Characterization and developmental expression patterns of the ubiquitin-conjugating enzyme UBC-2 in the nematode *Caenorhabditis elegans*

by

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ABSTRACT

The role of the essential ubiquitin-conjugating enzyme, UBC-2, during development in the nematode *Caenorhabditis elegans* was examined. The mutant strain, *let-70*, which corresponded to *ubc-2*, was L3 larval lethal, and indicated that UBC-2 was required post-embryonically. Additionally, a maternal contribution of UBC-2 was necessary for embryonic development. Detailed analysis of *let-70(s1132)* and *let-70(s689)* mutant phenotypes revealed defects in muscle cell positioning, attachment and sarcomere assembly. Intestinal cell attachment and maturation were also affected. A small number of *let-70(s689)* mutants developed into sterile adults with gonadal defects. In these mutants, the somatic gonad and vulva did not develop properly, only a small number of germ cells were produced, and oocytes and spermatocytes failed to mature.

UBC-2 protein was undetectable in *let-70(s1132)* and *let-70(s689)* mutants. The *let-70(s689)* mutation altered the splice donor site of the fourth intron. In affected animals, the fourth intron was not removed from the *ubc-2* pre-mRNA, and the transcript was subject to *smg*-mediated mRNA surveillance. The mutant UBC-2 protein produced in *let-70(s1132)* appeared to be highly unstable.

The *let-x(s2293)* strain was identified in a non-complementation screen for additional alleles of *let-70* and was a large deletion that spanned several essential genes, including *ubc-2*.

The UBC-2 expression pattern during development was determined by immunofluorescence staining and examination of transgenic animals carrying a UBC-2::GFP fusion construct. UBC-2 expression was largely tissue general in embryonic stages. Post-embryonically, UBC-2 remained tissue general, although a large amount of protein became concentrated in the nucleoli of a number of cells, including intestinal, body wall muscle, hypodermal, somatic gonad, germline, pharyngeal muscle, pharyngeal-intestinal valve and several neurons. In addition, UBC-2 localized to the dense bodies or M-lines of body wall muscle, and a few neuronal cell processes. In *let-70* mutants, this expression pattern was lost.

Somatic and germline expression of an extrachromosomal array that contained *ubc-2* could rescue *let-70(s689)*, but not *let-70(s1132)* mutants. A cold-sensitive allele of *ubc-2* was constructed which was also capable of rescuing *let-70(s689)* mutants in a temperature-dependent manner.

ubc-2 forms part of a polycistronic unit with two other genes, *apg-7* and Y5F2A.4. Both *ubc-2* and Y5F2A.4 are exclusively SL1-spliced while *apg-7* is SL1- and SL2-spliced.

Comparison of the *ubc-2* polypeptide sequence between *C. elegans* and *C. briggsae* revealed 100% identity; however, *C. briggsae* UBC-2 was unable to rescue *let-70* mutants. A high degree of synteny exists between the genomic sequences surrounding the *C. elegans* and *C. briggsae ubc-2* genes, with the exception of sequences immediately surrounding the *ubc-2* operon.

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LIST OF ABBREVIATIONS

Å	angstrom
APC	adenomatous polyposis coli
APC/C	anaphase promoting complex/cyclosome
apr	APC-related
Arm	armadillo
ATP	adenosine triphosphate
β-cat	β-catenin
β-TrCP	β-transducin repeat-containing protein
BLAST	basic local alignment search tool
bp	base pairs
Cdc	cell division cycle
CDK	cyclin-dependent kinase
cDNA	complementary deoxyribonucleic acid
deb	dense body component
DNA	deoxyribonucleic acid
dpy	dumpy
Dsh	disheveled
DTT	dithiothreitol
E1	ubiquitin-activating enzyme
E2	ubiquitin-conjugating enzyme
E3	ubiquitin ligase
ECL	enhanced chemiluminescence
ECM	extracellular matrix
EGF	epidermal growth factor
ER	endoplasmic reticulum
F1	first filial generation
F2	second filial generation
FAC	focal adhesion complex
FAK	focal adhesion kinase
FGF	fibroblast growth factor
Fz	frizzled

ges	gut esterase
GFP	green fluorescent protein
gon	gonad development abnormal
GSK-3β	glycogen synthase kinase-3β
hlh	helix-loop-helix
HM	silent mating type loci
IκB	inhibitor of NF-κB
ILK	integrin-linked kinase
kb	kilobase
kDa	kiloDalton
let	lethal
lev	levamisole resistant
lin	abnormal cell lineage
MAPK	mitogen activated protein kinase
ml	milliliter
mm	millimeter
mM	millimolar
mom	more of MS
mRNA	messenger ribonucleic acid
mu	map unit
mua	muscle-attachment defective
mup	muscle-positioning defective
Net	nucleolar silencing establishing factor and telophase regulator
NFκB	nuclear factor κB
NLS	nuclear localization signal
NMD	nonsense-mediated mRNA decay
OD	optical density
PAGE	polyacrylamide gel electrophoresis
pat	paralyzed, arrested elongation at two-fold
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PKC	protein kinase C

pop	posterior pharynx-defective
Rad	radiation sensitivity abnormal
RENT	regulator of nucleolar silencing and telophase
rol	roller
rpm	revolutions per minute
SCF	Skp1-Cdc53/CUL1-F-box protein
SDS	sodium dodecyl sulfate
SL	spliced leader
Slimb	supernumerary limbs
smg	suppressor affecting message stability
Tris	tris(hydroxymethyl)aminomethane
UBC	ubiquitin-conjugating enzyme
Ubp	ubiquitin protease
UCH	ubiquitin C-terminal hydrolase
µg	microgram
µl	microliter
unc	uncoordianted movement
UV	ultraviolet
Wg	wingless
wrm	worm arm motif
YAC	yeast artificial chromosome

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This thesis is dedicated to the memory of my father, Raymond Douglas Stevens, who passed away while this work was in progress. All the love and caring you gave to me will remain in my heart forever.

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However far you go, you will
never find the boundaries of the soul.
Heraclitus

I. INTRODUCTION

1. UBIQUITIN-MEDIATED PROTEIN DEGRADATION-AN OVERVIEW

Ubiquitin-mediated protein degradation is believed to be the major non-lysosomal proteolytic system in eukaryotic cells. Ciechanover *et al.* (1978) showed that intracellular protein degradation was an ATP-dependent process that required a co-factor, which was later determined to be ubiquitin (Wilkinson *et al.*, 1980). This process involves the attachment of ubiquitin to a specific protein, with the attached ubiquitin then serving as a signal for degradation. Target protein recognition and the attachment of ubiquitin is controlled by a multi-step enzymatic pathway that includes a ubiquitin-activating enzyme, a member of the ubiquitin-conjugating enzyme family and one or more ubiquitin protein ligases. A multiubiquitin chain is formed on the protein and is recognized by a large multisubunit protease called the 26S proteasome which degrades the target protein and releases peptides and free ubiquitin.

2. THE UBIQUITIN SYSTEM

2.1 Ubiquitin

Ubiquitin is a small 76 amino acid protein that is present in all eukaryotic cells. It is considered to be one of the most conserved proteins known in eukaryotes, with yeast and human ubiquitin differing by only three conservative amino acid substitutions (Özkaynak *et al.*, 1984). Such a high degree of evolutionary conservation indicates that the entire ubiquitin molecule has been under intense selective pressure.

The crystal structure of ubiquitin has been determined to 1.8-Å resolution (Vijay-Kumar *et al.*, 1987). It is a compact, globular protein with a protruding C-terminal glycine-glycine that is involved in covalent interactions with lysine ε-amino groups on other proteins.

Ubiquitin genes are encoded in two forms: As polyubiquitin, in which the ubiquitin coding regions are lined up head to tail, or as fusions to ribosomal proteins (Finley *et al.*, 1989; Özkaynak *et al.*, 1984). The ubiquitin fusions are translated as single units and are rapidly processed by hydrolases called ubiquitin C-terminal hydrolases (UCH) or ubiquitin specific proteases (Ubp), which cleave ubiquitin at the C-terminus (Jonnalagadda *et al.*, 1989; Liu *et al.*, 1989). The polyubiquitin precursors that are produced contain additional amino acid residues

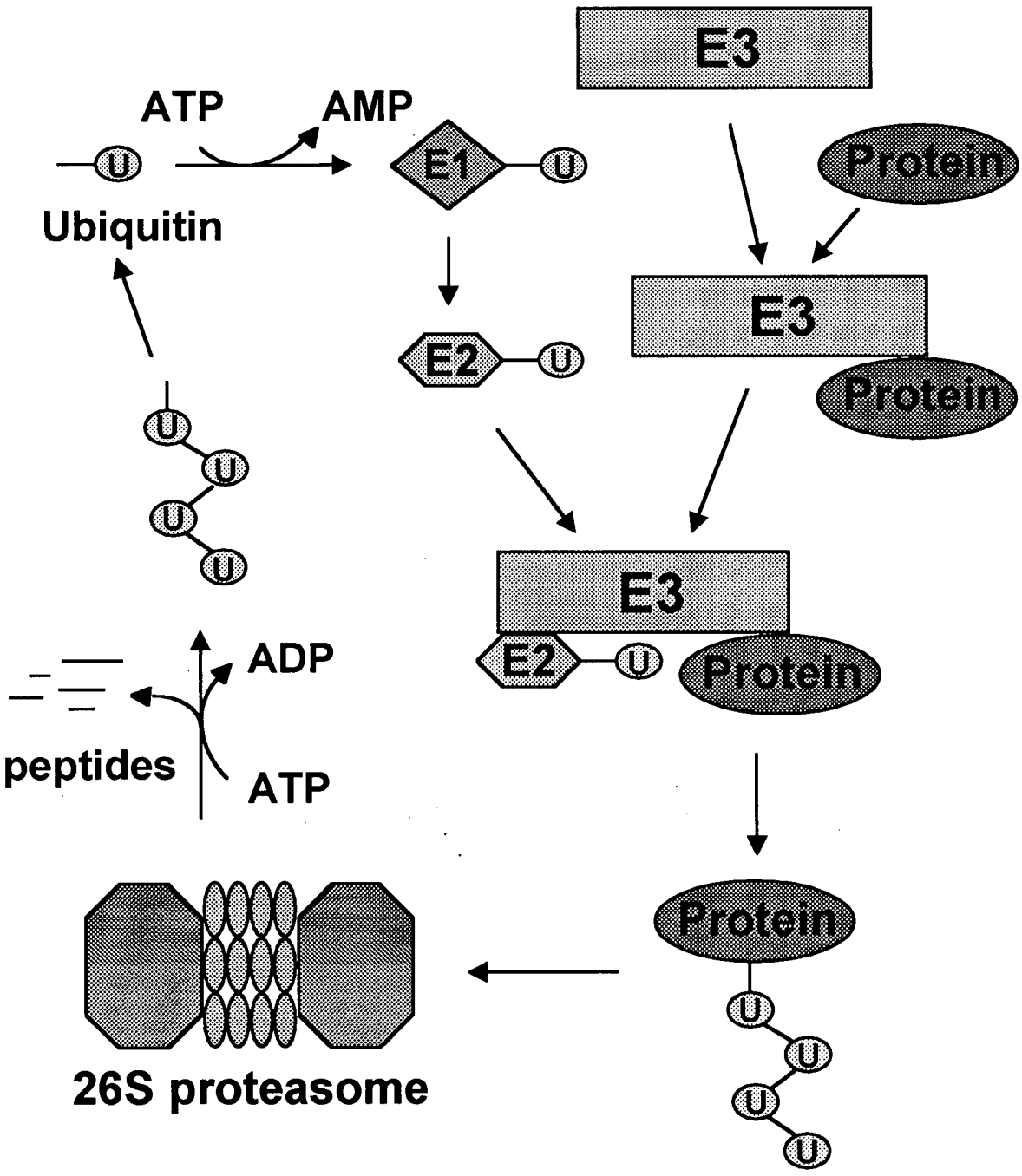
on the C-terminus that prevent conjugation to proteins (Finley and Chau, 1991). These residues are removed by ubiquitin hydrolases prior to activation of the ubiquitin moiety.

2.2 Ubiquitin conjugation

Ubiquitin is covalently attached to proteins in a multi-step enzymatic process (Figure 1). The ubiquitination of a protein targets that protein for degradation by the 26S proteasome. Ubiquitin itself is first activated by a ubiquitin-activating enzyme (E1). In a reaction that requires ATP hydrolysis and proceeds via an adenylate intermediate, a high-energy thioester bond is formed between the C-terminal glycyl residue of ubiquitin and a specific cysteinyl residue in the E1 (Haas *et al.*, 1982). Ubiquitin is then transferred to a specific cysteinyl residue of a ubiquitin-conjugating enzyme (E2; Hershko *et al.*, 1983; Pickart and Rose, 1985). E2 enzymes can transfer ubiquitin directly to protein substrates *in vitro* (Pickart and Rose, 1985); however, it is believed that ubiquitin conjugation *in vivo* requires accessory factors for substrate recognition. These factors, called ubiquitin ligases (E3) can function in different ways. One class functions as a single protein or as a multi-protein E3 complex and serves to bring E2 enzymes and substrates in close proximity to facilitate the ubiquitination of the substrate directly by the E2 enzyme (Bailly *et al.*, 1994; Hershko, 1997). Another group of E3s has the ability to accept ubiquitin as a thioester and thus acts as a catalytic intermediate in the transfer of ubiquitin to the target protein (Scheffner *et al.*, 1995).

Ubiquitin attaches to the target protein by an isopeptide bond that forms between the carboxyl-terminal glycine of ubiquitin and the ε-amino group of a lysyl residue in the target protein (Hershko, 1991; Hershko *et al.*, 1983; Pickart and Vella, 1988). Once the first ubiquitin is attached to the substrate protein, ubiquitin itself can become a target for further ubiquitination, resulting in the formation of a multiubiquitin chain (Chau *et al.*, 1989). Polyubiquitin chains linked through K48-G76 isopeptide bonds represent the predominant *in vivo* targeting signal in the ubiquitin pathway (Bachmair *et al.*, 1986; Finley *et al.*, 1994; Johnson *et al.*, 1992). There is evidence, however, that at least four other lysyl residues are sites for multiubiquitin chain initiation. The functional significance of most of these chain initiation sites remains to be determined. In yeast, K29-linked diubiquitin and K63-linked chain synthesis that is mediated by the E2s Ubc4 and Ubc5 have been observed (Arnason and Ellison, 1994; Spence *et al.*, 1995). The K63 linkages are involved in the stress response as well as DNA repair. Ubc2/Rad6-mediated polyubiquitin chains that initiate at K6 are formed

Figure 1. Major enzymatic steps in the ubiquitin proteolytic pathway. Ubiquitin is activated through the ATP-dependent formation of a high-energy thioester bond with E1 and transferred to an E2. A protein substrate is bound to a specific ubiquitin ligase, E3, and an intermediary complex is formed between E3, the protein substrate and E2-ubiquitin. Ubiquitin is transferred to a lysyl residue of the protein substrate, followed by formation of a multiubiquitin chain. The 26S proteasome utilizes ATP to degrade the protein substrate into peptides, and ubiquitin is recycled by the action of isopeptidases.


E3
ATP
AMP
U
E1
U
Protein
Ubiquitin
E3
E2
U
Protein
U
U
U
U
E3
ADP
E2
U
Protein
peptides
ATP
Protein
U
U
U
U
26S proteasome

on histones *in vitro* (Baboshina and Haas, 1996), although this type of linkage has not been demonstrated *in vivo*. In mammalian systems, the keratinocyte-specific enzyme $E2_{EPF}$ has been shown to initiate polyubiquitin chain formation of N-end rule substrates at K11 (Baboshina and Haas, 1996; Liu *et al.*, 1996). The existence of these distinct linkages, and the recognition that at least one may be involved in a specific cellular process, raises the possibility that alternative polyubiquitin chain linkages may represent a novel mechanism for specifying the fate of ubiquitinated proteins.

2.3 Degradation of ubiquitinated proteins

Most multiubiquitinated proteins in the eukaryotic cell are degraded by the 26S proteasome which contains a 20S catalytic core and two 19S caps that act as regulatory complexes (Orino *et al.*, 1991; Peters *et al.*, 1991; Peters *et al.*, 1998). The crystal structure of the 20S proteasome from the archaeon *Thermoplasma acidophilum* revealed a hollow, cylindrical shaped structure composed of four stacked rings (Löwe *et al.*, 1995). The two inner rings are composed of β type subunits while the outer rings are α subunits. The β subunits contain catalytic sites positioned within the central channel of the proteasome that have chymotrypsin-like, trypsin-like and peptidyl-glutamyl-peptide hydrolysing activities (Löwe *et al.*, 1995; Seemüller *et al.*, 1996). The α rings on either end of the proteasome form small pores of 13Å in diameter which presumably allow only unfolded proteins to travel through into the inner channel of the β subunits, thus preventing random entry and degradation of proteins by the 20S proteasome.

The 19S regulatory complex is believed to confer substrate recognition, substrate unfolding and the ability to transfer polypeptide chains to the active sites within the inner channel of the proteasome (Peters *et al.*, 1998). Of the 15-17 subunits that make up the 19S regulatory complex (Dubiel *et al.*, 1995), the function of only a few have been elucidated. Six subunits are AAA ATPases (Confalonieri and Duguet, 1995; Glickman *et al.*, 1998), one subunit (S5a) binds polyubiquitin chains and presumably functions in substrate selection (Deveraux *et al.*, 1994) and one subunit is a deubiquitinating enzyme capable of cleaving the polyubiquitin chain from the substrate protein, thus preventing the degradation of ubiquitin (Glickman *et al.*, 1998).

