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Characterization of the *let-653* gene in *Caenorhabditis elegans*

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Abstract A mutation in the *let-653* gene of *Caenorhabditis elegans* results in larval death. The lethal arrest is concurrent with the appearance of a vacuole anterior to the lower pharyngeal bulb. The position of the vacuole is consistent with a dysfunction of the secretory/excretory apparatus. Germline transformation rescue experiments were able to position the *let-653* gene to two overlapping cosmid subclones. Sequence data generated from both cDNA and genomic DNA subclones indicated that *let-653* encodes a mucin-like protein. Our characterization suggests that a mucin-like protein is essential for effective functioning of the secretory/excretory apparatus within *C. elegans*.

Key words *Caenorhabditis elegans* · *let-653* · Mucin · Excretory/secretory apparatus

Introduction

Mucins are normally secreted as mucus, the role of which is believed to be to provide a protective barrier between living cells and their environment. The high molecular weight mucin glycoproteins have been widely studied in both mammals and amphibians (for a review see Strous and Dekkler 1992). By forming highly viscous, aqueous solutions mucin proteins protect epithelial cells from physical damage, dehydration and infection.

The protective action of mucin protein is a function of its viscous rheological properties. Mucins acquire these properties by undergoing extensive glycosylation. The steric interactions of the clustered O-linked oligosaccharides with the protein backbone provide a stiff and highly extended confirmation. The extensive glycosylation of mucin proteins can result in over two-thirds of the dry weight of the protein consisting of carbohydrate chains. The rheological properties of mucins are also determined to a great extent by their ability to form intermolecular disulfide bridges resulting in oligometric forms of mucin.

Mucins have been implicated in the pathology of a number of diseases. In cystic fibrosis the rheological properties of mucin change as it undergoes an increased level of glycosylation (Cheng et al. 1989). The link between the defective Cl^- transport activity in cystic fibrosis and this increase in glycosylation is, however, not understood (Anderson et al. 1991).

Mucin proteins have also been implicated in the metastatic potential of carcinoma cells. In many human carcinomas production of the mucin protein MUC-1 is increased and the glycosylation pattern is altered (Zotter et al. 1988). In this case the protective coating properties of mucin are seemingly reversed, as the aberrant expression of the mucin prevents the detection of the cancer cells by interfering with immune surveillance by T lymphocytes.

Mucins exhibit little sequence similarity at the DNA and amino acid level but share a number of characteristic features. These features include a central threonine- and serine-rich region, often consisting of tandemly arranged repeats, which is subject to heavy O-glycosylation. The regions flanking the threonine/serine-rich region are cysteine rich and are subject to N-glycosylation.

The existence of mucin-like proteins in the free-living soil nematode *Caenorhabditis elegans* has not previously been demonstrated. However the sinuses of the secretory/excretory tract, which are in contact with the outside environment, represent structures that could

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require the protective function of mucin-like glycoproteins. Little is currently understood about the function of the *C. elegans* excretory/secretory apparatus. This apparatus consists of a binucleate gland cell and a large excretory cell, which is connected to the outside via the excretory duct cell and excretory pore cell. The gland cell has been shown to possess secretory clusters of many kinds (Nelson et al. 1983) although no secretory products of this cell have as yet been identified. Functions that have been proposed for the excretory/secretory systems include the secretion of peptidases involved in molting (Singh and Sulston 1978), and the excretion of metabolic waste, and osmoregulation (Weinstein 1952; Croll et al. 1972; Nelson and Riddle 1984). In the last case, the internal hydrostatic pressure and osmotic potential of the nematode is proposed to be regulated by the flow of fluid through the excretory/secretory sinuses.

The inference that the secretory/excretory apparatus has a role in osmoregulation is supported by laser ablation experiments conducted by Nelson and Riddle (1984). In these experiments it was shown that the destruction of the excretory cell, its pore cell or its duct cell resulted in the animals becoming bloated with fluid, and eventually dying. Laser ablation of the gland cell, however, seemed to cause no obvious developmental or behavioral defects.

