The role of the ELAV homologue EXC-7 in the development of the Caenorhabditis elegans excretory canals

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Abstract

The exc mutations of Caenorhabditis elegans alter the position and shape of the apical cytoskeleton in polarized epithelial cells. Mutants in exc-7 form small cysts throughout the tubular excretory canals that regulate organismal osmolarity. We have cloned the exc-7 gene, the closest nematode homologue to the neural RNA-binding protein ELAV. EXC-7 is expressed in the canal for a short time midway through embryogenesis. Cysts in exc-7 mutants do not develop until several hours later, beginning at the time of hatching. We find that the first larval period is when the canal completes the majority of its outgrowth, and adds new apical cytoskeleton at a rapid rate. Ultrastructural studies show that exc-7 mutant defects resemble loss of βH-spectrin (encoded by sma-1) at the distal ends of the excretory canals. In addition, exc-7 mutants exhibit synergistic excretory canal defects with mutations in sma-1, and exc-7 binds sma-1 mRNA. These data imply that EXC-7 protein may affect expression of sma-1 and other genes to effect proper development of the excretory canals.

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Introduction

The Caenorhabditis elegans excretory canals form an attractive model for studying the maintenance of tubule structure (Buechner, 2002). This osmoregulatory epithelium consists of a pair of narrow tubules that stretch the length of the animal. Near the pharynx, the tubules are connected to each other and to an excretory pore to allow elimination of excess fluid. Significantly, these tubules are entirely the product of a single cell (White, 1988) (Fig. 1A). In considering the processes needed to maintain tubular shape, a unicellular tubule provides an attractive alternative to multicellular structures, since there is no need to take into account cadherin-mediated cell–cell contact or cellular proliferation. Furthermore, the elaborate shape of the excretory canal cell facilitates isolation of mutants in several important steps of morphogenesis. Mutations that have subtle effects on other epithelia or neuronal processes cause easily identified, penetrant defects in excretory canal morphology (Hedgecock et al., 1990). These include defects in axon pathfinding and basement membrane structure.

We have examined defects that occur at the apical (luminal) surface of the excretory canals. Twelve exc mutations have previously been identified that affect the narrow tubular structure of the canals (Buechner et al., 1999). Mutations in most of these genes cause formation of large, septate, fluid-filled cysts at characteristic positions along the canals, and also cause more subtle structural defects in other epithelia. Cloned exc genes encode proteins that make up or regulate the apical actin cytoskeletal network, including βH-spectrin (McKeown et al., 1998), mucin (Jones and Baillie, 1995), and a guanine nucleotide exchange factor specific for CDC-42 (Gao et al., 2001; Suzuki et al., 2001).
Unlike other exc genes, mutant alleles of the exc-7 gene cause formation of small cysts throughout the entire length of the lumen rather than large, variable-sized cysts. exc-7 canals are also generally shortened and often end in a bolus of cysts and endosomes. The defects of exc-7 mutants exhibit synergistic effects with two other genes affecting canal structure: exc-3 and sma-1 (Buechner et al., 1999). Although exc-3 has not been cloned, sma-1 encodes a $\beta_H$-spectrin found at the apical and lateral surfaces of several epithelia in C. elegans (McKewon et al., 1998). $\beta_H$-Spectrin is also responsible for apical morphogenesis in Drosophila epithelia (Thomas and Williams, 1999; Zarnescu and Thomas, 1999). The exc-7 gene therefore encodes a protein that may interact with $\beta_H$-spectrin to maintain and regulate the structure of the apical epithelial surface.

We report here the cloning of the exc-7 gene and show that it encodes the closest nematode homologue to Drosophila ELAV. Members of the ELAV family of RNA-binding proteins bind to AU-rich elements in the 3'UTR of various mRNAs (Abe et al., 1996a,b; Levine et al., 1993) and shuttle that mRNA from the nucleus to the cytoplasm through interaction with nuclear export proteins (Brennan et al., 2000; Gallouzi and Steitz, 2001; Keene, 1999; Yannoni and White, 1999). ELAV binding has generally been found to prevent the rapid degradation of mRNA (Brennan and Steitz, 2001; Fan and Steitz, 1998; Ford et al., 1999; Jain et al., 1997; Peng et al., 1998). Deficiency of ELAV decreases the amount of protein synthesized from ELAV-bound mRNAs (Koushika et al., 1996; Wang et al., 2000), while overexpression of ELAV causes increased levels of regulated proteins (Antic et al., 1999).