The proteasome is a dynamic structure that alters its function depending on the needs of the cell. One of its functions is in antigen presentation. In mammals two β subunits, LMP2

and LMP7 are induced by γ-interferon and specifically replace the β subunits, δ and MB1, respectively (Fruh *et al.*, 1994). This modification causes a change in the cleavage activities of the proteasome by enhancing the chymotrypsin-like and trypsin-like activities. In addition, a ring shaped particle called PA28, which is also induced by γ-interferon, can assemble onto the ends of the 20S proteasome (Gray *et al.*, 1994; Groettrup *et al.*, 1996). This activator complex acts to increase the rate of antigenic peptide production by the proteasome.

In addition to its role in protein degradation and antigen presentation, the proteasome is also implicated in protein processing. Inactive protein precursors such as NF-κB and the *Drosophila* transcriptional regulatory protein Cubitus interruptus (Ci) are spliced by the 26S proteasome into active protein products (Palombella *et al.*, 1994; Ingham, 1998).

2.4 Recycling of ubiquitin

The final step in the ubiquitin pathway is the regeneration of free and reusable ubiquitin. This is carried out by UCHs or isopeptidases. The linkage between the protein substrate and ubiquitin molecule is cleaved by UCHs (Eytan *et al.*, 1993). This process occurs at the 26S proteasome and utilizes ATP. Isopeptidase T preferentially cleaves the G76-K48 linkage between ubiquitin moieties in multiubiquitin chains (Hadari *et al.*, 1992). Its function appears to be the disassembly of multiubiquitin chain remnants following proteolysis of the substrate protein via the 26S proteasome.

A surprisingly large number of UCHs have been identified, although comparatively little is known about them. All deubiquitinating enzymes are thiol proteases (Mayer and Wilkinson, 1989) which are capable of binding ubiquitin (Wilkinson *et al.*, 1995). It is likely that they serve distinct functions at discrete times and places in the cell. Some deubiquitinating enzymes are involved in specific developmental processes. The *fat facets (faf)* gene product is a deubiquitinating enzyme that is required in *Drosophila* for early development and normal eye formation (Huang *et al.*, 1995). In yeast, a deubiquitinating enzyme, Ubp3 is involved in gene silencing at telomeres and the silent mating type loci (Moazed and Johnson, 1996).

3. ENZYMES OF THE UBIQUITIN SYSTEM

3.1 Ubiquitin-activating enzymes

E1s carry out the ATP-dependent activation of the C-terminal glycyl residue of ubiquitin in a two step process. An ubiquitin adenylate is formed by displacement of PPi from

ATP and the ubiquitin is then transferred to a thiol ester site on E1, and AMP is released (Ciechanover *et al.*, 1981). Genes encoding E1 enzymes have been cloned from various organisms including yeast (McGrath *et al.*, 1991), wheat (Hatfield *et al.*, 1990) and human (Handley *et al.*, 1991). With the exception of three related E1 genes present in wheat (Hatfield and Vierstra, 1992), the enzyme exists as a single-copy gene in other organisms. The E1 enzyme is roughly 100 kDa and contains the nucleotide-binding motif, GXGXXG, where X is any residue (McGrath *et al.*, 1991). The putative active site cysteinyl residue that is required for thioester formation with ubiquitin is located in the center of the amino acid sequence (Hatfield and Vierstra, 1992). E1 functions as a homodimer (Ciechanover *et al.*, 1982), and likely forms a complex with E2 and E3 proteins in the ubiquitination of substrate proteins.

3.2 Ubiquitin-conjugating enzymes

E2 enzymes accept activated ubiquitin from E1 through high energy thioester bond formation and transfer the ubiquitin moiety to the substrate protein, either directly or via a third protein, the ubiquitin ligase. E2s form a large enzyme family. In yeast, 13 genes coding for ubiquitin conjugating enzymes (UBC genes) have been identified (Hershko *et al.*, 1983; Pickart and Rose, 1985), and in *C. elegans*, 20 UBC genes are known (D. Jones, pers. comm.). Research suggests that at least 20 UBC genes are present in other species as well (Haas and Siepmann, 1997). Sequence comparison of E2s revealed that all share a highly conserved catalytic domain termed the UBC domain. Within the UBC domain, a specific cysteinyl residue is required for ubiquitin-E2 thioester formation (Sommer and Jentsch, 1993; Sung *et al.*, 1990). The active site region has a consensus sequence of FHPNIXXXGXICLDL that is nearly identical in all E2s. In addition to the active site cysteine, the consensus sequence contains a conserved HPN tripeptide which is required for proper folding of the core region of the E2 (Haas and Siepmann, 1997). The conserved histidine of the tripeptide forms a triad with two conserved tyrosinyl residues that are spaced seven amino acids apart in the carboxyl terminal region. The loss of interaction of this triad through mutation at any of the three sites results in a dramatic decrease in the stability of the protein, suggesting that the triad is critical in maintaining the tertiary structure of the E2 (R.H. Bohnsack, pers. comm.).

The N-terminal region of the UBC domain is a conserved basic motif that, when deleted, results in decreased thioester formation, suggesting that this domain is involved in E1 binding (Sullivan and Vierstra, 1991). The C-terminal region may be involved in binding of E3

ubiquitin ligases since deletion of the carboxyl terminus of human UbcH2B results in loss of E3α binding (R.H. Bohnsack, pers. comm.).

E2s can be divided into four classes based on their structure. Class I E2s are small proteins of approximately 16 kDa that consist of only the UBC domain. This class includes yeast Ubc4, Ubc5, Ubc7 and Ubc9, *Drosophila UbcD1* and *C. elegans* UBC-2.

Class II E2s contain C-terminal extensions in addition to the UBC domain. Yeast class II E2s include Ubc1, Ubc2 (Rad6), Ubc3 (Cdc34), Ubc6 and Ubc8. *C. elegans* UBC-1 is also a class II enzyme (Leggett *et al.*, 1995). The extensions may mediate substrate specificity or intracellular localization. The short acidic C-terminal tail of Rad6 is required *in vitro* for the recognition of basic substrates such as histones (Sung *et al.*, 1988), and *in vivo* for sporulation (Morrison *et al.*, 1988). The tails of Cdc34 and *C. elegans* UBC-1 are involved in self-association to form higher order quaternary structures (Leggett and Candido, 1997; Ptak *et al.*, 1994). The C-terminal extension of Ubc6 anchors the enzyme to the endoplasmic reticulum (ER) membrane with the catalytic domain facing the cytosol (Sommer and Jentsch, 1993). Ubc6 is believed to form part of an ER degradation pathway for integral membrane proteins.

Class III enzymes have, in addition to the UBC domain, N-terminal extensions. While no class III E2s have been identified in yeast, they have been found in higher eukaryotic organisms. These include *Drosophila UbcD2* and human UbcH6, UbcH7, UbcH8 and UbcH9 (Matuschewski *et al.*, 1996; Nuber *et al.*, 1996). In humans, these extensions are enriched in serine and threonine residues which may function as phosphorylation sites to provide substrate specificity. Members of this family can partially substitute for Ubc4 in yeast, suggesting that they have Ubc4-related functions.

The Class IV enzymes contain both C- and N-terminal extensions and include rabbit $E2_{230K}$ and murine UbcM1 (S. Jentsch, pers. comm.; Berleth and Pickart, 1996). UbcM1 has a short C-terminal extension and a long N-terminal extension. The overall organization of the enzyme resembles E3 enzymes with HECT domains (see Section I.5.7), suggesting that UbcM1 may combine E2 with E3-like properties within the same molecule.

3.3 Ubiquitin-protein ligases

E3s are loosely defined as proteins that participate with E1 and E2s in the ubiquitination of proteins that are otherwise not recognized by E2s (Ciechanover, 1994; Hershko and Ciechanover, 1992). It has become increasingly clear that E3s play a major role in substrate

recognition in the ubiquitin pathway. Recently, a number of E3 ligases have been identified that fall into two groups. One type of E3 functions as part of an E1-E2-E3 thiol relay (Scheffner *et al.*, 1995). The E3 accepts ubiquitin from an E2, forming a thioester bond with ubiquitin at a specific cysteinyl residue. The ubiquitin is then transferred to a substrate protein that is also bound to the E3.

Other E3 ligases do not bind ubiquitin. They tend to function as a scaffold protein for both the E2 and the substrate, bringing them in close proximity to facilitate transfer of ubiquitin (Dohmen *et al.*, 1991; Patton *et al.*, 1998). It must be pointed out that a specific E3 may interact with several E2s, just as a specific E2 can interact with many E3s. This interchangeability of components aids in target specificity. Many laboratories are currently studying E3 ligases, and it is likely that many more types of E3s await discovery.

4. THE UBC4 BRANCH OF UBIQUITIN-CONJUGATING ENZYMES

In yeast, three E2s encoded by UBC1, -4, and -5 comprise a functionally overlapping E2 subfamily that has been designated the "UBC4 branch" of E2s. These enzymes are responsible for the degradation of most short-lived and abnormal proteins (Seufert and Jentsch, 1990). In addition, UBC4 and UBC5 expression increases on exposure to heat shock, *ubc4ubc5* double mutant strains are sensitive to a variety of stresses and deletion of all three E2s is lethal (Seufert and Jentsch, 1990; Treier *et al.*, 1992). In general, these E2s, together with specific E3s, are thought to recognize abnormal proteins and prevent their accumulation by targeting them for degradation. However, it is unclear what structural features of an abnormal protein are recognized by the E2s.

In *C. elegans*, UBC-2 is the functional homolog of yeast Ubc4 and is able to rescue the growth defect of yeast *ubc4ubc5* double mutants (Zhen *et al.*, 1993). This suggests that UBC-2 in *C. elegans* may also function to degrade short-lived and abnormal proteins. Interestingly, *ubc-2* is an essential gene that is required during larval development (Zhen *et al.*, 1996). Comparison of the 20 known *C. elegans* E2s to the *ubc-2* sequence does not reveal any closely related genes that may form part of a subfamily with *ubc-2* (D. Jones, pers. comm.). One gene with high similarity to *ubc-2* was previously identified, but was subsequently determined to be a pseudogene (Zhen, 1995).

The *Drosophila* UBC4 homolog, *UbcD1*, is also an essential gene (Treier *et al.*, 1992). Deletion mutants are embryonic lethal, while weak alleles cause lethality during the pupal stage

and disrupt telomere behavior in both mitotic and meiotic cells. Some mutant alleles produce sterile males with abnormal telomere behavior in meiotic cells (Cenci *et al.*, 1997). Thus, *UbcD1*, like *C. elegans ubc-2*, has specific functions during development. In addition, both E2s are unique in the UBC4 branch in that they are essential genes.

The UBC4 branch of E2 enzymes has expanded during the evolution of higher organisms. In *Arabidopsis*, multiple isoforms of UBC8 exist and it is believed that the expansion of the family has led to the specialization of isoforms in terms of spatial and temporal expression and in the specificity of binding partners. Three homologs of UBC4 have been found in the rat, two of which are germline specific, with one being expressed only in spermatids (Wing *et al.*, 1996). In humans at least three isoforms of UbcH5 have been identified (UbcH5A, UbcH5B and UbcH5C; Jensen *et al.*, 1995), all of which are capable of transferring ubiquitin to the E3 ligase E6-AP *in vitro*, although only UbcH5C appears to perform this function *in vivo*. The UbcH5B isoform is specific for the degradation of IκB and the processing of NF-κB (see Sections I.5.4 and I.5.5). Thus, an interesting feature of human UBC4 family members is that each appears to bind to specific targets through interaction with specific E3s. Subtle differences in amino acid sequence within the UbcH5 family may dictate E3 specificity.

5. ROLES OF THE UBC4 BRANCH OF UBIQUITIN-CONJUGATING ENZYMES

Although the enzymatic pathway for ubiquitin conjugation is well characterized, the ways in which proteins are selected for degradation are only beginning to be elucidated. The degradation of substrates appears to be mediated by specific degradation signals which are sequence or structural features of the substrate. These signals include lysyl residue(s) that can be ubiquitinated as well as elements that are recognized by a specific E2/E3 complex. The proteolysis of a number of proteins is phosphorylation-dependent, such that the ubiquitination apparatus only recognizes and modifies the substrate in specific phosphorylation states. Some substrates must be dephosphorylated, while others require phosphorylation at specific residues to be recognized by the degradation system.

One family of E3 ubiquitin ligases that recognize phosphorylated substrates are F box-containing proteins that form part of the SCF (Skp1-Cdc53/CUL1-F-box protein) complex (Feldman *et al.*, 1997; Skowrya *et al.*, 1997). The phosphorylation-dependent destruction of substrate proteins via SCF complexes has been described for yeast proteins that regulate the G1

phase and G1/S transition of the cell cycle, the inhibitor protein IκB, and β-catenin (Feldman *et al.*, 1997; Skowrya *et al.*, 1997; Spencer *et al.*, 1999; Winston *et al.*, 1999; Maniatis, 1999). In many of these systems, the E2 responsible for ubiquitination of the substrates is a member of the UBC4 branch of conjugating enzymes.

A large number of proteins that are targeted for degradation by the ubiquitin-dependent proteolysis system have been identified in recent years. This includes many transcriptional regulators, cyclins, integral membrane proteins and cell cycle inhibitors (reviewed in Peters *et al.*, 1998). In fact, the list of known substrate proteins is far too large for all of them to be described. Therefore, only those ubiquitin pathways and substrates which are known to be mediated by E2s of the UBC4 branch, or have some relation to UBC4-dependent systems, will be discussed.

5.1 Stress response

Ubiquitin degradation plays an essential role in the eukaryotic stress response. In yeast, UBC4 and UBC5 are stress inducible (Seufert and Jentsch, 1990). UBC7 is also induced by cadmium, which is known to elicit a stress response (Jungmann *et al.*, 1993). At elevated temperatures, *ubc4ubc5* null mutants are inviable, and express heat shock proteins under non-stress conditions. *ubc7* null mutants are only affected when treated with cadmium. This suggests that Ubc4/Ubc5 and Ubc7-mediated proteolysis are involved in the stress response induced by heat shock and cadmium, respectively.

The *C. elegans* gene, *ubc-2*, which is a functional homolog of UBC4, is not heat shock inducible (Zhen *et al.*, 1993). However, UBC-2 is expressed at all life stages and, at least during embryonic and larval stages, is expressed in all tissues (Zhen *et al.*, 1996). Thus, a role for UBC-2 in the stress response cannot be ruled out. Rather than increased expression of UBC-2, perhaps existing pools of UBC-2 are re-localized under stress conditions.

Arnason and Ellison (1994) have shown that K63 of ubiquitin is essential to the stress response and that the formation of the K63-linked polyubiquitin chains is mediated by UBC4 and UBC5. The functional role for these alternatively linked chains in the stress response has not been determined.

5.2 Cell cycle progression

For cells to divide, two critical processes must occur: DNA must be accurately replicated and it must be accurately segregated to daughter cells. The cell cycle control

mechanism regulates the progression of the cell from one phase to another by ensuring that each process has occurred correctly before continuing on to the next. Eukaryotic cells cycle through a DNA replication phase (S) and a mitotic phase (M) which are interspersed with gap phases that occur before (G1) and after (G2) replication. The transition from one phase to the next is controlled by the activation or inactivation of cyclin-dependent protein kinases (CDKs; reviewed in Morgan, 1995). In yeast, there is only one CDK (Cdc28), while in higher eukaryotes there are several. The key regulator of CDKs, and thus of cell cycle transitions, are cyclins. Cyclin levels fluctuate during the cell cycle and are controlled by ubiquitin-mediated proteolysis (Chun *et al.*, 1996; Hershko, 1997).

Two major pathways of ubiquitin-dependent degradation are involved in cell cycle progression. One pathway utilizes the SCF complex (Skp1-Cdc53/CUL1-F-box protein) and regulates the transition from G1 to S phase, while the second utilizes the anaphase promoting complex/cyclosome (APC/C) complex and triggers anaphase and exit from mitosis.

5.2.1 Role of ubiquitin-dependent degradation in the G1/S transition

The transition from G1 to S is controlled by a Cdc34-dependent proteolytic pathway. Cdc34 is a ubiquitin conjugating enzyme also known as Ubc3 (Goebl *et al.*, 1988). In this pathway, Cdc34 is responsible for the degradation of an inhibitor protein Sic1 (Schwob *et al.*, 1994). In G1 cells, Sic1 binds to and inhibits the Cdc28-Clb kinase complex, thus preventing initiation of DNA replication. Sic1 is phosphorylated by Cdc28-Cln2p kinase, which allows the ubiquitination and subsequent destruction of Sic1 by the Cdc34 pathway (Verma *et al.*, 1997).

Cdc34 is associated with a large multiprotein SCF complex which includes Cdc4, Cdc53 and Skp1 (Feldman *et al.*, 1997). The SCF^{Cdc4} complex constitutes an E3 ligase and functions to facilitate the ubiquitination of Sic1 by Cdc34 (Lisztwan *et al.*, 1998; Lyapina *et al.*, 1998; Mathias *et al.*, 1998; Patton *et al.*, 1998). Cdc4 contains an amino acid motif termed the F-box and a series of WD repeats which are involved in protein-protein interactions (Bai *et al.*, 1996). Cdc4 binds the substrate protein Sic1 and also associates with Skp1. Skp1 is in turn bound to the cullin protein Cdc53 which recruits Cdc34 to the complex. Thus, the whole complex serves to bring the E2 and the substrate protein together to facilitate transfer of ubiquitin to the substrate.

SCF has become the general name for a collection of E3s that have been identified in many systems, and which bear striking similarity to the yeast SCF complex (Spencer *et al.*, 1999; Winston *et al.*, 1999). The SCF complex is composed of two evolutionarily conserved factors, Skp1 and a member of the Cullin family of proteins. In addition, the complex contains an F-box protein and a ubiquitin-conjugating enzyme. The F-box proteins are a diverse family of proteins that are capable of recognizing different substrates through specific protein-protein interaction domains such as WD or leucine-rich repeats (Bai *et al.*, 1996). An examination of genome databases has revealed over 400 F-box proteins, with 20 in yeast and over 100 in *C. elegans* (Koepp *et al.*, 1999). Members of the Cullin family of proteins are thought to recruit the E2 while the adapter protein, Skp1, tethers the proteins to the complex.

