in the region relative to a panel of deficiencies. Using PCR primers specific to 11 clones, McKim amplified DNA from dead embryos or larvae homozygous for each deficiency in order to determine whether a given clone was in a region deleted by a given deficiency. DNA sequences represented by K08B11 and T18F3, a cosmid approximately 100 kb to the left of K08B11 in the same contig, were found to be in the same deficiency interval as unc-34 (deleted by \textit{sDf32}}
and sDf34 but not sDf28 or sDf39). R09A1 was positioned to the right of unc-34 by this technique, in a region deleted by sDf28 as well as sDf32 and sDf34. F56E12, a cosmid adjacent to the cosmid F59C5, which was placed 0.5 map units to the right of unc-34 by RFLP mapping, was found by PCR analysis to be in a deficiency interval consistent with the RFLP data. Finally, clones overlapping the cosmid C26D7, identified by Southern blot experiments as representing DNA deleted by both sDf34 and sDf39, were also shown to be absent from both deficiency chromosomes by McKim's PCR mapping.

Based on the estimated 0.13 map unit distance between unc-34 and the nearest identified mapped cosmid, R09A1, rough estimates of the range in which unc-34 was likely to be found were made. R09A1 and K08B11 are joined by a 310 kb YAC, Y50H12. Based on YAC-cosmid hybridization, the two cosmids are likely to be about 230 kb apart. If K08B11 lies 0.05 map units to the right of unc-34 and R09A1 is 0.13 map units to the right of the gene, then the 230 kb separating K08B11 from R09A1 represent 0.08 map units, so K08B11 would be expected to lie about 140 kb to the right of unc-34. Overlapping cosmids were known to extend approximately 150 kb to the left of K08B11, sufficient to cover the left end of the likely unc-34 range. If K08B11 lies to the left of unc-34, then the gene is probably within the region corresponding to the YAC.

Clones representing this entire region, from C05E4 to R09A1, were pooled for germline transformation experiments (Fig. A-1). Cosmid DNA was injected at 25-50 μg/ml along with pRF4, a plasmid encoding the dominant rol-6(su1006) mutation (Kramer et al., 1990) at 40 μg/ml into either unc-34(e566) homozygotes or unc-34(e566)/+ heterozygotes, and F2 lines of Rol animals were analyzed for the presence of unc-34 and rescue
of the Unc phenotype. Homozygous unc-34 Rol lines were established and their locomotory ability relative to lines of N2 and unc-34 transformed with pRF4 alone was examined.

Prior to the positioning of R09A1 to the right of unc-34, two transgenic lines with R09A1 and one line with the overlapping set R09A1, T19F10, and C49D7 were examined. None showed any rescue of unc-34.

Examination of transgenic lines with cosmids adjacent to K08B11 also failed to show rescue. Pools of overlapping cosmids representing the entire set of cosmid clones in this portion of the contig were tested: K08B11 and T02B11 (6 lines; K08B11 could not be isolated in its full-length form); T03D5 and K09C6 (5 lines); T03D5, K09C6, and C44B1 (3 lines); T02B11, K09C6, and C09H11 (6 lines); and C53B12, C24D11, and C05E4 (4 lines). These cosmids were all within the same deficiency interval as unc-34 (Kim McKim, personal communication), so it is possible that unc-34 could lie to the left of the cosmids tested.

If unc-34 lies to the right of K08B11, it should be contained within the YAC Y50H12, which extends between T03D5 and a cosmid mapped to the right of unc-34, R09A1. DNA from Y50H12 was prepared by pulsed-field gel electrophoresis and injected into unc-34(e566)/+ hermaphrodites. Three Rol unc-34 lines were obtained, but no rescue of unc-34 was apparent. The amount of YAC DNA available was low, and so the number of copies of any given locus injected was much lower than was normal for a cosmid injection. In addition, rescuing YACs have been found to be much less stable than cosmids, and so the F2 and F3 animals scored for rescue may have lost the YAC DNA. Later attempts to rescue unc-34(e566) with higher concentrations of Y50H12 produced one transiently-rescued unc-34 animal (D. Ginsberg, personal communication).
Recent germline transformation with a smaller YAC unavailable at the time of these experiments, Y10G5, showed that this YAC contains unc-34(e566) rescuing activity (D. Ginsberg, personal communication).

Discussion

Two independent mapping experiments using two different techniques positioned the cosmid R09A1 close to unc-34 on the right and the cosmid K08B11 close to unc-34 on either the left or the right. Both experiments indicated that unc-34 must be between R09A1 and C26D7, a range of 600 kb, and estimates based on recombination mapping suggest the range is likely to be closer to 400 kb. Successful rescue of unc-34 with a YAC in the identified region indicates that these mapping results were correct. The YAC overlaps K08B11, the cosmid mapped closest to unc-34, and extends into the region between K08B11 and R09A1 not represented by cosmid clones.

Previous failure to rescue unc-34 with the YAC Y50H12 could have arisen from the low DNA concentrations used, or peculiarities of the YAC itself. The relative small size of the rescuing YAC, 200 kb, and the development of techniques for subcloning YACs by recombination in yeast, should facilitate identification of smaller subclones containing unc-34.

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References


Figure A-1

Physical and genetic maps of the unc-34 region. Cosmid and YAC clones positioned with respect to unc-34 and unc-60 and used in mapping and transformation experiments are shown below the genetic map.
Figure A-1

V(L)
jet-450  jet-396  jet-447  jet-448  jet-453  unc-34  jet-326  unc-60

1.1 mu

0.13 mu

0.5 mu

TCUNC60C contig

K08B11  R09A1  F59C5

K08B11  R09A1  C49D7  T19F10

T02B11  T03D5  T03D5

C44B1  C09H11  C53B12

C24D11  C05E4
Figure A-2.

RFLP mapping of K08B11 and R09A1 with respect to unc-34 and unc-60. A. Southern blot probed with radiolabeled cosmid K08B11. All unc-34-non-unc-60 recombinants retain the N2 variant of the K08B11 polymorphic bands, indicating tight linkage to unc-34. Recombinant lines (from left): unc-34-non-unc-60-1,2,3, (5 not visible), 6, 12, (15 not visible), 17, 18, 20, 21, 23, 24. B. The same filter used in A is probed with R09A1. Two recombinants show the EM1002 pattern, indicating that R09A1 is close to unc-34 on the right.