While the biochemical role of ELAV proteins is becoming clearer, their biological functions in development have remained elusive. Most ELAV proteins are found in the nervous system (Good, 1997; Gouble and Morello, 2000; Park et al., 2000), but others appear to be expressed ubiquitously (Ma et al., 1996). In Drosophila, mutations in ELAV cause embryonic lethality and widespread disruption.
of the nervous system (Campos et al., 1985), but the reason for this disruption is unknown. Here, we show that expression of the C. elegans ELAV homologue exc-7 is expressed earlier in canal development than is apparent from its loss-of-function phenotype; cysts develop several hours later, during late embryogenesis and the first larval stage. We show that this is also the period when the canals grow most rapidly, and therefore canal apical surface synthesis is greatest. Cyst ultrastructure resembles the effects of loss of βH-spectrin, and EXC-7 binds to the 3′ UTR of sma-1 mRNA. These results imply that early synthesis of exc-7 protein regulates the ability of βH-spectrin to form the long excretory canals during postembryonic growth of this polarized epithelial cell.

Materials and methods

DNA

Cosmids corresponding to the region on LG II between let-23 and sqv-8 were supplied by the Sanger Center, Cambridge. Cosmids were prepared for microinjection into nematodes by means of standard techniques (Mello and Fire, 1995). A 3.5-kb fragment (containing predicted genes E02H1.5 and E02H1.6), a 4.9-kb fragment (E02H1.1 and .3), and a 17.4-kb fragment (F35H8.5, .6, and .7) were isolated from cosmids E02H1 via digestion with BamHI (New England Biolabs) and gel purification. Primers specific to predicted gene F35H8.5 (5′-ATGACGAGCGAGATCCACCACC-3′ and 5′-CATAACAAACGGGAGTCAGTCC-3′) and F35H8.6 (5′-ATGGATCCATTGTTTTTCTTTCCCTT-3′ and 5′-ATGGATCCCATTTGTTTTTCTTTCCCTT-3′) were used to amplify genes from the 17.4-kb fragment. Amplified DNA was digested with EcoRI and cloned into pBluescript IIKS(+) (Stratagene). DNA was microinjected into the distal gonadal syncytium of young adult exc-7(rh252) hermaphrodites, together with plasmid pHF4 containing the dominant genetic marker rol-6 (Kramer et al., 1990).

Computer analysis of DNA sequence, including CLUSTALW comparisons, was performed by using Vector NTI software package 5.0 (Informax). BLAST analysis used the National Center for Biotechnology Information and C. elegans Genome Project Internet servers. Since the RRM regions are largely similar between RNA-binding genes, CLUSTALW analysis was initially performed on the more variable ELAV hinge region between the second and third RRMs. Subsequent analysis of the entire genes yielded essentially the same result.

Nematode culture and genetics

C. elegans strains were maintained by use of standard culture techniques on lawns of Escherichia coli strain OP50 grown on nematode growth medium (NGM) plates (Sulston and Hodgkin, 1988). sma-1(e30), unc-42(e270), dpy-6(e14), and dpy-6(e14) animals were supplied by the Caenorhabditis Genetics Center, Minneapolis; sma-1(ru18) was the gift of Judith Austin; a vha-1::gfp construct was the gift of T. Oka and M. Futai; and exc-7(ok370) was generated by the C. elegans knockout project, Oklahoma City (http://www.mutantfactory.ohsu.edu/).

exc-7(rh252);sma-1(e30) and exc-7(ok370);sma-1(ru18) double mutants were constructed by mating heterozygous sma-1 males to exc-7 animals containing an easily scored homozygous marker [unc-42(e270)] located near sma-1 on LG V, and picking motile progeny (exc-7/+; sma-1/unc-42). Progeny of these heterozygotes were scored by means of DIC microscopy to find homozygous exc-7 animals, and the progeny of these were screened for animals (1/4 of the total) no longer containing the unc-42 marker, which are sma-1 homozygotes. Similar strategies were used to construct exc-7(rh252);exc-3(rh207) and exc-7(rh252);exc-6(rh103) homozygotes using dpy-6 and dpy-1, respectively, as markers easily scored via dissecting microscope.