In this report, we present a characterization of the *let-653* gene of *C. elegans*. We describe the phenotype resulting from a mutation in *let-653*, and present the sequence of the gene. Our analysis has shown that *let-653* encodes a protein with mucin-like features and we also provide evidence that a mutation in the *let-653* gene causes dysfunction within the excretory/secretory system, ultimately resulting in death.

Materials and methods

Genetics

The *C. elegans* strains were maintained on petri plates containing nematode growth medium streaked with *Escherichia coli* strain OP50 (Brenner 1974). Mutations used were *unc-31(e169)* (Brenner 1974) *unc-22(s7)* (Moerman and Baillie 1979) and *let-653* (*s1733*) (Clark and Baillie 1992). Lethal mutation-bearing chromosomes were balanced over the reciprocal translocation *nT1(IV;V)* (Ferguson and Horvitz 1985).

Microscopy

Arrested larvae were photographed using Nomarski optics as described in Sulston and Horvitz (1977). Worms were mounted on 5% agar pads containing 10 µl S buffer (Brenner 1974). Photographs were taken using Kodak technical Pan film at ASA 32 on an Olympus AHBS3 microscope. To prevent *unc-22(s7)* homozygotes from twitching during photography 1 µl of 10 mM sodium azide solution was added to the mounted specimen.

Germline transformation

Cosmid clones used in this study were obtained from A. R. Coulson (Coulson et al. 1986) at the MRC Laboratory of Molecular Biology, Cambridge, England. Transformation experiments were performed using an inverted Zeiss microscope with Nomarski optics. DNA was injected into the gonadal syncytium as described by Mello et al. (1991). DNA preparations for germline transformation were mixed with the plasmid pRF4 containing the dominant Roller allele *rol-6(su1006)* (Kramer et al. 1990). The Roller gene acted as a marker for transformation. Cosmid DNA was isolated by the alkaline lysis procedure (Sambrook et al. 1989) and purified by CsCl density centrifugation. Plasmid DNA for germline transformation was prepared by the alkaline lysis miniprep method outlined in Sambrook et al. (1989) omitting the phenol/chloroform purification step. No further purification was found to be necessary, apart from centrifugation of the DNA solution at 13 000 rpm for 20 min prior to use, to pellet cellular debris. DNA for germline transformation was suspended in TE buffer (10 mM TRIS-HCl, 1 mM EDTA pH 8).

General molecular biology methods

Restriction enzyme digestions were carried out under conditions recommended by the enzyme manufacturers (Pharmacia or BRL). Subcloning was carried out by a shotgun method. Vector and target DNA were ligated together as outlined by Snutch (1984). The vector used was pBluescript I (SK$^-$) (Stratagene). Completed ligation reactions were transformed into the *E. coli* host DH5α (Hanahan 1983). Probes were ^{32}P labeled by random priming (Feinberg and Vogelstein 1983). A partial restriction map of C46F3 was generated by determining overlapping *Eco*RI and *Pst*I subclones from C46F3 using Southern hybridization. Southern hybridization was also used to determine the overlap between B0033 and C46F3.

DNA sequencing was carried out using an Applied Biosystems (ABI) Model 373A automated sequencing machine utilizing dideoxynucleotide chain termination chemistry. Protocols and reagents were supplied by ABI. cDNA clones used for sequencing were isolated from a lambda ZAP library (Stratagene) constructed by Barstead and Waterston (1989). Exonuclease III-generated deletions (Henikoff 1987) of genomic or cDNA clones for sequencing were prepared using the Erase-a-Base system (Promega).

DNA and protein sequence analysis

Nucleotide sequences were aligned, formatted and translated using the Eyeball Sequence Editor (ESEE) program (Cabot and Beckenbach 1989). Amino acid sequences predicted using ESEE were used to search entries in the SwissProt and PIR databases using the BLAST algorithm (Altschul et al. 1990). Computations were performed at the National Centre for Biotechnology Information (NCBI) using the BLAST network service. Hydropathy plots were calculated using the algorithm of Kyte and Doolittle using the program GREASE (Pearson and Lipman 1988). Secretory signal detection was carried out using the method of von Heijne (1986) incorporated into the EGCG package (Peter Rice, personal communication).