5.2.2 Ubiquitin-dependent proteolysis and the anaphase promoting complex

During mitosis, two events are controlled by ubiquitin-mediated proteolysis: sister chromatid separation and exit from telophase into G1 (reviewed in Peters *et al.*, 1998). Both events utilize a large multisubunit E3 ligase complex, the APC/C, which in yeast is composed of 12 subunits (Zachariae *et al.*, 1998b). The formation of thioester intermediates was not detected with the APC/C, suggesting that the APC/C serves a scaffolding function rather than acting as a final ubiquitin donor in the reaction (King *et al.*, 1995).

The targets of the APC/C include both A and B-type cyclins and a number of inhibitor proteins that regulate cell cycle progression (Koepp *et al.*, 1999; Wolf and Jackson, 1998). Many of the APC/C targets contain a conserved 9-residue motif called the "destruction box" which is believed to signal ubiquitin-mediated degradation by the APC/C (Glotzer *et al.*, 1991). It is not clear, however, whether the destruction box on its own represents a recognition site or whether it must be presented within a larger structure for recognition.

The transition from metaphase to anaphase is controlled by the sequential degradation of inhibitors by the APC/C (Cohen-Fix *et al.*, 1996). Cdc20 is expressed during G2 and binds to the APC/C during metaphase. The association of Cdc20 with APC/C allows ubiquitination of the anaphase inhibitor Pds1 (Lim *et al.*, 1998). Destruction of Pds1 removes the block to anaphase allowing sister chromatid separation to proceed.

Exit from mitosis requires the degradation of B-type cyclins by the APC/C. This activity depends on the activator protein Cdh1 (Fang *et al.*, 1998; Zachariae *et al.*, 1998a). Cdh1 is constitutively present but binds the APC/C only during mitotic exit and G1, points in

the cell cycle where mitotic cyclins are degraded. During most of the cell cycle, Cdh1 is inhibited by Cdk phosphorylation. Cdh1 is activated by the phosphatase Cdc14 which functions in three parallel processes to ensure the destruction of B-type cyclins and mitotic exit (Visintin *et al.*, 1998). Cdc14 acts during telophase to dephosphorylate and activate both Cdh1 and a transcription factor Swi5. Active Swi5 stimulates the transcription of the inhibitor Sic1. Cdc14 also dephosphorylates Sic1 which protects Sic1 from degradation by the SCF (see Section I.5.2.1). The increased levels of Sic1 block Cdc28/Clb activity, while at the same time, B-type cyclins are degraded by the APC/C. These processes, acting in concert, lead to exit of cells from mitosis.

The timing of mitotic exit is regulated by the ability of Cdc14 to access its substrates (Shou *et al.*, 1999; Visintin *et al.*, 1999). Through G1 and early mitosis, Cdc14 is localized in the nucleolus through its association with the nucleolar protein Cfi1/Net1. During telophase, Cdc14 is released from the nucleolus and spreads through the nucleus and into the cytoplasm where it functions to dephosphorylate its substrate. Thus, the activity of Cdc14 is regulated through its physical location within the cell.

While the identity of the E2 that works in concert with APC/C is unknown, biochemical experiments indicate that Ubc4 is a possible candidate (King *et al.*, 1995), and *Drosophila* UbcD1 is required for proper telomere behavior (Cenci *et al.*, 1997). However, in *Xenopus* a novel E2 called Ubcx was shown to be involved in APC/C-dependent degradation (Yu *et al.*, 1996). In the presence of E1 and purified APC/C, Ubc4 or Ubcx can mediate the ubiquitination of cyclin. In support of a role for Ubcx in the APC/C, mutations in the homolog of Ubcx in fission yeast (UbcP4; Osaka *et al.*, 1997) and human (UbcH10; Townsley *et al.*, 1997) cause metaphase arrest.

5.3 Degradation of the transcription factors c-Fos and c-Jun

The transcriptional activator AP-1 is involved in cell proliferation following stimulation by mitogenic factors (reviewed in Angel and Karin, 1991). The active form of AP-1 is composed of two subunits, Jun and Fos. Both c-Jun and c-Fos are ubiquitinated *in vivo* (Stancovski *et al.*, 1995; Treier *et al.*, 1994) and the ubiquitination of c-Fos is greatly enhanced by interaction with c-Jun (Tsurumi *et al.*, 1995). For c-Jun to be degraded, it must be dephosphorylated (Musti *et al.*, 1997) and there is evidence which suggests that c-Fos must also be dephosphorylated for ubiquitination to occur (Okazaki and Sagata, 1995). When c-Jun is

phosphorylated by the MAPK signalling pathway, ubiquitination and degradation of c-Jun is suppressed, and genes regulated by c-Jun and c-Fos are transcribed. The ubiquitination of both c-Fos and c-Jun requires UbcH5 (Musti *et al.*, 1997; Stancovski *et al.*, 1995). An E3 enzyme specific for c-Fos, called E3-Fos, has also been identified (Stancovski *et al.*, 1995).

5.4 Processing of NF-κB

NF-κB is a member of the Rel family of dimeric transcription factors and regulates the expression of multiple genes involved in immune and inflammatory responses (reviewed in Thanos and Maniatis, 1995; Verma *et al.*, 1995). It is a heterodimer formed from the p50 (NF-κB1) and p65 (Rel A) subunits. The p50 subunit is generated from a 105 kDa precursor called p105. The relatively stable p105 is ubiquitinated and partially degraded to produce active p50. Unlike other substrates of the proteasome which are degraded to small peptides, only the C-terminus of p105 is degraded, leaving the functional p50 subunit (Palombella *et al.*, 1994). The C-terminus is characterized by a series of ankyrin repeats and a PEST domain (Ghosh *et al.*, 1990). Ankyrin repeats are involved in protein-protein interactions (Gilligan and Bennett, 1993), and the PEST domain is believed to be a degradation signal (Rechsteiner and Rogers, 1996). Ubiquitination of p105 requires a member of the UbcH5 subfamily (Coux and Goldberg, 1998). Rabbit $E2_{25K}$, another UBC4 branch member, can substitute for UbcH5 in the reaction. A novel 320 kDa E3 is also necessary for p105 processing (Orian *et al.*, 1995). A small E3 (E3-κB) was identified by Coux and Goldberg (1998) and is involved in p105 processing. It is likely that E3-κB is a component of the 320 kDa activity.

5.5 Ubiquitin-dependent degradation of IκB and the *Drosophila* homolog, Cactus

The transcription factor NF-κB, itself, is also activated by the ubiquitin-proteasome pathway (Baeuerle and Henkel, 1994; Li *et al.*, 1995; Palombella *et al.*, 1994; Roff *et al.*, 1996). In quiescent cells, NF-κB is held in a latent state in the cytoplasm through attachment to a member of the IκB family of inhibitors, IκBα. In response to external stimuli such as viruses, cytokines and antigens, IκBα is degraded in a ubiquitin-dependent manner, allowing the nuclear translocation of NF-κB which leads to a variety of transcriptional responses (Baeuerle and Henkel, 1994).

IκBα contains ankyrin repeats and a PEST domain, and bears striking similarity to the C-terminus of p105 (see Section I.5.4). It is interesting that both the C-terminus of p105 and IκBα function to inhibit the nuclear translocation of NF-κB.

Following stimulation of cell surface receptors, a signal transduction cascade is initiated that leads to the activation of an IκB kinase complex. This complex phosphorylates NF-κB-bound IκBα at two specific serine residues in its N-terminal regulatory domain, serine 32 and 36 (Brown *et al.*, 1995; Chen *et al.*, 1996). The addition of these phosphates triggers the recognition of IκBα by the E3 ubiquitin ligase, β-TrCP (Spencer *et al.*, 1999; Winston *et al.*, 1999). The targeting component for E3 binding is a short, phosphorylation-dependent recognition element in IκBα, DS*GLDS* (where S* represents a phosphoserine) and does not include the ubiquitin conjugation site.

The E3 ubiquitin ligase that binds to the IκBα degradation determinant is the F box and WD domain protein β-TrCP (Spencer *et al.*, 1999; Winston *et al.*, 1999). When β-TrCP recognizes the phosphorylated degradation signal on NF-κB-bound IκBα, it is associated with Skp1, Cul1 and UbcH5C in a large complex (Spencer *et al.*, 1999; Winston *et al.*, 1999). The $SCF^{\beta\text{-}TrCP}$ complex promotes the UbcH5C-dependent conjugation of ubiquitin to IκBα. The multiubiquitinated IκBα is then targeted to the 26S proteasome for degradation.

The *Drosophila* IκB homolog, Cactus, also undergoes signal-induced degradation (Belvin *et al.*, 1995). A number of parallels exist between IκB and Cactus. The *Drosophila* NF-κB homolog Dorsal is sequestered in the cytoplasm in early embryos through interaction with Cactus. During dorsal-ventral patterning of the early *Drosophila* embryo, Dorsal is activated specifically on the ventral side of the embryo by the Toll receptor-signalling pathway (reviewed in Morisato and Anderson, 1995). The pathway culminates in the destruction of Cactus, allowing nuclear translocation of Dorsal and activation of downstream genes such as *twist* and *snail* (Geisler *et al.*, 1992). The E3 involved in this process is Slimb, a homolog of β-TrCP, which is a component of an SCF complex in *Drosophila* (Spencer *et al.*, 1999).

5.6 The Wnt/Wg pathway

The E3 ubiquitin ligase β-TrCP/Slimb degrades β-catenin/Armadillo via the Wnt/Wg signalling pathway. This pathway is conserved between *C. elegans*, *Drosophila* and vertebrates (Rocheleau *et al.*, 1997; Thorpe *et al.*, 1997), and is involved in a number of

developmentally regulated processes such as cell fate decisions, segment polarity and axis formation, respectively. Inappropriate activation of the Wnt pathway has also been implicated in human cancers (He *et al.*, 1998).

In the absence of Wnt signalling, the serine/threonine kinase glycogen synthase kinase-3β (GSK-3β) is activated. GSK-3β, in a complex with adenomatous polyposis coli (APC) and axin, binds to β-catenin and phosphorylates two serine residues on its N-terminus (Orford *et al.*, 1997). The phosphorylation sites are part of a sequence that bears a striking resemblance to the IκBα degradation signal. In a process reminiscent of IκBα degradation, phosphorylated β-catenin is ubiquitinated and subsequently degraded (Aberle *et al.*, 1997; Ikeda *et al.*, 1998). The $SCF^{\beta\text{-}TrCP}$ complex is believed to mediate this process (Maniatis, 1999).

When the Wnt pathway is activated, GSK-3β is inhibited (Cook *et al.*, 1996), resulting in the accumulation of β-catenin which translocates to the nucleus and interacts with an HMG-box transcription factor, Tcf/LEF-1. This promotes the activation or repression of a number of developmental genes (reviewed in Willert and Nusse 1998; Payre *et al.*, 1999).

5.7 Ubiquitination of p53

The tumor suppressor protein p53 is a transcription factor that regulates the expression of genes involved in cell cycle arrest and apoptosis (reviewed in Ko and Prives, 1996; Vogelstein, 1990). The current model of p53 function suggests that it prevents the accumulation of somatic mutations by either signalling cell cycle arrest to allow time for damage repair, or by eliminating the damaged cell by apoptosis. Mutations within the p53 gene are frequently associated with human cancer.

Degradation of p53 is increased in cells infected with human papilloma virus (Scheffner *et al.*, 1990). A protein encoded by the virus, E6, is responsible for the increased degradation of p53. E6 acts by binding to a cellular protein called E6-associated protein (E6-AP). The E6/E6-AP complex functions as an E3 protein ligase by accepting ubiquitin from an E2 and transferring it to p53, thereby promoting the ubiquitination and subsequent degradation of p53 (Scheffner *et al.*, 1995). The E2 enzymes that transfer ubiquitin to E6-AP are UbcH5A, 5B, 5C, 6 and 7 (Jensen *et al.*, 1995; Nuber *et al.*, 1996; Scheffner *et al.*, 1994). The first four are members of the UBC4 branch, while UbcH7 is not, indicating that distinct E2 families can direct ubiquitin transfer to a specific E3 protein.

E6-AP is member of a large family of related proteins called HECT proteins (homologous to E6-AP carboxyl terminus) (Huibregtse *et al.*, 1995). There are at least 20 HECT proteins in humans (Schwarz *et al.*, 1998) and six in *C. elegans* (Peters *et al.*, 1998). HECT proteins are defined by an essential conserved C-terminal domain of approximately 350 amino acids (Huibregtse *et al.*, 1995). This domain contains the active site cysteine necessary for ubiquitin thioester bond formation and plays some role in substrate specificity. HECT domain proteins define a class of E3 ubiquitin ligases that function as part of a thioester cascade leading to ubiquitination of the target protein.

In the absence of E6, E6-AP does not direct the ubiquitination or degradation of p53 (Huibregtse *et al.*, 1993). However, under normal circumstances, p53 is a target of the ubiquitin system. The ubiquitin ligase Mdm2 has been shown to function *in vivo* and *in vitro* with UbcH5 to ubiquitinate p53, leading to its destruction via the 26S proteasome (Honda *et al.*, 1997). Mdm2 is, as expected, a member of the HECT family of E3 proteins.

6. C. elegans AS A MODEL ORGANISM

Most studies of ubiquitin-mediated protein degradation have been carried out in yeast or in mammalian tissue culture. To examine the role of the ubiquitin system during development in a multicellular organism, the free-living soil nematode, *C. elegans*, was utilized, since it has many characteristics that make it an attractive model organism for developmental, genetic and molecular studies. It has a short life cycle of about 3-4 days at 20°C where it develops from an embryo through four larval stages (L1-L4) to an adult (White, 1988). It can be easily grown in the laboratory on nutrient growth media spread with bacteria as a food source. The population consists primarily of self-fertilizing hermaphrodites although males arise spontaneously through non-disjunction. Males can be maintained by mating to hermaphrodites, thus allowing for genetic crosses. A single mated hermaphrodite will produce around 300 progeny.

The animal is transparent with a relatively simple anatomy consisting of less than 1000 cells. The developmental pattern is invariant and the complete cell lineage, including cell migration, programmed cell death and neurocircuitry is known (Sulston, 1983; Sulston and Horvitz, 1977; Sulston *et al.*, 1983). Additionally, mutants can be readily obtained using various mutagens. The mutants can be easily mapped and the genes isolated. Methods of target-selected gene inactivation using transposon insertion and excision or chemical mutagens have been developed (Rushforth *et al.*, 1993; Plasterk *et al.*, 1998). DNA transformation in *C.*

elegans, both integrative and extrachromosomal, provide an efficient means to employ reverse genetics and to study gene expression and cell biology (Mello and Fire, 1995).

The genome is relatively small, being only five times that of yeast (Herman, 1988). Most of the genome has been cloned and ordered as a series of cosmid and YAC (yeast artificial chromosome) clones (Coulson *et al.*, 1988). The *C. elegans* genome sequencing project is essentially complete, so any sequence or gene can be easily obtained and manipulated (The *C. elegans* Sequencing Consortium, 1998). AceDB (a *C. elegans* database), an program that compiles and displays data on *C. elegans*, including sequence and gene information, is currently available online at http://genome.wustl.edu/gsc/ace/acedocs/ace.html.

Many of the phenotypes observed in this study make it necessary to provide additional information about *C. elegans* embryogenesis and development of the gonad, intestine and body wall muscle.

6.1 Embryonic and larval development

Embryogenesis lasts approximately 800 minutes post-fertilization at 22°C (Wood, 1988). During the first 100 minutes, six founder cells, AB, MS, E, C, D and P4 are produced by a series of asynchronous divisions (Sulston *et al.*, 1983). Three of these, AB, MS and C give rise to a mixture of hypodermal, neuronal and muscle cells. D gives rise to body wall muscle cells, E produces intestine exclusively, and the germline develops from descendants of P4. A number of cell migrations and programmed cell deaths take place in the descendants of these founder cells during development.

Gastrulation occurs at around 100 minutes and is followed by a period of cell division and organogenesis which continues until about 350 minutes. From 350-650 minutes, morphogenesis takes place. During this time, the embryo undergoes transformation from a ball of about 550 cells into a cylindrical worm. The volume and cell number of the animal does not change much during this time, but there is a four-fold increase in length. As the embryo elongates within the confines of the eggshell, it is forced to fold up on itself. A series of morphological stages can be identified based on the extent of elongation. These stages include comma (around 390 minutes), 1.5-fold (around 420 minutes), 2-fold (around 450 minutes) and 3-fold (around 520 minutes). The first twitching movements, indicative of muscle function, are observed at around 400 minutes and pharyngeal pumping begins at about 760 minutes.