The following cosmids, microinjected singly or in combination, did not restore the exc-7(rh252) phenotypes: C06C3, AH6, M176, R09D1, T19H5, F42A8, C01G6, T15H9, F35H8, T26C5, D2013, ZK1067. Microinjection of the 4.9- and 3.5-kb BamHI fragments of E02H1, and of PCR-amplified F35H8.6 also did not rescue. Canal and tail whip phenotypes were rescued in multiple rol-6-expressing lines created by microinjection of cosmids E02H1, as well as by microinjection of the 17.4-kb BamHI fragment of E02H1, and by microinjection of PCR-amplified F35H8.5. We note that cosmids F35H8 did not rescue the mutant phenotype when injected either alone or in combination with cosmids E02H1; F35H8 may contain another gene toxic to C. elegans when present in high copy number.

RNAi

dsRNA was PCR-amplified from the cloned exc-7 gene with 5′-CCAGGCTTTACACTTTATGC-3′ and 5′GGGCC-CTTTGCATTACGC-3′ primers. RNA was synthesized from the amplified DNA through use of the MEGAscript T3 and T7 kits (Ambion) and measured via formaldehyde PAGE. The transcripts were mixed in equimolar amounts and annealed at 65°C, then microinjected into N2 wild-type young adult hermaphrodites (Mello and Fire, 1995). Progeny of injected animals were examined for evidence of the Exc-7 canal and tailwhip phenotypes, as well as for any other abnormal phenotypes that might be produced.

In vitro RNA binding analysis

cDNA fragments corresponding to amino acids 2-455 of EXC-7 and amino acids 14-385 of mHuD were PCR-amplified and cloned into plasmid pGEX-3X (Amersham Pharmacia Biotech) and transformed into E. coli XL1-blue. GST-fusion proteins were induced with 1 mM IPTG for 4 h.
and then affinity-purified over glutathione–Sepharose. RNA corresponding to the sma-1 UTR was synthesized via in vitro transcription with SP6 (sense strand) and T7 (antisense strand) RNA polymerases in the presence of α-32P-labeled GTP, and purified by means of denaturing PAGE. The 10-μl binding reaction mixture contained: buffer (20 mM Hepes–NaOH, pH 7.9, 200 mM KCl, 5% glycerol, 0.1% Triton X-100, 1 mM DTT, 0.1 mM PMSF), 100 μg/ml yeast RNA, labeled RNA (1 × 10⁶ cpm), and 500 ng of each GST fusion protein. The mixture was incubated for 20 min at 20°C followed by UV light irradiation (600 mJ/cm²) on ice. The irradiated sample was mixed with 1 μl of RNase A (10 mg/ml), incubated for 30 min at 37°C, and then electrophoresed on 10% SDS–PAGE. Label transfer to each fusion protein was visualized by use of a Fuji BAS 2000 Image Analyzer.

Microscopy

Nematodes were examined by means of DIC and/or fluorescence microscopy (Sulston and Hodgkin, 1988) with a 63× objective on a Zeiss Axioskop and photographed in both b/w (DIC) and/or color (fluorescence) with a Magna-Fire Electronic Camera (Optronics). Images were merged electronically (PhotoPaint, Corel) to show the position of GFP fluorescence.

For electron microscopy, L4 larvae and young adults were cut in midbody and fixed immediately in buffered (100 mM Hepes, pH 7.5) 3% glutaraldehyde, followed by post-fixation in buffered 1% OsO₄ (Hall, 1995; Sulston and Hodgkin, 1988). After encasement in 1% agar, samples were dehydrated and embedded in Polybed 812 resin (Polysciences). Serial sections, ca. 70 nm, were poststained in uranyl acetate followed by lead citrate.