Results

The *let-653* phenotype

The larval stage at which *let-653* (*s1733*) homozygotes underwent lethal arrest was estimated to be late L1 or early L2. This result was determined during a study of 13 *let-653(s1733)* homozygotes, 5 of which were ob-

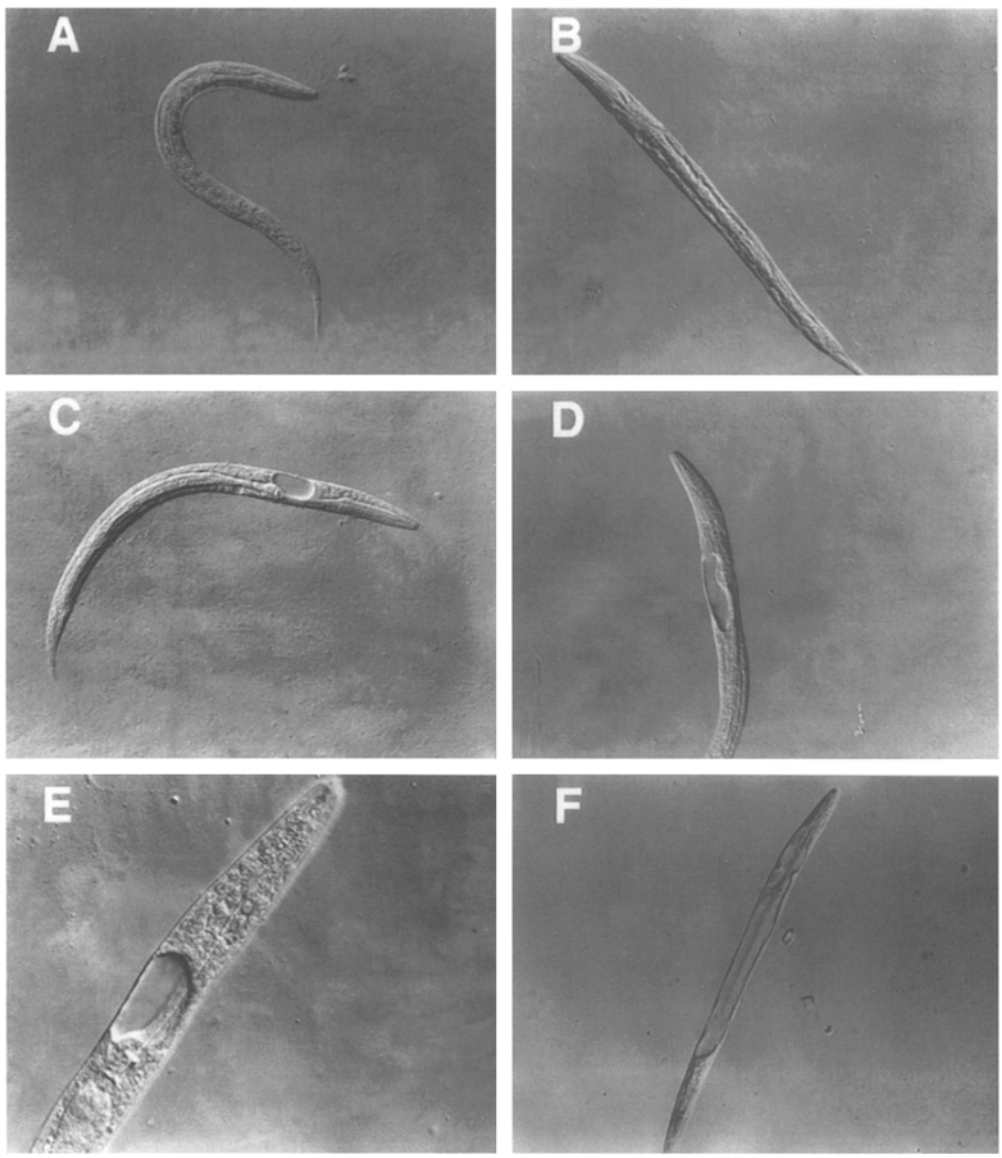


Fig. 1A–F Nomarski photographs of the Let-653 phenotype. All photographs were taken at 20° C. The *let-653(s1733)* homozygotes shown were derived from strain BC3261. **A** Wild-type L1 larva. **B** A *let-653(s1733)* larva with vacuole just beginning to form. **C, D** *let-653(s1733)* larvae with progressively larger vacuoles. **E** Close-up of a vacuole from a *let-653(s1733)* individual. The presence of the vacuole can be seen to cause displacement of the pharynx against the body wall. **F** A *let-653(s1733)* larva in which the vacuole has extended along almost the entire length of the body

served to undergo an L1 molt. Growth prior to the lethal arrest was found to be retarded when the larval size of homozygotes was compared with that of the background phenotype *Unc-22 Unc-31* as determined by Clark (1990) (data not shown). Coincident with the lethal arrest is the appearance of a vacuole slightly anterior to the lower pharyngeal bulb (Fig. 1). It should be noted that the phenotype of *let-653* was not examined in the absence of the linked mutations *unc-22* and *unc-31*, either of which could have a synergistic or suppressive effect on the phenotype.