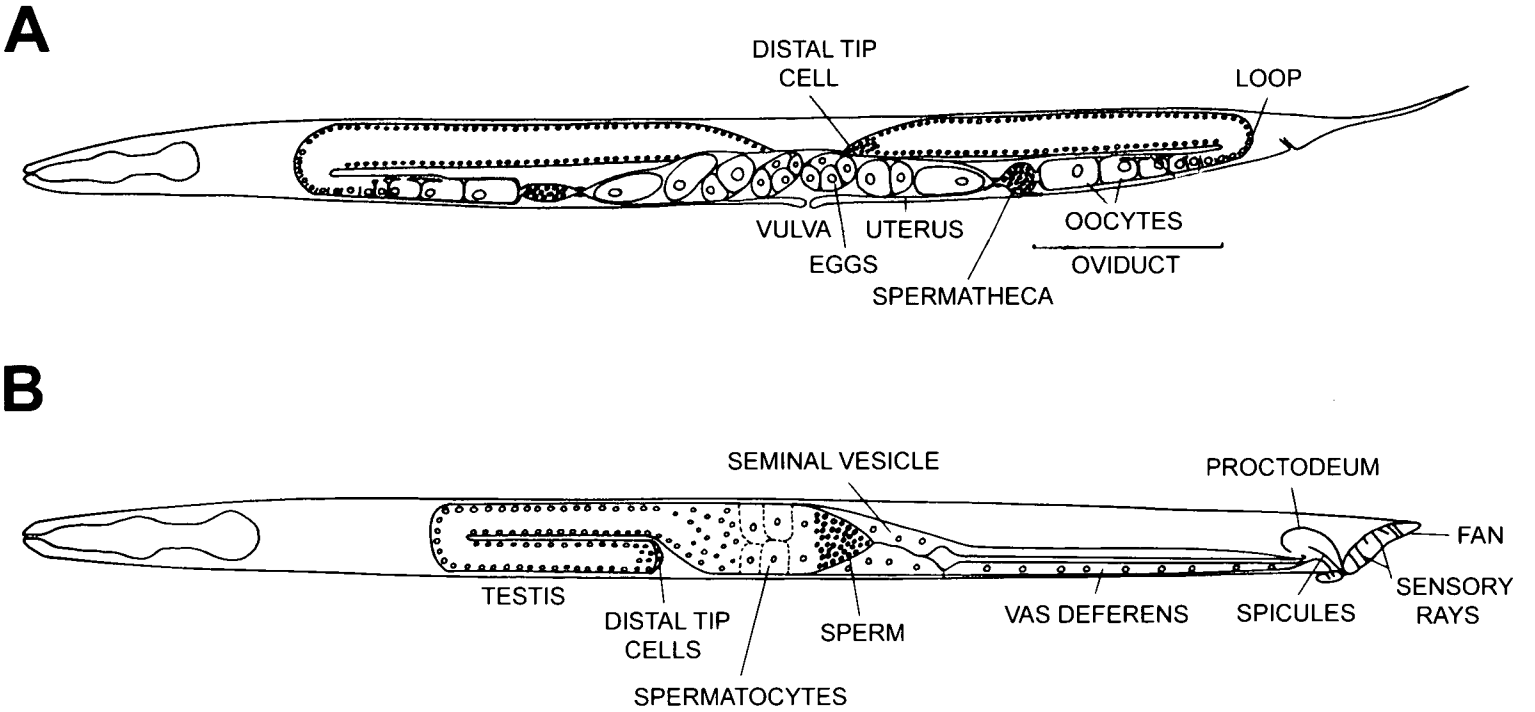
Embryos hatch as L1 larvae consisting of 558 cells in the hermaphrodite and 560 cells in the male. Over the next 50 hours, larval development proceeds through three additional larval stages, L2, L3 and L4, during which they undergo further cell divisions. The larval stages are punctuated by molts. During each molt, a new cuticle is synthesized under the old one which is shed. The cuticle is different both structurally and molecularly at each stage.

6.2 Gonadal development and anatomy

The *C. elegans* L1 larva hatches with a gonad primordium consisting of four cells: Z2 and Z3 are precursors of the germline, and Z1 and Z4 are somatic gonad precursors (reviewed in Kimble and Ward, 1988). During gonadogenesis, distal tip cells produced from Z1 and Z4 control the elongation of the growing gonadal arms. The hermaphrodite reproductive system consists of two tubular gonads, one extending anteriorly and the other posteriorly from the middle of the animal. Each lobe is U-shaped, consisting of a distal (to the uterus) ovary, a more proximal oviduct and a spermatheca connected to a common uterus centered around the vulva (Figure 2; Kimble and Ward, 1988). Somatic cells of the gonad consist of one distal tip cell located at the apex of the gonad arm, two somatic epithelial cells in the distal arm and a contractile epithelial sheath, or oviduct, surrounding the proximal arm. The germline nuclei located most distally are mitotic and, as they travel proximally, progress through meiosis and reach diakinesis in the oviduct prior to fertilization. The distal arm consists of a central anucleate core surrounded by a layer of germline nuclei. An incomplete plasma membrane separates each nucleus, while the cytoplasm is continuous. Thus, the ovary is syncytial in the distal arm. Differentiation of the germline cells into sperm and oocytes begins in the loop region. The proximal arm of the gonad is the site of gametogenesis. Hermaphrodites transiently produce about 150 sperm before switching to oocyte production. Developing oocytes travel single file along the proximal arm and arrest in diakinesis of meiosis I. The oocyte is fertilized as it passes through the spermatheca to the uterus. The oocyte nucleus completes meiosis after fertilization, two polar bodies are extruded and the two pronuclei move towards one another. The pronuclear membranes break down and embryonic cell divisions begin.

The male reproductive system consists of a single U-shaped tubular testis that is connected to the cloaca by the seminal vesicle and vas deferens (Figure 2). Two distal tip cells are located at the apex of the testis. As in hermaphrodites, germline cells are mitotic distally

Figure 2. Organization of the hermaphrodite and male gonad. A) Hermaphrodite gonad structure. The distal tip cell, ovary, oviduct, oocytes, spermatheca, uterus, vulva and developing eggs are indicated. B) Male gonad structure. The distal tip cells, testis, spermatocytes, sperm, seminal vesicle, vas deferens, proctodeum, fan and sensory rays are indicated. From White, 1988.


A
DISTAL TIP CELL
LOOP
VULVA
UTERUS
OOCYTES
EGGS
OVIDUCT
SPERMATHECA
B
SEMINAL VESICLE
PROCTODEUM
FAN
SENSORY RAYS
TESTIS
DISTAL TIP CELLS
SPERM
VAS DEFERENS
SPICULES
SPERMATOCYTES

and progress through meiosis as they move proximally. Sperm mature at the proximal edge of the proximal arm and spermatids travel through the vas deferens to the seminal vesicle. Spermatids transferred to hermaphrodites during copulation complete maturation in the uterus where they form pseudopods and crawl to the spermatheca. The male tail contains several structures specialized for copulation. The tail forms into a fan with nine pairs of sensory rays and two copulatory spicules. These structures are used to locate the hermaphrodite vulva and for sperm transfer.

6.3 Intestinal structure

In *C. elegans*, the intestine consists of a tube of 20 cells surrounding an interior lumen (White, 1988). A dense layer of microvilli is located on the apical surface of these cells. All but the six anterior-most and any of the four posterior-most cells undergo nuclear division during L1 lethargus and become binucleate, resulting in 30-34 nuclei in adults. The nuclei are large and circular, with a prominent nucleolus. The intestinal cells also undergo endoreduplication of their DNA during larval growth to reach a DNA content of 32n in adults (Hedgecock and White, 1985).

The intestine connects to the pharynx via the pharyngeal-intestinal valve. This structure is made up of six cells (White, 1988). One cell connects to the muscle cell at the posterior end of the pharynx, two are attached to the anterior intestinal cells and a layer of three cells lies in between. All of the cells are coupled to each other by desmosomes.

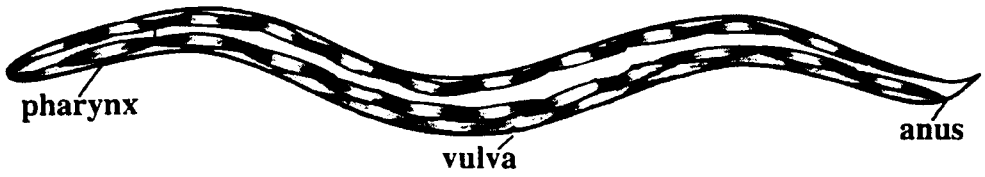
Intestinal cells are multi-functional. They secrete digestive enzymes into the lumen and absorb nutrients. Yolk protein synthesis occurs exclusively in the intestinal cells. The yolk proteins are secreted into the pseudocoelom and transported to the gonad to be incorporated into developing oocytes (Kimble and Sharrock, 1983). Intestinal cells are one of the major storage organs of the nematode body and contain a large number of granules with diverse functions. Some of these granules contain yolk proteins. A subset of gut granules are strongly autofluorescent under 300-400 nm light and appear to be lysosomes (Clokey and Jacobson, 1986). Some granules contain lipid, protein, and/or carbohydrate deposits.

6.4 Muscle structure and development

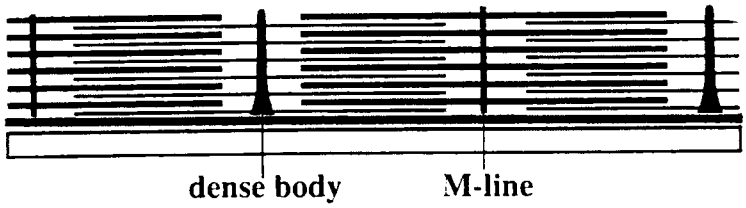
C. elegans body wall muscle extends from nose to tail and is arranged in four quadrants located left and right subdorsally and left and right subventrally (Figure 3). Each quadrant consists of a double row of elongated spindle-shaped muscle cells that run the length of the

Figure 3. *C. elegans* anatomy and muscle organization. A) Schematic of the position of body wall muscles in *C. elegans*. B) Diagrammatic view of the myofilament lattice, showing thin and thick filaments and their respective attachment structures, the dense bodies and M-lines. C) Schematic of a dense body with the components, including cuticle, hypodermis, basement membrane, cell membrane, integrin, talin, vinculin, α-actinin and actin indicated. Adapted from Mullen, 1998.

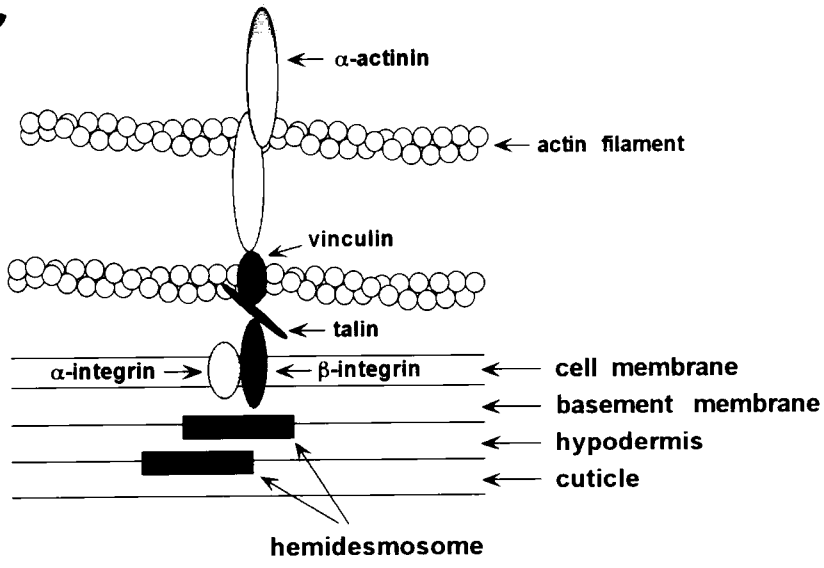
A


pharynx
vulva
anus

B


dense body
M-line

C


α-actinin
actin filament
vinculin
talin
α-integrin
β-integrin
cell membrane
basement membrane
hypodermis
cuticle
hemidesmosome

animal. Muscle cells are attached to the hypodermis through an extracellular matrix termed the basement membrane. This physical link between body wall muscle and the hypodermis through the basement membrane is critical in transducing the force of contraction to the cuticle of the nematode for locomotion.

6.4.1 Muscle composition

The basic structural unit of *C. elegans* muscle is analogous to the sarcomere of vertebrate muscle (reviewed in Waterston 1988). Thick filaments containing myosin are centrally placed and overlap with two sets of actin-containing thin filaments. The thick filaments are aligned by a structure called the M-line, and thin filaments are attached to dense bodies (Figure 3). Both dense bodies and M-lines are anchored to the basement membrane and serve as sites for the attachment of muscle cells to the hypodermis (Francis and Waterston, 1985).

Dense bodies and M-lines are attached to the basement membrane through transmembrane complexes. M-line complexes contain transmembrane receptors called integrins (Francis and Waterston, 1985) and the *unc-89* product (Benian *et al.*, 1996). Dense bodies are similar in structure to vertebrate focal adhesion complexes (Burridge *et al.*, 1997). They are anchored to the basement membrane through integrins (Figure 3; Gettner *et al.*, 1995). Integrins are heterodimeric molecules made up of one α- and one β-subunit. In *C. elegans*, α-integrin (*pat-2*) and β-1 integrin (*pat-3*) form the transmembrane integrin receptor (Williams and Waterston, 1989). Integrins are essential for mediating cell-extracellular membrane and cell-cell interactions. They also function to activate signalling pathways that regulate gene expression and can influence cell fate decisions (Burridge *et al.*, 1997).

Intracellularly, integrins are associated with vinculin (*deb-1*; Barstead and Waterston, 1989), talin (Moulder *et al.*, 1996) and α-actinin (*atn-1*; Barstead and Waterston, 1991; Francis and Waterston, 1985) to make up the dense body. These molecules in turn associate with the actin-containing thin filaments of the sarcomere. In vertebrates, a number of other cell adhesion and signalling molecules have been identified that are also associated with integrin (Burridge *et al.*, 1997). Extracellularly, integrins anchor dense bodies to the basement membrane.

Basement membranes are thin sheets of specialized extracellular matrix (ECM) material that are closely associated with cell membranes. They are important in cell adhesion, migration and differentiation. During morphogenesis, they are required to provide a surface for cell

attachment while tissues are organized, and to initiate cell polarity through interaction with cellular proteins and the cytoskeleton. In *C. elegans*, basement membranes cover the pseudocoelomic faces of the hypodermis, pharynx, intestine, gonad, and body wall muscles (White *et al.*, 1976).

Basement membranes are composed of type IV collagen, laminin, and proteoglycans (Paulsson, 1992; Yurchenco and Schittny, 1990). *C. elegans* genes for most of the basement membrane components have been identified. Type IV collagen is a heterotrimer of two α1 (*emb-9*) and one α2(IV) (*let-2*) chains (Sibley *et al.*, 1993). Laminin is a trimeric molecule, composed of three disulfide-bonded subunits, αβγ, that binds to integrin (Timpl and Brown, 1994). In *C. elegans*, the α chain (*epi-1*) and β chain (*lam-1*) have been identified (K. Joh, pers. comm.). Proteoglycans are a large family of proteins in vertebrates. The *C. elegans unc-52* gene encodes the homolog of the mammalian basement membrane heparan sulfate proteoglycan, perlecan (Rogalski *et al.*, 1993). Perlecan binds to many basement membrane components and to integrins. The association of integrin with perlecan (*unc-52*) is required for anchoring dense bodies and M-lines (Mullen, 1998). Another component of basement membranes is SPARC, a glycoprotein that can modulate the interaction of cells with the ECM (Lane and Sage, 1994; Schwarzbauer and Spencer, 1993).

Locomotion is accomplished by transmission of the force of contraction to the cuticle of the animal. For this to occur, the myofilament lattice is attached to the cuticle via a series of links between the basement membrane, the hypodermis and the cuticle. In vertebrates, adhesion of the hypodermis to the basement membrane and the cuticle is mediated by hemidesmosomes (Reviewed by Garrod, 1993). Hemidesmosomes are believed to play a key role in signal transduction pathways that lead to basal cell proliferation and epidermal differentiation. The transmembrane complex of hemidesmosomes is composed of integrin and BPAG2 (Hopkinson *et al.*, 1992; Stepp *et al.*, 1990). The protein HD1 is involved in attachment of the transmembrane complex to the intermediate filaments of the epidermal cell (Hieda *et al.*, 1992). Within the basement membrane, integrins are believed to be associated with kalinin (also called nicein) which binds laminin (Rouselle *et al.*, 1991).

In *C. elegans*, hemidesmosomes are associated with dense bodies in body wall muscle and the pharyngeal-intestinal valve (Waterston, 1988). They anchor the hypodermis to the basement membrane and the cuticle. Tonofilaments located in hypodermal cells adjacent to muscle cells connect to hemidesmosomes. Many of the components of hemidesmosomes have

not been identified, although the monoclonal antibody MH5 appears to react specifically with a hemidesmosomal protein (Francis and Waterston, 1991). Several other monoclonal antibodies, MH4 and MH46 appear to be hypodermal proteins that are involved in adhesion of the hypodermis to the basement membrane. Myotactin (*let-805*) is involved in maintaining the connection between muscle cells and hemidesmosomes and may be a component of hemidesmosomes (Hresko *et al.*, 1999). The transmembrane protein *mup-4* is believed to interact directly with the basement membrane and/or the cuticle to anchor the hypodermis to the ECM (L. Hong, pers. comm.). This function suggests that *mup-4* is also a component of hemidesmosomes.