Results

**exc-7 encodes the closest homologue to C. elegans ELAV**

The exc-7 allele rh252 was first identified through its effects on the structure of the lumen of the excretory canals (Buechner et al., 1999). The luminal (apical) membrane is surrounded by a thick cytoskeleton (Nelson et al., 1983) and is easily visible by means of DIC microscopy (Fig. 1B). The basolateral membrane is less easily observed. This membrane abuts the hypodermis and is connected to it by gap junctions. In exc-7 animals, the canals contain numerous small cysts. In addition, the mutant canals extend only about half their normal length and terminate in a bolus of somewhat larger cysts (Fig. 1C). Close examination now finds that the structure of another epithelial tissue, the tapering spike of the hermaphrodite tail, is also variably but not penetrantly affected. The wild-type tail spike of the hermaphrodite normally tapers to a smooth point (Fig. 1D), but in mutant adults, the tail spike often forms a characteristic bobbed shape (Fig. 1E). The adult male tail forms a fan instead of a spike; in exc-7 males, the tail fan appears unaffected, and exc-7 homozygous males mate normally.

The exc-7 gene was previously mapped between the let-23 and sqv-8 genes, a region encompassed by 15 cosmids (C. elegans Sequencing Consortium, 1998). We rescued both the canal and tail spike defects through microinjection of the cosmid E02H1 and of the PCR-amplified fragment of the predicted gene F35H8.5 contained within
this cosmid. This gene was previously identified as a homologue of the ELAV family of RNA-binding proteins, and was named \textit{elr-1} (Fujita et al., 1999) (Fig. 2A). ELAV proteins contain 3 RNA-recognition motifs (RRM) of approximately 80 amino acids each, with a hinge region between the second and third RRMs (Good, 1997). A BLAST search of the \textit{C. elegans} database revealed 10 genes with this structure. Since RRMs are found in many proteins, we performed ClustalW analysis specifically of the hinge sequence to determine the relatedness of the various ELAV homologues (Fig. 2B). This analysis indicates that the EXC-7 protein is more closely homologous to the \textit{Drosophila} ELAV protein and to vertebrate Hu proteins than are any of the other \textit{C. elegans} ELAV-like proteins. ClustalW analysis of the entire protein sequence also showed EXC-7 to be the closest \textit{C. elegans} homologue to \textit{Drosophila} and vertebrate ELAV proteins.

We found that the \textit{rh252} mutation is a 222-bp deletion that eliminates the first RNA-binding domain from the protein (Fig. 2A). Amplification via RT-PCR from primers at the predicted 5' and 3' ends of the \textit{exc-7} gene yielded a single band (Fig. 2C). Sequencing this band revealed that the gene structure had been correctly predicted. Similar amplification from \textit{rh252} animals yielded a product that uses a cryptic splice site in lieu of one that had been deleted. This product allows the remainder of the protein to be synthesized, but this product does not contain the first RNA-binding motif, which is the part of the protein most highly conserved between species. In addition, we had previously shown that the phenotype of \textit{rh252} homozygotes is identical to that of \textit{rh252} placed \textit{in trans} to a deletion (Buechner et al., 1999), as expected for a null allele. Finally, a second allele of \textit{exc-7} was isolated by the \textit{C. elegans} gene knockout project. This allele, \textit{ok370}, has the same excretory canal and tail spike phenotypes as does \textit{rh252}. PCR from this mutant shows that the last 2.5 exons, containing the third RRM and most of the hinge region, are missing (Fig. 2A).

The identity of F35H8.5 as the \textit{exc-7} gene product was further confirmed through the use of RNA-mediated interference (RNAi) to decrease \textit{exc-7} expression (Elbashir et al., 2001; Montgomery and Fire, 1998; Sharp, 1999). We microinjected into adult hermaphrodites a PCR-amplified 200-bp fragment of the gene corresponding to the gene-specific hinge region, to ensure no interference with the function of other ELAV homologues or other RNA-binding proteins in \textit{C. elegans}. More than 70% of the first-generation progeny of injected animals exhibited cystic canals and bobbed tail spikes (Fig. 1G). The affected canals were shortened to between one-third and one-half the wild-type length, with multiple small septate cysts, and ended in a bolus of cysts and endosomes, indistinguishable from that of \textit{rh252} animals. These sequence and RNAi results suggest that both \textit{rh252} and \textit{ok370} are null alleles of \textit{exc-7}.