Correlation of *let-653* with the physical map

Previously the transient rescue of *let-653(s1733)* by cosmid C29E6 had been reported (Clark and Baillie 1992). This rescue was confirmed by the rescue of *let-653(s1733)* with a stable extrachromosomal array containing cosmid C46F3, which overlaps extensively with C29E6 (Coulson et al. 1986). Cosmid B0033 was unable to rescue *let-653(s1733)*. A restriction map of the *let-653* candidate region was constructed (Fig. 2) and the plasmid subclones generated were used to rescue *let-653(s1733)*. A summary of the plasmid rescue experiments is shown in Table 1. Results from the plasmid rescue experiments show that rescue of *let-653* could only be achieved by extrachromosomal arrays that contained either plasmid subclones pCes1906, pCes-1940 and pCes1907 (*sEx41* and *sEx42*), or by extrachromosomal arrays that contained plasmid subclones pCes1940, pCes1906 and pCes1903 (*sEx47*). Extrachromosomal arrays containing only pCes1906 and pCes1940 (*sEx43*,

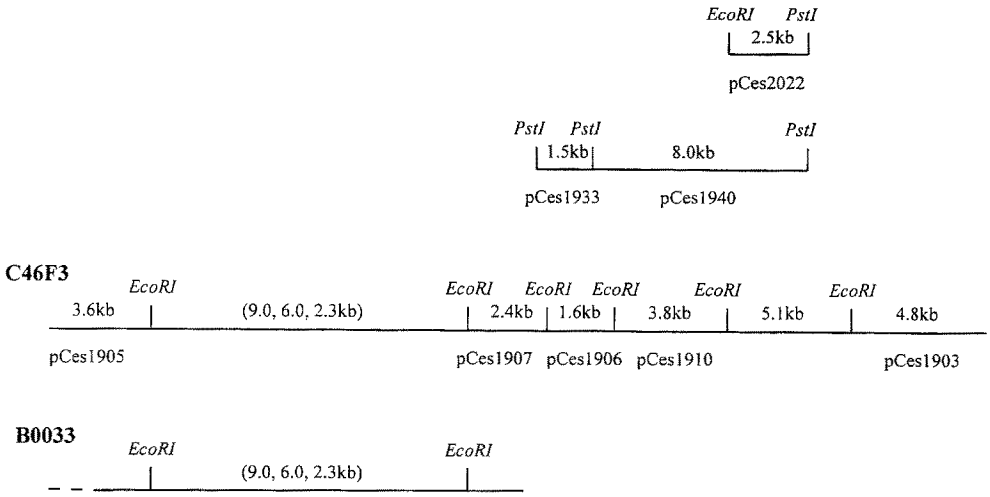


Fig. 2 A restriction map of the *let-653* candidate region. This map also shows the extent of overlap between cosmids B0033 and C46F3. *Parentheses* indicate *Eco*RI fragments that have not been ordered with respect to each other. Plasmid subclones are designated with the prefix pCes