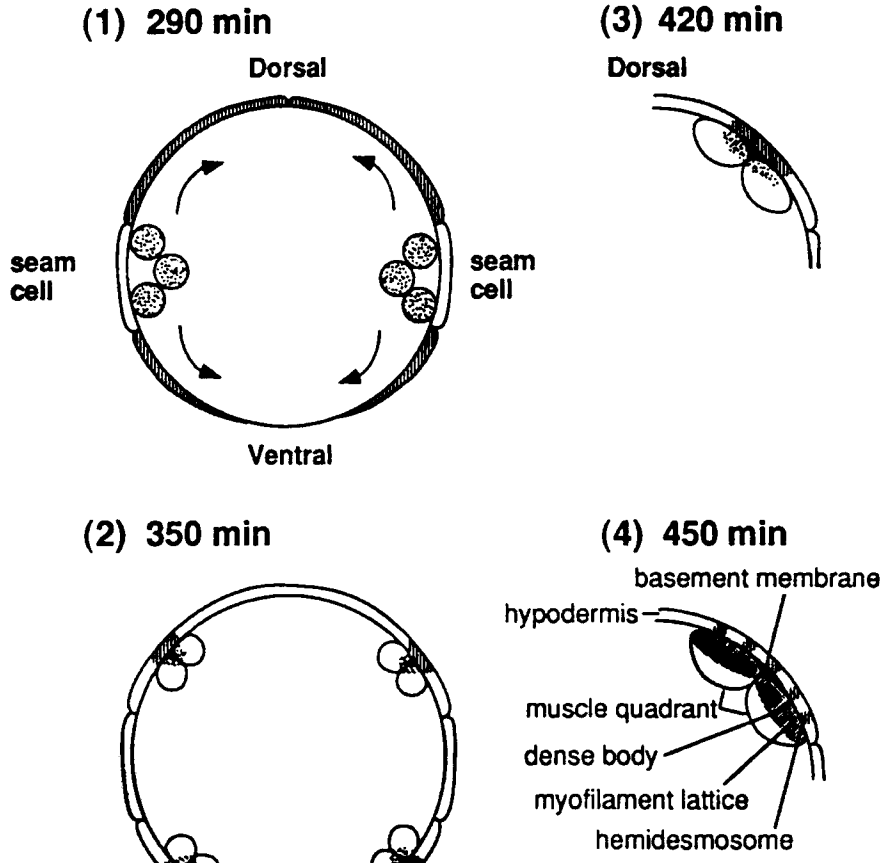
During post-embryonic development in *C. elegans*, the basement membrane and the cuticle undergo structural changes. The *unc-52* gene expresses tissue and temporal-specific perlecan isoforms (Mullen *et al.*, 1999) that must be incorporated into the basement membrane at different times during development. At the end of each larval stage, nematodes undergo a molt in which new cuticle is formed and old cuticle is shed. Thus, attachment of the cuticle to the hypodermis must be destroyed and reformed at each molt. The mechanisms involved in these processes are unknown.

6.4.2 Muscle development

Muscle development in *C. elegans* can be divided into four phases: cell fate decision, cell migration, myofilament lattice assembly, and muscle growth. During embryonic development, 81 of the 95 body wall muscle cells are born and establish attachments to each other and to the hypodermis. These attachments are maintained during larval molts.

Muscle structural proteins are first detected at around 290 minutes (Epstein and Bernstein, 1992). Two rows of myoblasts are initially located laterally along the seam cells (Figure 4; Sulston *et al.*, 1983). The myoblasts migrate onto dorsal and ventral surfaces, and organize into two dorsal and two ventral muscle quadrants by 350 minutes. Muscle components localize to the membranes where adjacent muscle cells contact one another and the underlying hypodermal cells (Hresko *et al.*, 1994). Hypodermal components accumulate in the regions adjacent to the muscle, and muscle cells flatten against hypodermis. At this point, muscle, basement membrane and hemidesmosome components become coextensive. The organization of sarcomeres begins at the basement membrane where two membrane-associated proteins, integrin and perlecan, colocalize and form part of the nucleation site for dense body and M-line

Figure 4. Embryonic muscle assembly in *C. elegans*. Summary of myofilament assembly in *C. elegans*, depicted in cross sections of embryos at various stages of development. 1) An embryo 290 minutes after first division. Myofilament components (dots) accumulated in muscle cells (circles) that are adjacent to seam cells. Muscle cells begin dorsal and ventral migrations. 2) 350 minute embryo. Myofilament components polarize to the plasma membrane at sites adjacent to other muscle cells and the hypodermis. Basement membrane and hemidesmosomal components are concentrated at these focal regions (black and hatching, respectively). 3) Close-up of muscle quadrant of 420 minute embryo. Muscle cells flatten and myofilament, basement membrane and hemidesmosome components are coextensive. 4) Close-up of muscle quadrant of 450 minute embryo showing organization of myofilament lattice and the extracellular anchorage. From Hresko *et al.*, 1994; Mullen, 1998; reviewed in Moerman and Fire, 1997.


(1) 290 min
Dorsal
seam cell
seam cell
Ventral
(3) 420 min
Dorsal
(2) 350 min
(4) 450 min
basement membrane
hypodermis
muscle quadrant
dense body
myofilament lattice
hemidesmosome

assembly. The assembly of dense bodies and M-lines is believed to occur by the stepwise addition of internal components from this nucleation site.

The organization of the hypodermal components into hemidesmosomes occurs coordinately with muscle assembly. The position of hemidesmosomes in the hypodermis is not dictated by the position of muscle structural components (Francis and Waterston, 1985).

The myofilament lattice is fully formed by 450 minutes (Hresko *et al.*, 1994) and larvae hatch with two sarcomeres in each muscle cell. During larval development, muscle cells increase in size, the number of sarcomeres per cell increases to around ten in adult muscle, and a few new body wall muscle cells are generated.

6.5 cis- and *trans*-splicing of *C. elegans* gene transcripts

C. elegans gene transcripts can undergo both *cis*- and *trans*-splicing (Krause and Hirsh, 1987; Blumenthal and Steward, 1997). The removal of introns by *cis*-splicing follows a process similar to that in other eukaryotes. Almost all introns begin with the dinucleotide GU (with the very rare use of GC) and end with the dinucleotide AG. This is referred to as the GU-AG rule. The 5' splice site consensus sequence G/GURAG (R=A or G) is well conserved in *C. elegans*. However, the 3' splice site consensus varies slightly from that in other eukaryotes, consisting of an extended, very highly conserved consensus sequence, UUUUCAG.

C. elegans also displays *trans*-splicing, in which a 22-nucleotide spliced leader (SL) sequence from a small nuclear RNA is attached to the 5' end of a mRNA precursor (Krause and Hirsh, 1987). The splice site for *trans*-splicing is the same as that for *cis*-splicing except that it lacks the 5' donor consensus site. A number of SL sequences have been identified. SL1 is the most common spliced leader used, and is attached near the 5' ends of most pre-mRNAs. SL2 is used at internal *trans*-splicing sites of polycistronic pre-mRNAs (Huang and Hirsch, 1989). Recently, SL3, -4 and -5 sequences have been identified, although the exact role of these additional SLs have not been elucidated (Ross *et al.*, 1995).

6.6 Operons in *C. elegans*

C. elegans contains numerous genes that are organized as operons (Blumenthal and Steward, 1997). In an operon, two or more structural genes are transcribed as a polycistronic message driven by a unique 5' promoter/enhancer region (Spieth *et al.*, 1993). The polycistronic message is converted to monocistronic mRNAs by conventional cleavage and polyadenylation of the 3' ends. Additionally, the pre-mRNAs are *trans*-spliced to a spliced

leader. The first gene of the polycistronic message is usually *trans*-spliced to SL1 while genes in downstream positions are generally SL2 spliced; however, they too, can be SL1 spliced (Spieth *et al.*, 1993; Zorio *et al.*, 1994).

7. UBC-2 CHARACTERIZATION

The *C. elegans* gene encoding the ubiquitin-conjugating enzyme, *ubc-2* has been cloned and partially characterized (Zhen, 1995). UBC-2 is a 16.7 kDa protein that belongs to a highly conserved gene family of class I E2s which includes yeast UBC4 and UBC5 (Seufert and Jentsch, 1990), *Drosophila UbcD1* (Treier *et al.*, 1992), *Arabidopsis* UBC8 (Girod *et al.*, 1993) and human UbcH5 (Jensen *et al.*, 1995; Scheffner *et al.*, 1994). *ubc-2* bears striking sequence similarity to yeast UBC4/5 and *Drosophila UbcD1*, at 90% and 98%, respectively. *C. elegans ubc-2* was shown to be the functional homolog of yeast UBC4, as it can rescue the growth defect of yeast *ubc4ubc5* double mutants (Zhen *et al.*, 1993).

Northern analysis indicates that *ubc-2* is constitutively expressed at all life stages. The protein is present at each life stage, although there is some variation in the pattern of UBC-2 expression during development (Zhen *et al.*, 1996). In transgenic animals that expressed *ubc-2/lac Z* fusions, the fusion protein was expressed in a tissue general pattern in embryonic and larval stages that became restricted to the nervous system in adults. This was confirmed immunofluorescent staining of adults using the anti-UBC-2 antibody. However, a detailed analysis of the UBC-2 staining pattern in wild type animals was not undertaken.

A complementation screen of previously identified essential genes in the vicinity of *ubc-2* revealed that *ubc-2* corresponds to *let-70*, a gene essential for larval development (Zhen *et al.*, 1996). Two recessive alleles of *let-70* were identified; both are L2/L3 larval lethal. The molecular nature of the mutations was determined (Figure 5). *let-70(s1132)* is a C to T transition that results in the substitution of histidine 75 for tyrosine. The *let-70(s689)* mutation is a G to A transition at the splice donor site of the fourth intron.

8. THE PRESENT STUDY

In this study, a detailed analysis of the *let-70(s1132)* and *let-70(s689)* mutant phenotypes was undertaken. As described in Section I.7, *let-70* had been previously shown to be L2/L3 lethal (Zhen *et al.*, 1996); however, the specific developmental defects that result in lethality were not examined. Thus, specific tissue and structural defects were examined using Nomarski optics, DAPI staining and immunofluorescent staining. To identify the putative null

phenotype of *let-70*, double-stranded RNA interference was employed. A complementation screen of unknown lethal mutants was undertaken to identify additional alleles of *let-70*, and one putative allele was partially characterized. The molecular nature of the *let-70(s689)* mutation was identified by mutant cDNA analysis. *let-70 smg-1* double mutants were constructed to determine if *smg*-mediated mRNA surveillance was a factor in the phenotype of *let-70(s689)* mutants. Through the use of immunofluorescent staining with UBC-2 antibodies and the construction of a transgenic line carrying a *ubc-2*::GFP fusion protein as an extrachromosomal array, the wild type UBC-2 expression pattern was determined. Once identified, the developmental expression pattern of UBC-2 was then compared with mutant UBC-2 expression patterns and the mutant phenotypes.

To determine the minimal rescuing fragment, attempts were made to rescue the *let-70* lethality by standard DNA-mediated transformation of worms using a number of *ubc-2* constructs that contained different amounts of upstream and downstream sequence. To provide robust expression in the germline and soma, an excess of random genomic DNA was included in the injection solutions. Using similar techniques, a putative temperature-sensitive allele of *let-70* was introduced into mutant *let-70* strains. The resultant transgenic lines were examined for the ability of to rescue *let-70* mutants in a temperature-sensitive manner.

To identify evolutionarily conserved sequences, the *C. briggsae* homolog of *let-70* was isolated and characterized. Approximately 30 kb of sequence surrounding *ubc-2* was compared between *C. elegans* and *C. briggsae*. The structure of the operon containing *ubc-2* was determined, and *trans*-splicing of the genes within the operon was examined.

```
  1 ATG GCT CTC AAA AGA ATC CAG AAG gtaaggtttcaacacttaacaacaataa 52
  1 M   A   L   K   R   I   Q   K                                 8

 53 aattattatgtacgtttcag GAA CTC CAA GAT CTC GGC CGT GAT CCA CCC 102
  9                      E   L   Q   D   L   G   R   D   P   P   18

103 GCA CAA TGC TCC GCT GGA CCA GTT GGT GAT GAT TTG TTC CAT TGG  147
 19 A   Q   C   S   A   G   P   V   G   D   D   L   F   H   W    33

148 CAA GCT ACG ATT ATG GGC CCA CCA GAG TCT CCC TAT CAG GGA GGT  192
 34 Q   A   T   I   M   G   P   P   E   S   P   Y   Q   G   G    48

193 GTC TTC TTC CTC ACT ATC CAC TTC CCA ACA GAC TAT CCA TTC AAA  237
 49 V   F   F   L   T   I   H   F   P   T   D   Y   P   F   K    63

238 CCA CCA AAG gtattgatcgaaattggaaaaaaataaatttaatttgttttcttcag  293
 64 P   P   K                                                    66

                          s1132  T (Y)
                                 ↕
294 GTT GCC TTC ACC ACT CGA ATT TAT CAT CCG AAC ATC AAT TCA AAC  338
 67 V   A   F   T   T   R   I   Y   H   P   N   I   N   S   N    81

339 GGA AGC ATC TGC CTT GAC ATT CTC CGT TCG CAG TGG TCG CCG GCT  383
 82 G   S   I   C   L   D   I   L   R   S   Q   W   S   P   A    96

384 CTG ACC ATT TCG AAA G gttgatattatcattattgttcgcgttctaacttttaa 437
 97 L   T   I   S   K   V                                       102

438 ttttcag TT CTG CTT TCG ATC TGC TCG CTG CTC TGT GAT CCA AAT   482
103           L   L   S   I   C   S   L   L   C   D   P   N    114

483 CCG GAT GAT CCA CTT GTG CCA GAG ATT GCA CGC ATC TAC AAG ACG  527
115 P   D   D   P   L   V   P   E   I   A   R   I   Y   K   T   129

          s689  a
                ↕
528 GAT CGT GAA AG gtgaatttagattttcttttaactactaaaaataatcgattttca 583
130 D   R   E   R                                               133

584 g G TAC AAT CAA TTG GCT AGA GAA TGG ACG CAA AAG TAC GCT ATG  627
134     Y   N   Q   L   A   R   E   W   T   Q   K   Y   A   M   147

628 TGA ggaggctaacaccattcatataagaacgcagccaaaccaatcaataaaccatgtgt 686
148 *                                                           148
```

Figure 5. Sequence of *let-70(ubc-2)* wild type and mutant alleles. Exon sequences are shown in uppercase, intron and 3' non-coding sequences in lower case. Protein sequence is indicated in upper case below the nucleotide sequence, in single letter code. The nucleotide and amino acid changes for *let-70(s689)* and *let-70(s1132)* are indicated. Possible stop codon for *let-70(s689)* is underlined.

II. MATERIALS AND METHODS

1. NEMATODE STRAINS AND CULTURE CONDITIONS

1.1 Culture and maintenance of strains

All nematode strains were cultured on NG plates (0.3% NaCl, 0.25% tryptone, 5 mg/ml cholesterol, 1 mM $MgSO_4$, 1 mM $CaCl_2$, 25mM KH_2PO_4 pH 6.0, 1.7% agarose) seeded with *Escherichia coli* OP50 as described by Brenner (1974). Animals were maintained at 20°C unless otherwise indicated. Nematodes were maintained on NG plates by transferring L4 larvae to new plates each generation. Large scale growth of *C. elegans* was performed in liquid culture (Sulston and Brenner, 1974) using frozen *E. coli* MRE-600 (purchased from the Fermentation Facility, University of Alabama at Birmingham) as a food source.

For short term storage, plates of worms were placed at 15°C and left to starve and develop into dauer larvae. Dauer larvae can survive without food for up to three months and can be revived by transferring to seeded plates (Riddle, 1988). Strains were frozen in liquid nitrogen for long term storage. A population of animals was grown to contain a high proportion of L2 larvae, washed off plates with M9 buffer (3% KH_2PO_4, 6% Na_2HPO_4, 5% NaCl, 1 mM $MgSO_4$), mixed with an equal volume of freezing buffer (30% glycerol in M9 buffer), aliquoted and frozen at -80°C for several days before transfer to liquid nitrogen. In general, only L2 larvae survive freezing (Riddle, 1988).

1.2 Strains utilized in this study

The genetic nomenclature used follows guidelines set by the *C. elegans* research community (Hodgkin, 1995). Appendix A lists the nematode strains used in this work.

All *let-70* mutants were balanced over *nT1 (IV;V)*, a translocation that recombinationally balances the right portion of LG IV and the left portion of LG V (Ferguson and Horvitz, 1985). The *unc-22(s7)* allele was used as a marker to identify individuals carrying a *let-70* allele (Moerman and Baillie, 1979). *unc-22(s7)* is a conditional semidominant mutation where *unc-22(s7)/+* individuals have a "twitcher" phenotype when placed in 1% nicotine solution but are otherwise phenotypically wild type. Homozygous *unc-22(s7)* individuals are unconditional twitchers, and wild type homozygotes are contracted and paralyzed in nicotine. In this study, heterozygous *let-70/nT1* individuals were identified and

selected by placing worms in a drop of 1% nicotine. After one minute, worms that were twitching were transferred to new plates.

For simplicity, nematode strains will be referred to by their first mutation and allele designation only, i.e. *let-70(s1132)*, henceforth. Linked mutations are presumed to be present unless otherwise noted. Transgenic worms will be referred to by their strain designation.

2. NEMATODE ANALYSIS AND GENETICS

2.1 Nematode matings

Nematode crosses were set up on mating plates, consisting of a small spot of OP50 bacteria in the center of an NG plate. Four L4 hermaphrodites were placed on the plate along with eight males. Matings were allowed to proceed overnight at 20°C and then hermaphrodites were transferred to separate plates. Plates were examined several days later for the presence of male progeny, indicating that mating had been successful.

2.2 Strain outcrosses

The three strains were outcrossed prior to analysis: 1) *let-70(s1132) unc-22(s7) unc-31(e169)/nT1(IV);+/nT1(V)*, 2) *let-70(s689) unc-22(s7)/nT1(IV);+/nT1(V)*, and 3) *let-x(s2293) unc-22(s7) lev-1(x22)/nT1(IV);+/nT1(V)*. Hermaphrodites were mated to N2 males and outcrossed hermaphrodite progeny were selected in 1% nicotine. The mating of nicotine-selected hermaphrodites to N2 males continued for 7-10 generations. At each generation, the plates were examined for homozygous arrested *let-70* worms to ensure that the *let-70* allele had not been removed from the strain. To rebalance the outcrossed *let-70* strains over *nT1*, nicotine-selected males were mated to *dpy-13(e184sd)/nT1(IV);unc-42(e270)/nT1(V)* hermaphrodites. *dpy-13* ("dumpy") mutants are shorter than wild type and *unc-42* worms exhibit a "kinker" phenotype. A number of nicotine-selected cross progeny were set to individual plates. Four days later, the plates were examined for *dpy-13* or *unc-42* progeny. Plates were retained if no dpy or unc progeny were seen. The population was maintained several generations to ensure that the *dpy-13* and *unc-42* mutations were not present. The outcrossed and rebalanced strains were utilized in all subsequent analyses.