\textit{EXC-7} is weakly expressed in the excretory canal cell prior to maximal canal growth

Previous examination of wild-type animals expressing an \textit{EXC-7::GFP} fusion protein revealed widespread expression...
of EXC-7 throughout the nervous system, especially in the acetylcholine-producing cells of the nerve cord (Fujita et al., 1999). We found that this construct rescues the Exc mutant phenotype (Fig. 3A and B), and so encodes normally functioning EXC-7 protein. Surprisingly, the exc-7::gfp construct shows no visible expression in the excretory canals of adults or larvae (Fig. 3C). This lack of expression was unanticipated, since the first larval stage (L1) is when canal morphology deviates in the exc-7(rh252) mutant. We therefore examined expression of EXC-7 during embryogenesis. No expression was seen until shortly before the birth of the excretory cell, approximately midway through embryogenesis (270 min after first cell cleavage). At this point, four cells in the head showed expression of the fusion construct (Fig. 3D). Expression was seen in the excretory cell shortly after its birth (Fig. 3E). This expression was weaker than that seen in surrounding neural tissue, and continued for only about an hour, until the approximate time when internal organs such as the intestines and pharynx become clearly delineated by basement membranes. exc-7 expression in the canals completely disappeared before the 1.5-fold stage, approximately 420 min after first cell cleavage, still 6 h before hatching (Fig. 3F and G). We saw little or no expression in the developing hypodermis or intestine during the period of continuing morphogenesis, but strong expression throughout the developing pharynx and nerve ring and in two nerve cells of the preanal ganglion (Fig. 3G).

The time of exc-7 expression in the excretory canals is much earlier than the L1 stage, the point at which defects become visible in mutant animals. In order to understand the role of EXC-7 protein in canal development, we examined the formation of the excretory canals in wild-type larval animals (Fig. 4). The excretory cell is the largest cell in C. elegans. Its nucleus is located beneath the pharynx in larvae and adults (Chitwood and Chitwood, 1974; Nelson et al., 1983). Shortly after it is born in embryogenesis, the excretory cell sends out left and right processes that both branch and continue growing to extend the full length of the organism. We observed embryonic and larval canal growth by use of a GFP-labeled marker for the apical canal lumen, the proton ATPase VHA-1 (Oka et al., 2001). This marker is first strongly expressed in embryos at the two-fold stage, after exc-7 expression has ceased, basement membranes have formed, and tissue differentiation has begun. At this time, canal left–right extension is complete, and growth has begun in the anterior and posterior directions (Fig. 4A and B). Surprisingly, we found that the canals extend slowly during embryogenesis (Fig. 4C) and extend only halfway along the length of the animals at hatching, just past the V3 hypodermal seam cell (Fig. 4D). The canals continue to extend actively during the first larval stage (Fig. 4E and F), reaching their full length just past the V6 seam cell by the middle of L1. The canals remain at full length through three more larval stages and through adulthood, extending passively as the animals grow. The diameter of the lumen also gradually widens together with the diameter of the animal as it matures. In exc-7 mutants, the canals generally maintain a length halfway along the hypodermis as the animal grows into an adult. This observation implies that passive extension of the canals continues in growing mutants, even...
though active extension of the canals ceases at about the
time of hatching in exc mutants. Several other exc mutants
(exc-1, exc-3, exc-5, sma-1) and other mutants affecting
canal growth (e.g., unc-34, unc-53, and unc-73) also char-
acteristically contain half-length canals (Hedgecock et al.,
1987; Stringham et al., 2002). As the canals complete out-
growth during L1 while the animal continues to grow in
length, the greater part of canal apical surface construction
must occur during this stage.

We extended these observations on larval canal growth
through ultrastructural examination of the lumen of 10 wild-
type embryonic and early L1 animals (Fig. 5). Several hours
before hatching, but after exc-7 expression has been down-
regulated, a channel forms containing a lining of very elec-
tron-dense material within the lumen. There is little if any
electron-dense material evident on the cytoplasmic face of
the lumenal membrane at this time. The cytoplasm contains
large amounts of endoplasmic reticulum (ER) and Golgi
bodies (Fig. 5A and B). Later, at about the time of hatching,
the nature of the canal lumen changes (Fig. 5C). The ex-
cretory canal cell connects to the duct cell through which
lumenal material is expelled, and the hollow canals begin to
stretch anteriorward and posteriorward. At the ultrastruc-
tural level, the very dense material disappears from inside
the lumen, myriad thin membranous channels (“canaliculi”)
branching from the lumen appear, and thick electron-dense
material becomes prominent within the cytoplasm. The
thick material appears to be attached to the luminal mem-
brane via $\beta_{H^+}$-spectrin (Buechner et al., 1999) encoded by
sma-1 (McKeown et al., 1998). $\beta_{H^+}$-Spectrin binds the elec-
tron-dense terminal web to the apical epithelial surface of
many epithelia (Thomas and Kiehart, 1994). During the L1
stage, the amounts of ER and Golgi bodies diminish as the
membranous canaliculi become denser (Fig. 5D). These
observations indicate that the deposition of apical cytoskel-
eton along the length of the excretory canal cell luminal
surface is greatest from the time of hatching until late in L1,
the same period when defects in exc-7(rh252) become man-
ifest.