Table 1 Summary of germline transformation rescue results. Construction of extrachromosomal arrays (denoted *sEx*) for the rescue of *let-653*. The strain used for germline transformation was *let-653(s1733) unc-22(s7) unc-31(e169)*; + /*nT1(V)* (*BC3261*). Rescue was achieved when fertile Unc-22 Unc-31 adults were obtained

Array	Plasmid	pCes concentration (ng/ml)	pRF4 concentration (ng/ml)	Strain	Rescue of *let-653*
sEx21	pCes1903	5.0	100	BC4273	Yes
	pCes1906	5.0			
	pCes1907	5.0			
	pCes1910	5.0			
	pCes1940	1.25			
sEx22	pCes1940	7.5	100	BC4423	No
sEx23	pCes1903	10	100	BC4424	No
sEx24	pCes1910	5.0	100	BC4429	No
	pCes1907	5.0			
sEx27	pCes1906	10	100	BC4539	No
sEx43, 44, 45	pCes1906	60	25	BC4591	No
	pCes1940	15		BC4590	
				BC4593	
sEx46	pCes1940	15	65	BC4595	No
	pCes1907	20			
sEx48	pCes1906	30	50	BC4596	No
	pCes1907	20			
sEx41, 42	pCes1906	30	40	BC4594	Yes
	pCes1940	15		BC4592	
	pCes1907	20			
sEx47	pCes1906	30	40	BC4597	Yes
	pCes1940	15			
	pCes1903	20			

sEx44 and *sEx45*) were unable to rescue *let-653* (*s1733*).

Isolation of a *let-653* cDNA

The requirement for both pCes1906 and pCs1940 to rescue *let-653(s1733)* suggested that these subclones would most probably contain the coding element of the *let-653* gene. As pCes1906 and pCes1940 represented overlapping subclones it was likely that the *let-653* gene spanned the right end of pCes1906 and the left end of pCes1940. An intact *let-653* coding element could then be reconstituted through homologous recombination between the two plasmids during the construction of the extrachromosomal array. The subclone pCes1910 (Fig. 2) was used as a probe to screen a mixed stage *C. elegans* lambda ZAP cDNA library. Approximately 25 000 phase were screened. One positive 3.4 kb clone designated pCes1942 was isolated. pCes1942 was found to hybridize to both pCes1906 and pCes1910 (data not shown).

Function of pCes1903 and pCes1907

In order to achieve rescue of *let-653(s1733)* using plasmids, the presence of either pCes1903 or pCes1907 was required within the extrachromosomal array. Neither pCes1903 nor pCes1907 show any overlap with either pCes1906 or pCes1940 and so are unlikely to recombine with their inserted sequences. This inference is supported by the fact that Southern hybridization experiments carried out during the construction of the restriction map for this region failed to detect any evidence of repeated sequences shared between these clones (data not shown). Therefore, the action of pCes1903 or pCes1907 is likely to be positionally independent of the sequences of pCes1906 and pCes1940 within the extrachromosomal array. This fact, and the ability of either pCes1903 or pCes1907 to allow the rescue of *let-653*, makes it unlikely that these contain any of the coding elements of *let-653*. The action of both pCes1903 and pCes1907 is consistent with that of transcriptional control or elements required for the appropriate expression of the *let-653* gene.

The *let-653* gene

Sequence data were generated from the cDNA clones and genomic subclones obtained from cosmid C46F3 (Fig. 3). The cDNA was found not to represent a full-length transcript. Thus the initial exon and intron were predicted from the genomic sequence. The *let-653* gene was found to contain ten exons and nine introns. The 5′ splice site of intron 6 contains GC in place of the usual GT (Blumenthal and Thomas 1988). This variation on the consensus has been reported many times previously (Jackson 1991); however, this is the first time that it has been reported in *C. elegans*.