2.3 Construction of transgenic *let-70* strains

Extrachromosomal arrays were placed in mutant backgrounds by mating to both *let-70(s1132)* and *let-70(s689)* strains. The transgenic strains that were used are listed in Appendices B and C. Stably transformed hermaphrodites with a 'roller' phenotype were mated to *let-70/+* males. F1 roller progeny that twitched in 1% nicotine were set to separate plates. These worms carried the extrachromosomal array and the genotype was *let-70/+*. The progeny of these worms were examined for animals that twitched (*let-70/let-70*) and rolled (carried the extrachromosomal array). Twitcher/roller F2 animals were set individually and their development was followed to determine if the *let-70* mutation was rescued by the transgene.

For the putative temperature-sensitive *let-70* transgenes, half of the selected F1 worms were placed at 15°C and selected F2's were also maintained at 15°C. The other half of the selected F1's were placed at 25°C for the duration of the experiments.

2.4 Construction of *let-70 smg-1* double mutants

To generate *let-70 smg-1* double mutants, *let-70(s689)* hermaphrodites were crossed to N2 males. Homozygous *smg-1* hermaphrodites were mated to *let-70(s689)/+* males and nicotine-selected cross progeny were set to separate plates and placed at 25°C. A large number of twitcher F2 progeny were set separately, and development at 25°C was followed. Of the twitcher F2 progeny, ¼ should be *let-70(s689) unc-22(s7)/ let-70(s689) unc-22(s7);smg-1/smg-1* homozygotes, while the rest should be *let-70(s689) unc-22(s7)/ let-70(s689) unc-22(s7);smg-1/+*.

2.5 Complementation crosses

Complementation tests were performed on lethal mutations that were tightly linked to *unc-22*. The procedure used to screen the mutants is shown in Figure 6. Hermaphrodites of the genotype *let-70(s689) unc-22(s7)/nT1(IV);+/nT1(V)* were mated to wild type N2 males. Heterozygous male progeny were picked and crossed to either *let-x unc-22(s7) lev-1(x22)/ nT1(IV);+/nT1(V)* (Marra, 1994) or *let-x unc-22(s7) unc-31(e169)/nT1(IV);+/nT1(V)* (M.E. Green and D.V. Clark, unpubl.) hermaphrodites. If no fertile twitcher progeny were found, then the lethal mutation was considered to be a putative allele of *let-70*.

Putative alleles of *let-70* were further tested for complementation by mating to *let-70(s1132)* animals following the protocol described above. The progeny were examined for fertile twitcher hermaphrodites.

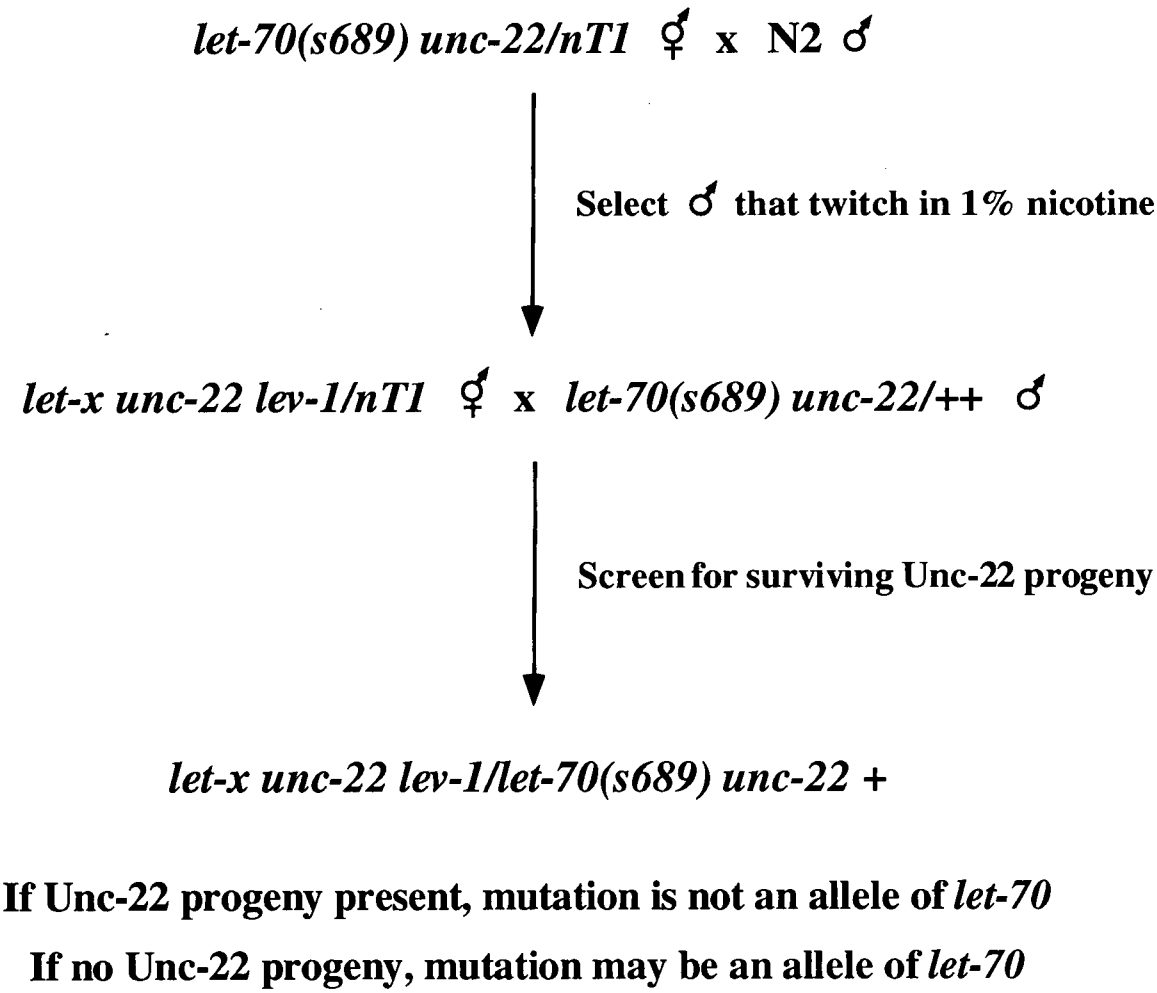


Figure 6. Non-complementation screen for *let-70* alleles. Heterozygous *let-70(s689) unc-22* males were mated to *let-x unc-22* hermaphrodites. The progeny were screened for surviving Unc-22 animals, indicating that the mutation is not an allele of *let-70*. If no Unc-22 progeny were present, the mutation was considered a putative allele of *let-70*, and was further examined.

2.6 DAPI staining

To DAPI stain a population of nematodes, animals were washed off a plate with M9 buffer and resuspended in acetone. After permeabilization for 4 minutes, animals were washed several times with M9 and resuspended in DAPI solution (33 ng/ml). An equal volume of mounting medium (2.5% DABCO, 90% glycerol, 0.5% sodium azide, 7% 10X TBE pH 8.0) was added and the stained animals were examined under UV fluorescence.

To DAPI stain individuals, animals were picked into a drop of dH_2O on a polylysine coated slide. Most of the dH_2O was evaporated, and drops of acetone were added such that the sample was incubated in acetone continually for 4 minutes. DAPI (33 ng/ml) followed by an equal volume of mounting medium was added. Animals were examined by UV fluorescence microscopy.

2.7 Microscopy

For phenotypic analysis, nematodes were examined on a Zeiss IM35 microscope equipped with fluorescence and Nomarski optics. Magnification ranged from 200-1000X. Photographs were taken using an attached 35 mm camera. To make figures, photographs were scanned into a Macintosh 8600/200 computer using an Umax Astra 122OS flat bed scanner with Vistascan (version 2.4.3) software.

Immunofluorescence was viewed on either a Zeiss Axioskop-2 or a Nikon Optiphot-2 microscope. The Axioskop-2 fluorescence was equipped with Attoarc, and magnification ranged from 200-1000X. Images were captured with a Sony 950 CCD camera using the Northern Eclipse (version 5.0) application.

The Optiphot-2 was equipped for both epifluorescence and confocal laser scanning microscopy (CLSM). Confocal images were captured on a BioRad MRC 600 system using the CoMOS 7.0a application. K1 and K2 filters were used for dual channel collection and each channel was captured separately. For most samples, the confocal aperture was set between 2 and 3 to provide the best compromise between brightness and confocality. The neutral density filter was usually set to 1, which attenuates light from the laser to 30% of its maximal intensity. A setting of 2 (3%) was used for some samples, depending on the brightness of the fluorescence. Scan speed was set to normal and a kalman filter (4 scans) was used to reduce background noise during collection.

For image capture, a Z-series was collected at 0.2 micron intervals for the full depth of the specimen. For embryos, collections were 400x400 pixel images. For larvae and adults, collections ranged from 400x500 to 700x500 pixel images.

Z-series were transferred to a Power Macintosh 8600/200 for data analysis and to generate projections. Z-series were imported into NIH Image 1.62 for viewing and manipulations such as making projections, rotating images and generating vertical sections.

Projections produced in NIH Image and all scanned or captured images were imported into Adobe Photoshop (version 4.0) for assembly into figures for publication. Images were cropped to the desired size and resolution, colorized, arranged and labelled. Figures were printed on a Codonics NP-1600 printer.

3. GENERAL DNA AND RNA TECHNIQUES

3.1 Restriction endonuclease digestion of DNA

DNA samples (1-10 μg) were digested with a variety of restriction endonucleases using conditions recommended by the manufacturers. Restriction enzymes were obtained from Promega, GIBCO/BRL and Pharmacia. Restricted samples were loaded directly onto horizontal agarose gels for analysis. To purify samples, the DNA band of interest was cut out of the gel and purified using the Qiaex II kit (Qiagen, Inc.). At times, the sample was purified directly after digestion by precipitating the DNA with three volumes of ethanol. The DNA was collected by centrifugation at 12,000 rpm for 15 minutes, washed in 75% ethanol, dried and resuspended in dH_2O or TE (10 mM Tris-HCl; 1 mM EDTA, pH 8.0).

3.2 Enzymatic manipulations of DNA

Ligations were performed using T4 ligase (Gibco/BRL) following the manufacturer's instructions. Nucleotide overhangs resulting from restriction digests were filled in using Klenow DNA polymerase (Pharmacia) following the manufacturer's directions.

3.3 Purification of plasmid DNA from *E. coli*

Five ml cultures of *E. coli* containing the plasmid of interest were grown overnight at 37°C in LB broth supplemented with 100 μg/ml ampicillin. Plasmid DNA was purified following the method of Morelle (1989), or using the Qiaprep spin miniprep kit (Qiagen Inc.) following the manufacturer's instructions.

3.4 Bacterial transformation

Purified plasmid DNA and ligation products were transformed into competent *E. coli* DH5α cells (GIBCO/BRL) following the manufacturer's instructions. Competent BL21(DE3) cells (Novagen) were prepared by $CaCl_2$ treatment and transformed by standard protocols (Sambrook *et al.*, 1989).

3.5 Polymerase chain reaction

Polymerase chain reactions (PCR) were performed in a 25 µl volume using PCR beads (Pharmacia) containing 10 mM Tris-HCl pH 9.0, 50 mM KCl, 1.5 mM $MgCl_2$, 200 µM of each dNTP and 1.5 units of *Taq* DNA polymerase. Primers were added to a final concentration of 2 pM. Oligonucleotides utilized in this study are listed in Appendix D. Reactions were overlaid with mineral oil. In general, the PCR conditions were 95°C for 3 minutes; 30 cycles of 95°C for 30 seconds, 60°C for 30 seconds, 72°C for 1 minute; 72°C for 10 minutes. The annealing temperature varied between 58°C and 62°C, depending on the length and GC content of the oligonucleotides being used for amplification. DNA samples (1-5 µl) were added to the reaction mixture during the initial period at 95°C. Reactions were carried out in the TwinBlock™ system thermal cycler (ERICOMP Inc.).

3.6 Nematode PCR

3.6.1 Single embryo PCR

For single embryo PCR, one egg was picked using a piece of sterile fishing line (10 lb. test) and placed in 5 µl PCR-lysis solution (50 mM KCl, 10 mM Tris-HCl pH 8.5, 2.5 mM $MgCl_2$, 0.45% Tween 20, 0.01% gelatin) supplemented with 1.0 µl Proteinase K (20 mg/ml). The mixture was incubated at 60°C for 1.5 hours followed by 95 °C for 15 minutes. The entire solution was used in PCR reactions.

3.6.2 Long Distance PCR

Long distance PCR was performed on genomic DNA and on pools of 2-3 homozygous *let-x(s2293)* arrested embryos that had been treated with Proteinase K. Reactions were run in 50 µl volumes containing 1X Boehringer Mannheim Buffer 3, 0.7 mM of each dNTP, 25 pmol of each primer and 2.6 units Expand *Taq* polymerase (Boehringer Mannheim). Reaction conditions were 94°C for 3 minutes; 30 cycles of 94°C for 15 seconds, 57°C for 45 seconds and

68°C for 3 minutes with a 30 second extension following each cycle; 72°C for 10 minutes. The PCR reactions were run on a Perkin Elmer DNA thermal cycler.

3.7 DNA sequencing

Sequencing of double stranded DNA by the dideoxy chain termination method (Sanger *et al.*, 1977) was performed using the T7 Sequenase version 2.0 DNA sequencing kit (U.S. Biochemicals) following the manufacturer's procedure. Samples were analyzed on Applied Biosystems (ABI) model 373 stretch or ABI Prism 377 DNA sequencers. This service was provided by the NAPS Unit Biotechnology Laboratory, University of British Columbia.

3.8 Genomic DNA isolation

C. elegans genomic DNA was prepared from fresh larvae and adults or frozen embryos. Approximately 1 gram of frozen embryos or 0.5 gram of fresh worms were resuspended in Proteinase K buffer (100 mM Tris-HCl pH 8.0, 50 mM EDTA, 200 mM NaCl, 1% SDS, 0.15 mg/ml Proteinase K). The samples were incubated for three hours at 65°C, extracted twice with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) then extracted once with an equal volume of chloroform. Three volumes of 95% ethanol were added to precipitate the DNA. DNA was collected by centrifugation at 10,000 x g for 30 minutes, washed with 70% ethanol, dried and resuspended in dH_2O.

For the genomic Southern blot, nematodes were grown in 250 ml liquid culture and genomic DNA was prepared using a salt extraction protocol. Worms in liquid culture were transferred to 250 ml centrifuge bottles, settled, and most of the solution was aspirated. Animals were resuspended in PK buffer (10 mM Tris-HCl pH 8.0, 10 mM EDTA, 1% SDS, 200 µg/ml Proteinase K), incubated at 37°C overnight with shaking, and NaCl was added to a final concentration of 1.44 M. Samples were mixed for 30 seconds, centrifuged at 3000 x g for 35 minutes and the supernatant was transferred to a new tube. Three volumes of 95% ethanol were added, and the solution was gently rocked to precipitate the DNA. DNA was spun onto a glass rod, transferred to 70% ethanol and collected by centrifugation at 10,000 x g for 30 minutes. The DNA was dried and resuspended in dH_2O.

3.9 Random primer extension

DNA probes (200 ng) were labelled with ^{32}P by random hexamer extension using the T7 QuickPrime kit (Pharmacia) following the manufacturer's directions. The labelled probe

was separated from unincorporated ^{32}P on a 1 ml Sephadex G-50 spin column, prepared in a 1 cc syringe. A small amount of sterile glass wool was placed in the bottom of the syringe and TE-equilibrated Sephadex G-50 was added to the syringe to a volume of 1 cc. The column was washed several times with TE by centrifuging at 2000 x g for 1 minute. The labelled probe was applied to the bed of the column, spun for 1 minute at 3000 x g and collected in a 1.5 ml tube. One µl was spotted on a glass fibre filter and incorporation of ^{32}P was measured by scintillation counting. In general, DNA probes labelled in this manner had a specific activity of 2.5-4 x 10^5 cpm/ng.

3.10 Genomic Southern blot

Southern blots were performed on genomic DNA and RT-PCR products. Genomic DNA (4 µg) was digested with various restriction endonucleases prior to separation by electrophoresis on 0.8%-1.5% agarose gels. RT-PCR products were loaded directly onto agarose gels. The DNA was transferred to Hybond N nylon membranes (Amersham) following the alkaline transfer method of Westneat (1988). ^{32}P-labelled DNA probes were generated by random primer extension (see Section II.3.9). Hybridizations were carried out overnight at 65°C in 7% SDS, 1 mM EDTA pH 8.0, 1% BSA, 0.263 M Na_2HPO_4. The membranes were washed twice in 2XSSC/0.1% SDS (300 mM NaCl, 30 mM for 15 minutes at room temperature then once in 2XSSC. The membrane was exposed on a PhosphorImage screen for several hours to several days. The image was scanned using a Molecular Dynamics PhosphorImager SI and digitized onto a MacIntosh 7600 using the IPLab gel scientific image processing application, version 1.5e.

3.11 Total RNA extraction

Total RNA was extracted from wild type *C. elegans*, wild type *C. briggsae*, homozygous *let-70(s1132)* and homozygous *let-70(s689)* animals. Approximately 300 worms were individually picked into 180 µl DEPC-treated H_2O. Acid-washed glass beads were added to fill a 100 µl volume and two volumes of lysis buffer (4 M guanidine isothiocyanate, 0.13% sarkosyl, 33 mM Tris-HCl, 0.5% β-mercaptoethanol, 6.7 mM EDTA, pH 8.0) were added. One volume of phenol:chloroform (1:1) was added and samples were mixed vigorously (Vortex) for four minutes and centrifuged at 10,000 x g for 10 minutes. The upper aqueous layer was transferred to a new tube and re-extracted with chloroform. RNA was precipitated by the

addition of 1/10 volume of DEPC-treated 3 M NaOAc and three volumes of 95% ethanol, collected by centrifugation, washed with 70% DEPC-treated ethanol, dried and resuspended in DEPC-treated H_2O.