We examined the effects of the exc-7(rh252) mutation at
the ultrastructural level in light of the above observations.
Along the length of the canals, the cytoplasmic electron-dense terminal web material surrounding the lumenal surface is closely apposed to the membrane in both wild-type and exc-7 mutant animals (Fig. 6A). Electron micrographs of mutants at the canal termini (Fig. 6B and C) show widening of the canals and a large bolus of cysts at the termini of the shortened canals. In addition, the cytoplasmic electron-dense material is disrupted and this material is not evenly distributed on the lumenal surface. Within the bolus of cysts at the termini of the canals, the terminal web material is detached from the apical membrane (Fig. 6C). The detachment at these points is strikingly similar to the electron-microscopic phenotype of sma-1 mutants (Buechner et al., 1999). These results suggest that loss of EXC-7 might hinder the synthesis or deposition of SMA-1 spectrin, so that there is not enough SMA-1 spectrin present to coat the lumenal surface of the canals evenly, especially at the rapidly growing canal tips.

**Interactions of exc-7 with other genes**

The rh252 mutation shows synergistic effects with several other exc mutations. Null mutations in exc-3 cause moderate widening and meandering of the shortened canals. A homozygous exc-7;exc-3 double mutant, however, exhibits severely cystic short canals (Fig. 7A). Similarly, mutations in the sma-1 βH-spectrin gene cause the canals to be wide, unseptate, and shortened (Fig. 7B). Doubly mutant exc-7;sma-1(e30) homozygotes also exhibit synergistic canal defects, with extremely enlarged cysts in very short canals (Fig. 7C). In contrast, no synergistic effect was seen between exc-7 and another mild exc mutant, exc-6, or with...
the severely cystic mutants exc-1 and exc-5 (data not shown). The e30 allele of sma-1 is a hypomorph (McKeown et al., 1998). We therefore made double mutants between exc-7 and the null sma-1 allele ru18; surprisingly, this construct also showed synergistic effects. Upon hatching, these animals resembled typical sma-1 animals, with somewhat enlarged canals that had extended the normal distance along the hypodermis (Fig. 7D). But by the end of the first larval stage, the lumen had swollen to a single large cyst (Fig. 7E). The appearance of synergistic defects with a null sma-1 allele indicates that EXC-7 is used for at least one pathway complementary to that of βH-spectrin. Since ELAV proteins affect the stability of many genes involved in disparate cellular processes (Antic et al., 1999; Chagnovich and Cohn, 1996; Fan and Steitz, 1998; Jain et al., 1997; Quattrone et al., 2001; Wang et al., 2000), the appearance of synergistic defects between exc-7 and at least two genes suggests that EXC-7 activity affects multiple genes required for canal lumen formation.

We examined by biochemical means the possibility that the EXC-7 protein regulates sma-1 mRNA processing. ELAV proteins bind to AU-rich regions of 3' UTRs of mRNA, and in particular to the sequences AUUUA (Levine et al., 1993). Several of these target sequences are found in the exc-7 UTR of both C. elegans and the related nematode species C. briggsae (not shown). RNA sequence corresponding to the sma-1 3'UTR was bound in vitro both by a full-length GST–EXC-7 fusion protein and by a full-length fusion of GST to the mouse homologue HuD fusion protein, but not by GST alone (Fig. 8). In contrast, RNA complementary to the sma-1 3'UTR was not bound. These genetic and biochemical data indicate that EXC-7 binds to sma-1 mRNA.