The predicted LET-653 protein was found to be 694 amino acids in length. An unmodified protein product would have a predicted weight of 76 965 Da. The LET-653 protein was found to possess a number of characteristics consistent with its being a mucin-like protein. (i) The LET-653 protein was found to possess a threonine/serine-rich domain (exon 7), which comprises 46% threonine and 13% serine. (ii) The threonine/serine-rich domain (exon 7) is also relatively rich in proline (10%), a feature of mucins that is believed to induce β-turn conformations allowing the close packing of O-linked oligosaccharides (Strous and Dekker 1992). (iii) Flanking the threonine/serine-rich region are two cysteine-rich regions, which contain six putative N-glycosylation sites. (iv) As in other mucins none of the predicted N-glycosylation sites were found to be present within the threonine/serine-rich region. (v) The hydropathy profile of LET-653 protein revealed a hydrophobic amino-terminus, which suggests that the LET-653 protein is, like many mucin proteins, secreted (Fig. 4) (Blobel and Dobberstein 1975). The amino-terminus was also predicted to contain a secretory signal using the method of von Heijne (1986) (Fig. 3).

The LET-653 protein did not show any significant homology at the sequence level to any previously determined proteins including mucin proteins. This lack of homology is not unexpected, however, as mucins characteristically do not share sequence similarity at the amino acid level.

Unlike the case in other mucin proteins, the threonine/serine-rich region of LET-653 does not consist of tandem repeats. There exist other examples of mucin-like proteins in which the threonine/serine-rich region is not made up of well conserved tandem repeats. The canine tracheobronchial mucin (Verma and Davidson 1993) and the integumentary mucin C.1 (FIM-C.1) of *Xenopus laevis* (Hauser and Hoffmann 1992) both possess threonine/serine-rich regions that do not consist of conserved tandem repeats.