3.12 First strand cDNA synthesis

First strand cDNA was synthesized from total RNA extracted from whole worms following the protocol provided with the reverse transcriptase enzyme (BRL Superscript II, Bethesda, MD). Briefly, 1 μg of RACE 36 primer was added to 10 μg total RNA. The mixture was heated to 70°C and immediately placed on ice. First strand buffer was added to a 1X concentration, along with 10 mM DTT and 0.5 mM of each dNTP. The reaction mix was heated to 42°C, Superscript II reverse transcriptase was added and the reaction incubated at 42°C for 50 minutes. The enzyme was denatured by heating to 70°C for 15 minutes. First strand cDNA prepared in this manner was used in PCR reactions.

3.13 Synthesis of double-stranded RNA

Double-stranded RNA was synthesized following the method of Fire et al. (1998). The coding region of *ubc-2* that had been subcloned into the phagemid pBSIIKS(+) (Promega) was used as a template for RNA synthesis. Template DNA was linearized with *Hind* III or *Pst* I and purified as described in Section II.3.1. All subsequent manipulations were carried out in an RNase-free environment. For sense-strand RNA synthesis, 1 μg of *Hind* III-restricted template DNA was incubated at 37°C for 2 hours in 1X TSC buffer (Promega) containing 0.125 mM each of rATP, rGTP, rCTP and rUTP, 5 mM DTT, 1.0 unit RNasin (Promega) and 10 units T7 polymerase. To make anti-sense strand RNA, 1 μg of *Pst* I-restricted template DNA was incubated at 25°C for two hours in the same solution as described above except that T7 polymerase was replaced with 10 units of T3 polymerase. The two reactions were combined and stopped by the addition of Stop buffer (1 M NH_4Ac, 10 mM EDTA, 0.2 % SDS) and 6 μg glycogen as a carrier. The solution was extracted once with an equal volume of phenol:choroform (1:1) and once with an equal volume of chloroform. The solution was incubated at 68°C for 10 minutes, then transferred to 37°C for 30 minutes to allow the RNA strands to anneal. The double-stranded RNA was precipitated by adding three volumes of ethanol, collected by centrifugation at 10,000 x g for 10 minutes, washed with 70% ethanol, dried and resuspended in RNase-free TE. Formation of predominantly double-stranded RNA

was confirmed by agarose gel electrophoresis of a small amount of sense-, anti-sense- and double-stranded material. Double-stranded RNA prepared in this manner was loaded directly into needles and injected into young adults.

3.14 Computer analysis

Comparison of *C. elegans* and *C. briggsae* genomic DNA sequences was performed using the Dotter application (Sonnhammer and Durbin, 1996) and Genefinder (P. Green and L. Hillier, unpubl.). The *C. elegans* M7 + Y5F2 sequence used for analysis was made by combining the sequence of cosmid M7 (Z68337) and YAC Y5F2A (Z98871). The sequence for analysis was reverse complemented for the alignments, as *ubc-2* was encoded in the opposite orientation to that presented in the ftp site. The *C. briggsae* G47J11 sequence was obtained from the Washington University Genome Sequencing Center web site: http://genome.wustl.edu/ gsc/index.html.

4. PROTEIN AND IMMUNOLOGICAL TECHNIQUES

4.1 Overexpression and purification of UBC-2 fusion protein

A *ubc-2* expression construct in pRSETC (Invitrogen) was used to express the 6X HIS-UBC-2 fusion protein. Previous experiments had shown that a small amount of expressed 6X HIS-UBC-2 was soluble (Zhen, 1995). The pRSETC-UBC-2 construct was transformed into BL21(DE3) cells and fresh transformants were inoculated into 10 ml of LB/ampicillin/ kanamycin medium and grown overnight at 37°C. One litre of the same medium was inoculated 1:100 with the overnight culture and incubated at 37°C with shaking until an OD_{600} reading of 0.7 was reached. Cells were induced with 2 mM IPTG, grown an additional two hours and harvested by centrifugation at 4,000 x g for 10 minutes.

Soluble 6X HIS-UBC-2 fusion protein was isolated under non-denaturing conditions at 4°C. β-mercaptoethanol was added to all solutions used in the purification process to a final concentration of 10 mM, to prevent oxidation of the active site cysteinyl residue. The cell pellet was dissolved in sonication buffer (50 mM NaH_2PO_4, 300 mM NaCl, pH 8.0), frozen in a dry ice/ethanol bath, thawed in cold water and sonicated on ice (30 second bursts/1 minute cooling for a total of 6 minutes). The sample was passed through a 21 gauge syringe needle several times to shear the DNA and centrifuged at 10,000 x g for 20 minutes to pellet the cell debris. The supernatant was gently mixed with Ni-NTA resin for one hour to allow the 6X

HIS-UBC-2 fusion protein to bind to the resin. The resin was washed several times with sonication buffer and Wash buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10% glycerol, pH 5.0). The protein-resin mixture was loaded onto a column and washed with Wash buffer until the A_{280} of the flow-through was less than 0.1. Bound proteins were eluted with a pH 5.0-pH 4.0 gradient in Wash buffer. Samples were dialyzed in Wash buffer to bring the pH of the solution up to pH 8.0. This procedure resulted in the purification of 4-5 mg of 6X HIS-UBC-2 fusion protein.

4.2 Preparation of *C. elegans* protein extract

To extract proteins from nematodes for SDS-PAGE analysis, 50 mutant or wild type individuals were picked into a drop of M9 buffer in the lid of a 1.5 ml microcentrifuge tube. The drop containing the worms was quickly spun into the tube and 1X Laemmli sample buffer (50 mM Tris-HCl pH 6.8, 100 mM DTT, 2% SDS, 10% glycerol, 0.1% bromophenol blue) was added. Samples were boiled for 20 minutes prior to loading onto a 12.5% SDS-PAGE gel.

4.3 SDS-polyacrylamide gel electrophoresis

Discontinuous SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was used to separate protein samples (Laemmli, 1970). Samples were dissolved in 1X Laemmli sample buffer, boiled for 5-20 minutes and loaded onto SDS-PAGE gels. Gels were poured and run using the Mini-PROTEAN II apparatus (Bio-Rad) according to the protocols described by the manufacturer.

4.4 Western blot analysis

Proteins were separated on SDS-PAGE gels and electroblotted onto Immobilon-P membranes (Millipore). Prior to transfer, the gel and membrane were soaked in transfer buffer (25 mM Tris-HCl pH 8.5, 192 mM glycine, 20% methanol) for 10 minutes. The gel and membrane were sandwiched between sheets of pre-soaked filter paper and placed in the transfer apparatus. The transfer was carried out at 250 mA for one hour or 50 mA overnight. After transfer, the membrane was rinsed with dH_2O.

4.5 Immunostaining of Western blots

Western blots were blocked with 10% milk powder in TBS-Tween (140 mM NaCl, 20 mM Tris-HCl, 0.05% Tween-20, pH 7.5) for one hour. Primary antibody was added (1:5000

dilution in TBS-Tween for anti-UBC-2 antibody) and the blot was incubated for one hour. The blot was washed three times in TBS-Tween, incubated in secondary antibody (1:10,000 dilution in TBS-Tween of peroxidase-labelled anti-rabbit antibody; Amersham) for 30 minutes and washed three times in TBS-Tween. The protein-antibody complexes were visualized using the enhanced chemiluminescence kit (ECL-Amersham).

5. IMMUNOFLUORESCENCE STAINING

5.1 Immunostaining of embryos

Embryos were prepared and stained as described by Goh and Bogaert (1991) with some modifications. To obtain mutant embryos, *let-70(s1132) unc-22(s7) unc-31(e169)/nT1(IV); +/nT1(V)* or *let-70(s689) unc-22(s7)/nT1(IV);+/nT1(V)* hermaphrodites were crossed to N2 males. Outcross hermaphrodite progeny were set to 100 mm NG plates and maintained until large numbers of embryos had accumulated. Outcrossing removed the *nT1* balancer from the mutant population and prevented the mis-identification of *nT1* aneuploids as *let-70* mutant embryos.

Worms and embryos were washed off plates and treated with an alkaline sodium hypochlorite solution (25% bleach, 15 mM NaOH) to dissolve worms and release any unlaid eggs. Embryos were washed several times in M9 buffer, fixed in 3% formaldehyde/PBS, washed in dH_20 and resuspended in -20°C methanol. The embryos were rehydrated through a methanol/TBS series (75, 50 and 25%), washed several times in TBS and incubated in block buffer (2% milk powder in TBS-Tween). Primary antibodies were added and embryos were incubated at 4°C overnight. Samples were extensively washed in TBS-Tween, resuspended in block buffer and fluorescently-labelled secondary antibodies were added. Samples were incubated at room temperature for two hours to overnight. In some experiments, DAPI (1μg/ml) was also added. Embryos were extensively washed in TBS-Tween and TBS, then resuspended in mounting medium.

The stained embryos were a mixed population of phenotypically wild type and mutant embryos at a 3:1 ratio. Thus, it was necessary to examine a large number of embryos to identify the mutant phenotypes. In general, 50 embryos at each developmental stage were examined with the assumption that ¼ of these would be mutant. If a variant staining pattern was seen in at least 8/50 embryos, then the difference was identified and described as a mutant phenotype. If fewer than 8/50 embryos exhibited a variant staining pattern, the phenotype was

not identified as different from wild type. In some cases the mutant phenotype was obvious and it was not necessary to score as many as 50 embryos. In those instances, 15-20 mutant embryos were scored and wild type embryos were disregarded.

5.2 Immunostaining of larvae and adults

5.2.1. Formaldehyde fixation method

Wild type and mutant larvae and adults were stained using a modification of the procedure of Finney and Ruvkun (1990). Outcrossed *let-70* hermaphrodites were produced as described in Section II.2.2. Briefly, animals were collected from 10-12 large NG plates with M9 buffer and incubated in 4% sucrose/1 mM EDTA, pH 8.0 for one hour. Worms were resuspended in -20°C Ruvkun fixation buffer (160 mM KCl, 40 mM NaCl, 20 mM EGTA, 10 mM spermidine HCl, 30 mM PIPES pH 7.4, 50% methanol). Formaldehyde (J.B. EM Services) was added to a final concentration of 2% and worms were frozen at -80°C until required. Worms were taken through several freeze/thaw cycles, incubated on ice for 30 minutes, washed in TTB (100 mM Tris-HCl pH 7.4, 1% Triton X-100, 1 mM EDTA) and incubated for 30 minutes at room temperature in 1% Triton X-100 in M9 buffer. Worms were then incubated in 1% β-mercaptoethanol for several hours at 37°C. This treatment reduces disulfide bonds in the nematode cuticle. Worms were washed and incubated in 50 mM H_3BO_3/25 mM NaOH/10 mM DTT/0.01% Triton X-100 for 15 minutes at room temperature to complete the reduction reaction. The thiol groups were oxidized by treatment in 50 mM H_3BO_3/25 mM NaOH/0.3% H_2O_2/0.01% Triton X-100 for 15 minutes at room temperature. Samples were washed, resuspended in AbA buffer (1% BSA, 0.5% Triton X-100 in PBS) and primary antibodies were added. Samples were incubated overnight at 4°C, extensively washed in AbB buffer (0.1% BSA, 0.5% Triton X-100 in PBS) and resuspended in AbA buffer. Fluorescently-labelled secondary antibodies and DAPI (1 μg/ml) were added. Samples were incubated two hours to overnight at room temperature, washed extensively in AbB buffer and resuspended in mounting medium. Immunostained animals (10-15 μl) were mounted on 2% agarose pads on slides and examined.

5.2.2 Freeze-fracture method

In some experiments, larvae and adults were immunostained using the freeze-fracture method of Albertson (1984). Outcrossed *let-70* hermaphrodites were produced as described in Section II.2.2. Staged worms were washed off 5-8 small NG plates with M9 buffer, collected

by gentle centrifugation (2000 rpm for 1 minute), resuspended in 4% sucrose/1 mM EDTA, pH 8.0 and collected once again. The sucrose solution was aspirated, leaving the worms in approximately 250 µl solution. 20-25 µl of worm suspension was pipetted onto a polylysine-coated slide and covered with a large (24x50 mm, thickness 2) coverslip. Slides were placed on a metal block that had been pre-cooled to -80°C and stored at -80°C until needed. Coverslips were quickly flipped off the slide with a razor blade and the slides were fixed in 100% methanol at –20°C for 4 minutes. Slides were transferred to –20°C acetone for 4 minutes, rehydrated through a graded acetone/TBS series (75, 50, and 25%) and washed in TBS. The area around the worms was carefully dried, and primary antibody in 1% BSA in TBS-Tween was pipetted onto the worms. Samples were incubated in a humidified chamber overnight at 15°C then washed extensively in TBS-Tween. The area around the worms was dried, fluorescently-labelled secondary antibodies were pipetted onto the slides and the samples were incubated in a humidified chamber for two hours at room temperature. Slides were extensively washed in TBS-Tween, 10-15 µl of mounting medium was pipetted onto the worms, and a large coverslip was placed on top.

5.3 Antibodies used in this study

For immunolocalization of UBC-2, the rabbit polyclonal antibody, anti-UBC-2, was used. Anti-UBC-2 had been produced by immunizing rabbits with recombinant UBC-2 followed by affinity and acetone powder purification (Zhen, 1995). One or more mouse monoclonal antibodies were included in the preparations as a counterstain to aid in the identification of cells and tissues. These included DM5.6 (a kind gift from D. Miller), ICB4 (a kind gift from G. Mullen), C4 (ICN Biomedicals, Inc.), a double stranded DNA antibody (Chemicon International Inc.) and K76 (developed by S. Strome and W.B. Wood and obtained from the Developmental Studies Hybridoma Bank, The University of Iowa, Department of Biological Sciences, Iowa City, IA 52242, under contract NO1-HD-7-3263 from the NICHD). The specificity and dilutions of the antibodies used are shown in Table 1.

The secondary antibodies used were FITC-conjugated AffiniPure $F(ab')_2$ fragment of Donkey Anti-rabbit IgG and Texas Red-conjugated AffiniPure $F(ab')_2$ fragment of Donkey Anti-mouse IgG (Jackson ImmunoResearch Laboratories Inc.). Secondary antibodies were diluted 1:200 for most experiments.

For the competitor assay, anti-UBC-2 antibody was pre-incubated in an excess of recombinant UBC-2 (see Section II.4.1). Two μg of UBC-2 was added to the primary antibody and the mixture was incubated at 4°C for one hour with shaking. The entire solution was added to the nematode samples for primary antibody staining.

Table 1. Antibody dilutions used for immunostaining and their specificity

Antibody name	Specificity	Dilution
anti-UBC-2	UBC-2	1:400
DM5.6	myosin heavy chain A	1:50
1CB4	intestine, sperm, sensory neurons	1:50
K76	P granules	1:20
C4	actin	1:50
anti-dsDNA	double-stranded DNA	1:50

6. METHODS RELATED TO TRANSGENIC STUDIES

6.1 Construction of *ubc-2*::GFP fusions

To make constructs pTS1.1 and pTS2.2, the coding region of GFP was inserted in-frame into the second exon of a *ubc-2* genomic clone. The pTS1.1 and pTS2.2 constructs are identical except that pTS1.1 contains the nuclear localizing signal (NLS) from SV40 upstream of the GFP coding sequence, and the pTS2.2 construct does not. Both fusions were produced in the pZM13 construct background, and contain the complete *ubc-2* coding sequence, including five exons and four introns, with 7 kb of sequence upstream of the *ubc-2* initiation codon and 6 kb of sequence downstream of the *ubc–2* poly-adenylation signal.

An *Apa* I-containing linker was generated and inserted between the *Pst* I and *Bam*H I sites of the upstream multiple cloning site (MCS) of the GFP vectors pPD95.70 and pPD95.79 (a kind gift from A. Fire). This introduced an *Apa* I site into the upstream MCS of the GFP vectors. The linker sequence is shown in Appendix E. The GFP vectors were digested with *Apa* I and the approximately 1.8 kb fragments containing the GFP sequence were purified. The pZM13 construct was originally a 13 kb *Bam*H I-*Bgl* II fragment in pBSIISK(+). A *Not* I-*Sal* I fragment containing the 13 kb *ubc-2* fragment was removed from pZM13 and ligated to pGEM-9Z(-) (Promega). The sub-cloning step was necessary to remove all vector-containing *Apa* I sites. The *Apa* I GFP fragments were inserted into the *Apa* I site of *ubc-2* to produce an

in-frame fusion of GFP to the second exon of *ubc-2*. Restriction digestion with *Xho* I identified clones containing the GFP coding sequence in the proper orientation within the *ubc-2* gene.

6.2 Construction of a temperature-sensitive *let-70* allele

In yeast, an allele of the cell cycle-related E2, *cdc34-1*, is temperature-sensitive with respect to its E2 activity (Goebl *et al.*, 1988). *cdc34-1* contains a missense mutation resulting in a proline to serine substitution at a residue that is conserved in all E2s examined to date (Ellison *et al.*, 1991). Furthermore, creation of the corresponding mutation in the yeast E2s, RAD6 and Ubc9, created temperature-sensitive alleles in these genes as well (Betting and Seufert, 1996; Ellison *et al.*, 1991). A putative temperature-sensitive alleles of *ubc-2*, pZM13.34, was constructed by Mei Zhen in pZM13 following conventional site-directed mutagenesis protocols (Sambrook *et al.*, 1989). For pZM13.34, the cytosine at nucleotide position 229 was changed to a thymine, which converted the proline at position 61 into serine (P61S). The nucleotide substitution made was based on the published sequence of *cdc34-1* (Ellison *et al.*, 1991).