Fig. 7. exc-7(rh252) interacts synergistically with other mutations. (A) exc-7(rh252);exc-3(rh207) double heterozygotes exhibit large cysts at the canal cell body (arrows). (B) sma-1(e30) mutants exhibit short, wide canals with no septations. (C) exc-7(rh252);sma-1(e30) double homozygotes show very large cysts at the excretory canal cell body (arrow). (C) Homozygous exc-7(ok370);sma-1(ru18) early L1 larval canal has normal length and diameter (arrowheads). Toward posterior tip of canal, cysts are beginning to form (arrow). (C) Canals in an older exc-7(ok370);sma-1(ru18) larva have extremely large cysts (arrows) surrounding the excretory cell body.

Fig. 8. EXC-7 binds to the sma-1 mRNA 3'UTR. UV-crosslinking experiment with GST–EXC-7 (lanes 1 and 2) and GST–mHuD (lane 3) fusion proteins. GST alone (lane 4) showed no RNA-binding activity. Each protein was incubated with either sma-1 3'UTR probe (lanes 1, 3, and 4), or complementary to that 3'UTR (lane 2). Arrows indicate expected positions of the indicated proteins.
Discussion

**EXC-7 regulates tubule development in C. elegans**

The *C. elegans* excretory canal cell provides a sensitive model for understanding development and regulation of tubulogenesis (Buechner, 2002). Although many of the molecules forming the epithelial cytoskeleton are known, the genetic processes controlling the rearrangements of these molecules are poorly understood. Formation and maintenance of narrow tubules is a particularly difficult cellular task, as the epithelial apical surface must form a concave curvature, often against a strong osmotic pressure (Saxén, 1987). In addition, the tubule must bend and enlarge as the creature moves and grows. These changes necessitate constant reformation of cytoskeletal structures at the luminal surface. Defects in the maintenance of tubular diameter cause the formation of fluid-filled cysts, as seen in the vertebrate polycystic kidney diseases (Bernstein, 1990; Van Adelsberg, 1999; Wilson and Burrow, 1999).

The Exc phenotype defines a series of gene products that determine apical surface structure in this single-celled tubular epithelium. \( \beta_{H}\) Spectrin plays a structural role on the cytoplasmic surface of the apical domain. *let-653* encodes a secreted mucin that may play a similar structural role on the luminal canal surface (Jones and Baillie, 1995). The guanine nucleotide exchange factor EXC-5 regulates the relative deposition of cytoplasmic material at the apical and basolateral surfaces in the excretory canals (Gao et al., 2001; Suzuki et al., 2001). We have shown here that the exc-7 gene, required for maintenance of the narrow diameter of the excretory canals during development, encodes the closest nematode homologue to the *Drosophila elav* and vertebrate Hu mRNA-binding proteins. The *rh252* mutation is rescued by injection of the predicted F35H8.5 gene, and the Exc-7 phenotype is mimicked by injection of F35H8.5 RNAi into wild-type worms. The ability of ELAV proteins to stabilize mRNA (Brennan and Steitz, 2001; Fan and Steitz, 1998; Ford et al., 1999; Jain et al., 1997; Peng et al., 1998) suggests that the structure of the apical surface of the canals is regulated at the translational level as well as posttranslationally.

The canals of *exc-7* mutants are generally only half-length, as are the canals in several other *exc* mutants and mutants affecting canal outgrowth (Buechner et al., 1999). A signal that attracts the canal tips to midbody has been proposed as a guidance cue for the canal (Stringham et al., 1998). We have observed, however, that in late embryogenesis, active osmotic regulation commences, as evidenced by the rhythmic filling and emptying of the excretory duct (Nelson and Riddle, 1984), and by the formation of canal cysts beginning at this time in several *exc* mutants. Since wild-type canals extend only to midbody at the time of hatching, half-length canals in *exc* mutants might reflect an inability of the canal to continue outgrowth once luminal swelling has damaged the canals, rather than a failure to express gene products regulating extension past the gonad. *rh252* and *ok370* are both likely null alleles, since they delete large regions of the protein essential for the function of ELAV proteins both in *Drosophila* (Lisbin et al., 2000) and vertebrates (Kasashima et al., 1999). It is surprising, however, that the null phenotype of this gene only shows effects in the canals and tailspike, and even in those tissues, the effects are subtle. Although *exc-7* is also expressed widely throughout the *C. elegans* ring ganglion and nerve cord (Fujita et al., 1999), mutation of this gene results in no obvious behavioral defects. In *Drosophila*, *elav* is expressed in all neurons, and absence of ELAV causes lethal widespread malformation of the nervous system (Campos et al., 1985). EXC-7 is clearly the closest homologue to ELAV in *C. elegans*, but the worm genome contains several other mRNA-binding proteins with the same basic organization of an N-terminal domain, two RRM, hinge region, and a third RRM. For example, the ELAV-like protein ETR-1 is necessary for muscle development (Milne and Hodgkin, 1999). EXC-7 is expressed only in a subset of *C. elegans* neurons, however, so many neurons must develop and function normally without the presence of EXC-7. If an ELAV-like function is required in all *C. elegans* neurons, this function must be duplicated by one of the other mRNA-binding proteins. Nematodes mutated for several of these genes might exhibit similar severe neurological defects as do *Drosophila elav* mutants.