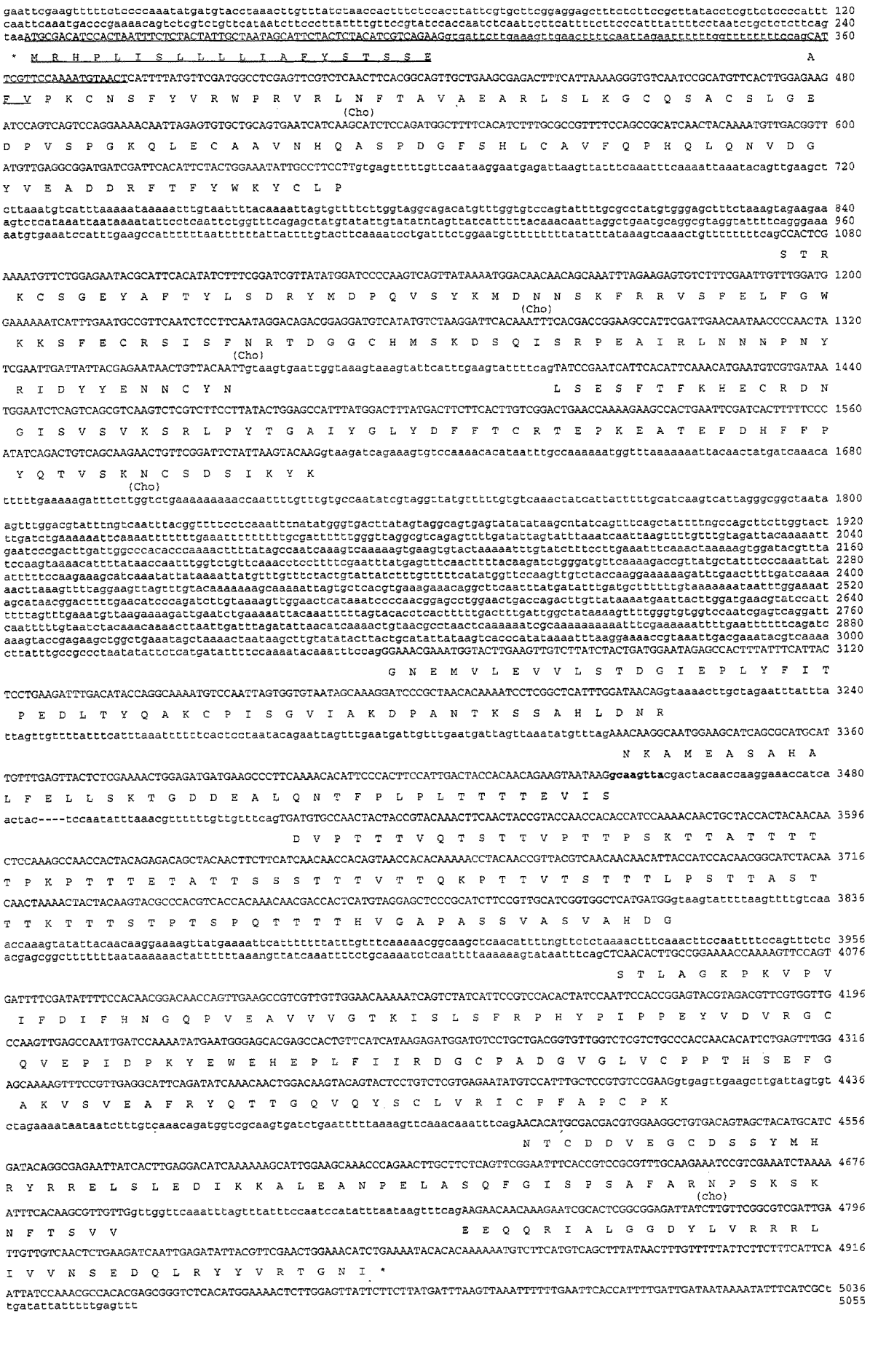
Discussion

In this paper we report the cloning and sequencing of the *let-653* gene. The predicted protein LET-653 possesses features similar to those possessed by members of the mucin glycoprotein family.

We have shown by germline transformation experiments that cosmid C46F3 contains the *let-653* gene. In addition, restriction enzyme fragments were used to rescue *let-653*. These subclones were then used to identify a cDNA clone that encoded the *let-653* gene transcript. Furthermore, we note that the *let-653* gene contains an intron splice site of a type not previously reported in *C. elegans* beginning with GC instead of the usual GT. We also report that mutations in the *let-653* gene appear to affect the operation of the secretory/excretory apparatus.

Phenotype of *let-653*

The *let-653(s1733)* phenotype is a L1/L2 lethal arrest coincident with the appearance of a vacuole slightly anterior to the lower pharyngeal bulb. The presence of this vacuole is suggestive of a dysfunction or malformation of the excretory/secretory apparatus (Matthew Buechner, personal communication). Such an inference is corroborated by the fact that the phenotype of the *let-653* gene can be phenocopied by laser ablation of the excretory/secretory apparatus. In experiments conducted by Nelson and Riddle (1984), laser ablation of the duct cell caused fluid accumulation and a swelling of the excretory cell, followed by large zones of the fluid filling the hypodermis. These defects are similar to the phenotype of *let-653(s1733)* homozygotes. Further evidence to suggest a dysfunction of the secretory/



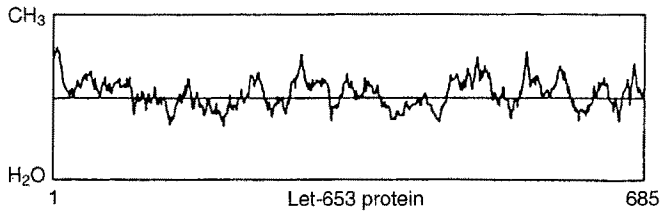


Fig. 4 Hydropathy profile of the predicted LET-653 protein

excretory apparatus in *let-653* mutants is provided by *lag-1*, *lag-2* and *lin-12:glp-1* mutants in which the excretory cell and ducts are absent (Lambie and Kimble 1991) and fluid accumulation is observed at the normal location of the excretory pore.

A model for *let-653* function

To propose a function for the LET-653 protein would be speculative at this point. However, some correlation between the protein and phenotype can be made. Mucins are secreted in order to protect regions where cells come into contact with the outside environment. The excretory duct and excretory cell represent structures where the protective properties of mucin could be beneficial. The presence of the mucin-like LET-653 protein within the secretory/excretory apparatus could provide a protective coating for the excretory sinuses and allow their normal structure to be maintained.

The control of *let-653* expression

The rescue of *let-653* required either pCes1907 or pCes1903 plasmid sequences to be present within the extrachromosomal array in addition to pCes1940 and pCes1906. Two different models explaining the requirement for these sequences can be proposed. In the first model, pCes1907 and pCes1903 both possess transcriptional control elements with an interchangeable or redundant function. Such a prediction is supported from the work of Okkema et al. (1993). In that work a screen was conducted for enhancers of the *C. elegans myo-2* gene. In all, 18 DNA fragments randomly derived from the genome were studied, representing a total of 62 kb of genomic DNA. Of the 18 fragments tested six were able to function as enhancers and were able to allow expression within muscle cells. However, when these enhancers were used to express *glp-1*, gene expression in non-muscle cells was observed. These results indicate that enhancer sequences are relatively abundant in genomic DNA and that much of the specificity of expression remains at the promoter. Thus we can propose that sequences from pCes1907 or pCes1903 are able to act as enhancers of the *let-653* gene, although they need not necessarily play a role in the wild-type expression of the *let-653* gene.

A second model for the action of pCes1907 and pCes1903 would require that they contain transcriptional promoter sequences. These promoter sequences within the extrachromosomal array would then produce a transcript encompassing the *let-653*˙ gene. A functional *let-653* mRNA could then be produced if the putative *trans*-splice acceptor site immediately upstream of the proposed *let-653* initiating methionine (Fig. 3) functioned as an SL2 spliced leader acceptor site (Speith et al. 1993). Such a model would predict that the *let-653* gene would normally be transcribed as part of a polycistronic mRNA. There does remain, however, the possibility that the true *let-653* gene lies within the 2.5 kb region of pCes1940 equivalent of pCes2022 and requires the presence of both pCes1906 and either pCes1907 or pCes1903 for appropriate expression. We consider this unlikely owing to the small size of this region and the fact that the left breakpoint of the deficiency *sdf9* has been found to lie within this 2.5 kb region (Marco Marra, unpublished results). The deficiency *sdf9* does not delete the *let-653* gene (Clark and Baillie 1992).

The *let-653* gene possesses a variant 5′ intron splice site

Of the 5′ variant intron splice sites previously reported in other organisms almost all have the GT dinucleotide at the first two bases of the intron replaced by GC. In vitro studies have shown that this substitution is the only one at this position that will allow the 5′ site to continue to be accurately cleaved, although these aberrant 5′ intron splice sites have been found to be processed more slowly than sequences possessing GT (Aebi et al. 1987). Previously in other organisms possessing GC instead of GT at this position the rest of the intron splice site shows an above average match to the consensus. In this *C. elegans* splice site this is also found to be true. The rest of this intron shows the best possible agreement with the *C. elegans* 5′ splice site consensus (Blumenthal and Thomas 1988) and this may in some way serve to counter the effects of the base substitution at the second position.

←

Fig. 3 The genomic sequence of the *let-653* coding region. The beginning of this sequence corresponds to the beginning of plasmid clone pCes 1906. The 3′ untranslated region of the *let-653* transcript extends 33 bp into clone pCes2022. Exons are denoted in *uppercase letters*. Introns are denoted in *lowercase letters*. The predicted 5′ exon and intron sequences are *underlined*. Putative *N*-glycosylation sites are denoted with (cho). The stop codon is represented by an *asterisk*. *Dashes* (–) represent gaps in the intron sequence data. Undetermined nucleotides are designated by 'n'. The 5′ intron splice site that does not conform to the *Caenorhabditis elegans* intron splice site consensus is shown in *bold type*. The secretory signal peptide predicted by the method of von Heijne (1986) is shown *underlined*. The *let-653* gene sequence is available under the EMBL accession number X91045

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