**EXC-7 subtly regulates developmental genetic expression**

EXC-7 binds to the 3’UTR of the *sma-1* mRNA encoding \( \beta_{H}\)-spectrin, and the time of *exc-7* expression in the excretory cell corresponds to that seen for *sma-1* (McKown et al., 1998). The defects in the *exc-7* mutant canal tips closely resemble defects evident throughout the canals in *sma-1(e30)* animals: The electron-dense cytoplasmic actin-based terminal web material is dissociated from the luminal membrane, as though \( \beta_{H}\)-spectrin has not been properly deposited at the apical surface (Bennett and Gilligan, 1993). The electron micrographs also show, however, that the spectrin-based terminal web is largely intact throughout much of the canal length. It is not surprising that the strongest effects of the *rh252* mutation occur at the growing tips of the excretory cell, even though EXC-7::GFP expression is predominantly visible in the nucleus. Vertebrate ELAV is similarly found in the nucleus, but shuttles mRNA to the cytoplasm through interaction with nuclear export proteins (Brennan et al., 2000; Gallouzi et al., 2001; Keene, 1999; Peng et al., 1998; Yannoni and White, 1999). Human ELAV homologues bind to microtubule-associated mRNA complexes (Antic and Keene, 1998). Since the canals contain ribosomes, ER, Golgi, and microtubules throughout their length, we conclude that in the *C. elegans* excretory cell, EXC-7 either increases *sma-1* mRNA expression, or more
efficiently shuttles *sma-1* mRNA to microtubule-associated complexes for transport to distant sites of translation.

*exc-7* is visibly but weakly expressed almost as soon as the excretory cell is born and begins to differentiate, similar to the period of ELAV expression in differentiating neurons (Robinow and White, 1988). Excretory cell expression is then downregulated several hours before embryogenesis is complete. The *exc-7* null mutant shows no phenotypic effects until the time of L1 larval development, however, when the canals extend relative to the rapidly-growing hypodermis (Fig. 4). The canal diameter also increases greatly during this stage, and the luminal dense material disappears at this time, to be replaced by the cytoplasmic spectrin-anchored terminal web (Fig. 5). Spectrin and other apical cytoskeletal components are therefore needed more during L1 than at any other period of development. Even in *sma-1 (ru18)* null mutants, the canals do not develop gross swellings of the lumen until this stage (Fig. 7). The presence of wild-type EXC-7 protein therefore allows mRNAs to be translated at later times than can occur in *exc-7* mutants.

The increased defects in canal structure seen when *exc-7* is mutated in a *sma-1* null background indicate that the loss of *EXC-7* function must affect other genes besides *sma-1*. Since *exc-7* mutations also show similar synergistic effects with *exc-3* mutations, *EXC-7* may also affect expression of this (as yet uncloned) gene. Since the observed defects of *exc-7* mutation are relatively mild, and the gene is expressed in many cells besides the excretory cell, *EXC-7* likely affects expression of additional genes, as do the vertebrate ELAVs (Antic et al., 1999; Chagnovich and Cohn, 1996; Fan and Steitz, 1998; Jain et al., 1997; Quattrone et al., 2001; Wang et al., 2000). Further studies on other genes causing synergistic effects with ELAV homologue mutations may identify other genes needed in large amounts during specific stages of *C. elegans* development that are regulated by *EXC-7* or other of the nine nematode ELAV homologues.

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