6.3 Preparation of DNA for injection into nematodes

6.3.1 Co-injection solutions

For DNA co-injections, plasmid and fosmid DNA was prepared using the Qiaprep Spin Miniprep kit (Qiagen, Inc.). Injection solutions contained a mixture of the DNA construct of interest and the plasmid pRF4 at a mass ratio of 1:50 or 1:200. The total DNA concentration was 100 μg/ml in dH_2O.

6.3.2 Injection mixtures containing carrier DNA

Injection mixtures containing carrier DNA were prepared following the protocol of Kelly *et al.* (1997). Samples were linearized by digestion with the appropriate restriction enzyme and, if required, overhanging ends were filled in using Klenow enzyme (see Sections II.3.1 and II.3.2). Samples were purified using the Qiaex II DNA purification kit or the Qiaspin PCR purification kit. Each injection mixture contained *Pvu* II-digested N2 genomic DNA (100 μg/ml), *Sma* I-digested plasmid pRF4 (2 μg/ml) and the linearized DNA construct of interest (2 μg/ml) in dH_2O. This generally gave a 1:3:100 mass ratio of reporter DNA:pRF4:genomic DNA.

6.4 Establishment of transgenic *C. elegans* strains

Injection needles were pulled from glass capillaries (Cat. No. 1B100F-6, World Precision Instruments Inc.) using a Frederic Haer & Co. micropipette puller and filled with the appropriate DNA mixture to be injected. Wild type or *let-70(s689) unc-22(s7)/ nT1(IV);+/nT1(V)* worms were injected into the syncytial gonad at a magnification of 400X using a Zeiss IM35 microscope equipped with Nomarski optics, and a Leitz micromanipulator according to the method of Fire (1986).

6.5 Selection of transformed progeny

Selection of progeny transformed with pRF4, a plasmid containing the *rol-6(su1006)* allele was by visual inspection for animals that roll. The antimorphic allele *su1006* encodes a mutant collagen and these mutants possess an altered body cuticle which forces the animal to roll on its right side (Kramer *et al.*, 1990).

Each selected F1 transgenic animal was placed on a separate plate for propagation and the F2 were examined for roller progeny to check for heritable transmission of the array. Clones that segregated the transgenic arrays were considered to be independent lines.

III. RESULTS

1. PHENOTYPE OF let-70 MUTANTS

Earlier studies indicated that *let-70* mutants arrested at the L2/L3 stage of development (Zhen *et al.*, 1996). In this study, a comprehensive examination of the mutant phenotype of *let-70(s1132)* and *let-70(s689)* homozygotes was undertaken. Specific cellular defects in mutant hermaphrodites and males were identified and compared to N2 and *unc-22* animals. The *unc-22* phenotype was utilized as a control since all *let-70* mutants were in an *unc-22* background. To generate *let-70* males, *let-70/nT1* hermaphrodites were crossed to *let-70/+* males and the resulting twitcher (*let-70/let-70*) male progeny were examined. Tissues and cellular structures were examined in live animals using Nomarski optics and in DAPI stained animals using UV fluorescence microscopy.

1.1 Phenotype of *let-70(s1132)* hermaphrodites

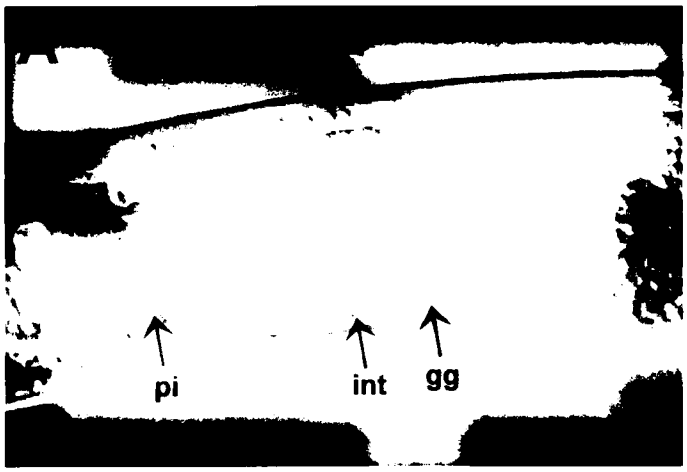
The *let-70(s1132)* hermaphrodite had a consistent phenotype (Figure 7). Most mutants arrested at L3 and were very thin and clear. Young larvae moved fairly well, becoming progressively more sluggish as they aged, and were usually paralyzed by L3 arrest. The pharynx of *let-70(s1132)* mutant larvae pumped and the animals were capable of feeding.

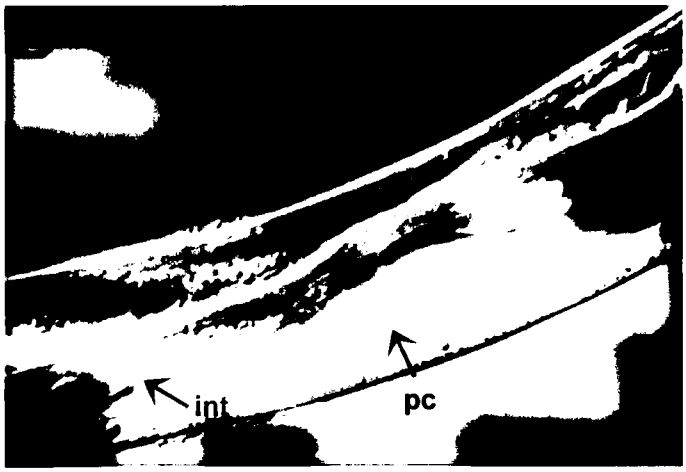
The development of the intestine was most strongly affected in *let-70(s1132)* mutants. Although the correct number of intestinal cells were present in these mutants, they were abnormal in appearance (Figure 7B). The intestinal cells were much smaller than normal and had an enlarged nucleus with a conspicuous nuclear membrane. The nucleolus, while prominent, was often abnormally shaped. Many intestinal cells were detached from the basement membrane, and as a consequence, the pseudocoelomic space between the intestinal cells and the basement membrane was enlarged. The intestinal cells contained very few gut granules. In general, more gut granules were located in the anterior intestine cells than in the posterior cells. The lack of gut granules in the intestinal cells may account for the clear appearance of the *let-70(s1132)* mutants. By Nomarski optics, the development of other cells and tissues did not appear to be strongly affected in *let-70(s1132)* mutants.

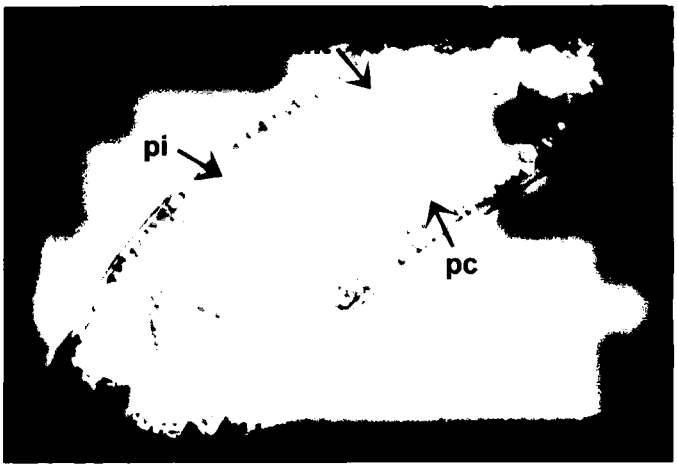
1.2 Phenotype of *let-70(s689)* hermaphrodites

The *let-70(s689)* mutant phenotype was more variable than the *let-70(s1132)* mutant phenotype. The arrest stage ranged from L3 to sterile adult, although animals which developed

Figure 7. Intestinal defects in *let-70(s1132)* and *let-70(s689)* mutant animals. A) Wild type adult viewed with Nomarski optics. The pharyngeal-intestinal valve (pi), intestine cell nucleus (int) and the numerous gut granules (gg) are indicated. B) *let-70(s1132)* terminal phenotype. Note the enlarged pseudocoelomic cavity (pc) and the absence of gut granules. C) and D) *let-70(s689)* mutant phenotypes. In panel C, note the separation of the intestine cells from the pharyngeal-intestinal valve and the enlarged pseudocoelomic cavity. In panel D, the intestinal lumen (lu) is enlarged and contains undigested bacteria. In each panel, anterior is to the left. Scale bar indicates 50 microns.


pi
int
gg


int
pc


pi
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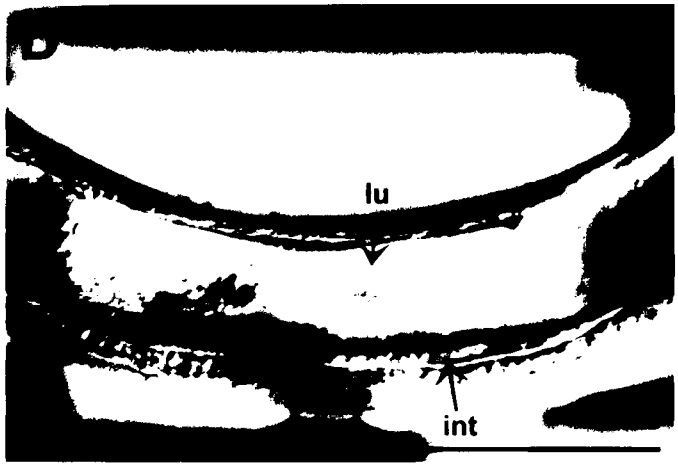
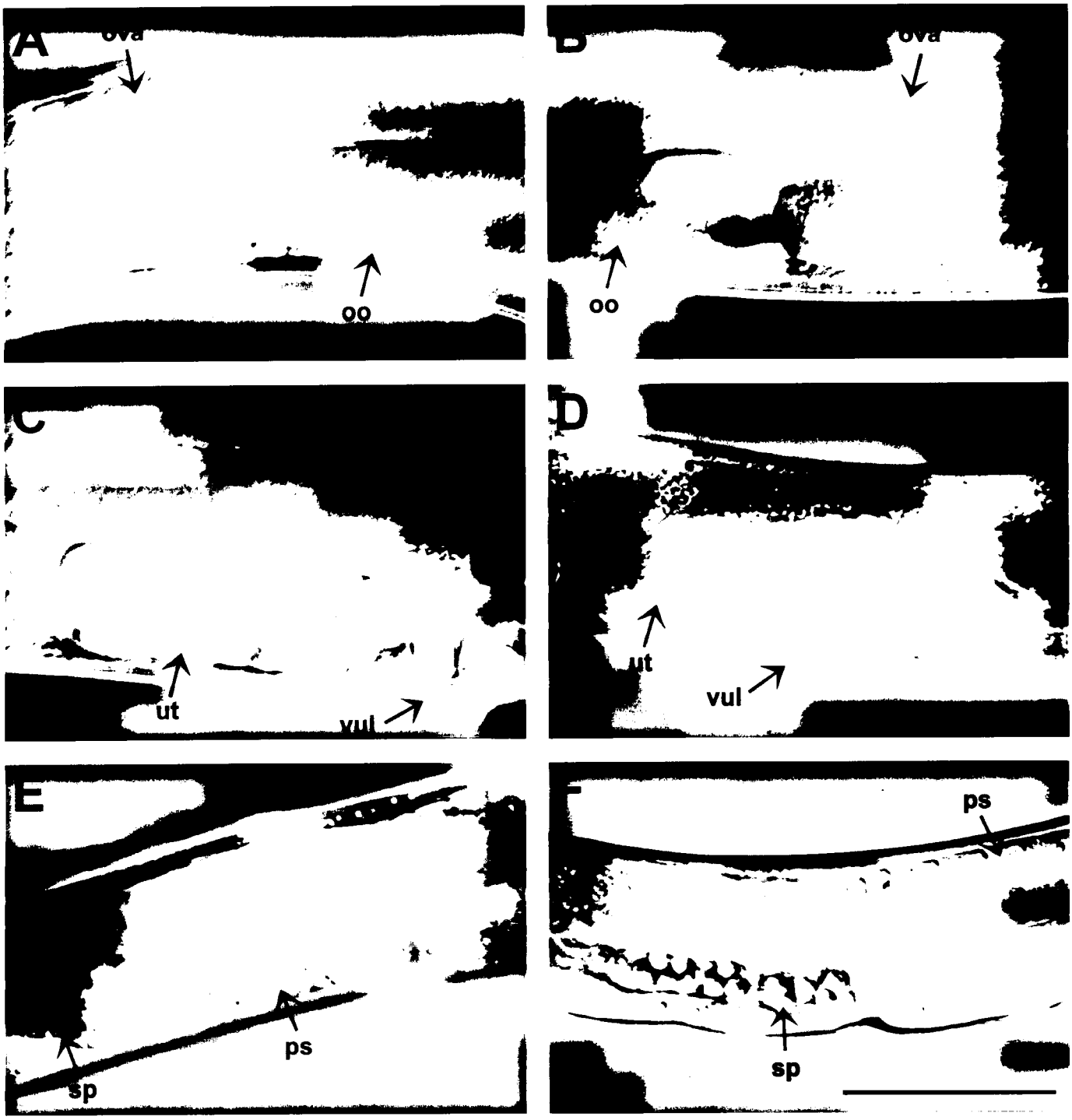

lu
int

beyond L3 were relatively rare. Most of the animals that arrested in L3 had phenotypes similar to *let-70(s1132)* mutants, although the defects were usually exacerbated. The intestinal cells were very small and were detached from the basement membrane, such that pseudocoelomic spaces encompassed most of the internal space of the animal. The apical border of microvilli was often not visible and there were very few gut granules. In many individuals, the intestine was partially detached from the pharyngeal-intestinal valve, with only a thin, ragged connection between the pharynx and the intestine (Figure 7C). In some animals, the intestine cells remained attached to the basement membrane, which resulted in the expansion of the intestinal lumen to form a large cavity within the animal that was often filled with bacteria (Figure 7D).

A small number of *let-70(s689)* individuals developed into sterile adults (Figure 8). In contrast to most *let-70(s689)* animals, the *let-70(s689)* adults had well developed intestinal cells and produced a large number of gut granules. However, these animals developed gonad defects that varied in phenotype and severity among individuals. The defects seen in *let-70(s689)* mutants affected almost all gonadal structures. The distal tip was enlarged and bulbous and the distal tip cell was often indistinguishable from the surrounding germline nuclei. In many individuals, the gonadal arms did not reflex and the arm continued to grow straight outward toward the anterior or posterior end of the animal. In some mutants, the distal arm reflexed or bent several times during outgrowth. In addition, the ovary contained fewer germ cells than normal, and these did not mature properly. Maturation of the germ cells was initiated at a position more distal than in wild type animals such that oocytes often formed within the loop region rather than within the oviduct (Figure 8B). The oocytes were often elongated ovals rather than the normal cuboidal shape. The oocytes were not fertilized and did not migrate into the uterus; however, an accumulation of oocytes within the oviduct and gonadal arm was not detected. Germ cell maturation appeared to arrest after only a few oocytes were produced. Only a small number of sperm were produced as well. The spermatheca and uterus of *let-70(s689)* mutants were a mass of undifferentiated cells rather than the well defined structure that is generally seen in wild type nematodes (Figure 8D). In many individuals, the development of the vulva was affected such that a proper vulval opening was not produced. In summary, *let-70(s689)* mutant hermaphrodites that developed into sterile adults exhibited defects in the formation of the somatic gonad and in the maturation of the germline.

Attempts were made to mate early adult *let-70(s689)* homozygotes with N2 males to

Figure 8. Gonadal defects in *let-70(s689)* mutant animals. A) and C) The anterior gonadal arm of a wild type hermaphrodite viewed with Nomarski optics. The distal ovary (ova) contains mitotic germline nuclei that progress through meiosis at the loop in the gonadal arm and develop into oocytes (oo). The oocytes are fertilized as they travel through the spermatheca into the uterus (ut), and embryos are released through the vulva (vul). B) The anterior gonadal arm of a *let-70(s689)* hermaphrodite. D) Uterus and vulva of a *let-70(s689)* hermaphrodite. E) Wild type male with primary spermatocytes (ps) and spermatids (sp) indicated. F) *let-70(s689)* mutant male. In each panel, anterior is to the left. Scale bar indicates 50 microns.


A
ova
oo
B
ova
oo
C
ut
vul
D
ut
vul
E
ps
sp
F
ps
sp

determine whether wild type sperm could rescue the arrest stage of these mutants. Mated *let-70(s689)* mutant hermaphrodites did not produce fertilized eggs, nor did they lay any eggs. The phenotype of mated *let-70(s689)* animals was similar to that of unmated individuals, although several mated individuals developed vulval protrusions, presumably as a result of the mating process.

1.3 Phenotype of *let-70* males

Mutant *let-70(s1132)* males arrest at L3 or early L4. The intestine was affected in a manner similar to mutant hermaphrodites: the cells were small, lacked a microvillus border and contained very few gut granules. The distal tip of the gonad arm was bulbous in shape and the gonad arm itself was slightly misshapen (data not shown).

The mutant *let-70(s689)* males arrested between L3 and young adult. The L3 arrest was similar in phenotype to *let-70(s1132)* males. The most common arrest stage seen was mid-L4. The vas deferens had extended to, but had not connected with, the cloaca. The specialized male tail structures had differentiated and the number and location of sensory rays and spicules appeared normal (data not shown). The distal tip of the testis was bulbous and slightly misshapen, although in general, the testis structure was properly formed. Both maturation of the germ cells and primary spermatocyte formation were initiated closer to the distal end of the gonad than in wild type males (Figure 8F).

2. THE let-70(s689) MUTATION IS A SPLICING ERROR

Previous characterization of the *let-70(s689)* allele indicated that the splice donor site of the fourth intron was altered (see Section I.7; Zhen *et al.*, 1996). This suggested that the splice site of the fourth intron of *ubc-2* might not be recognized by the spliceosome and that subsequently, the fourth intron might not be removed from the pre-mRNA. To determine if the fourth intron was spliced from the message, total RNA was isolated from wild type and homozygous *let-70(s689)* mutant animals and cDNA was synthesized. The coding region of *ubc-2* was amplified from first strand cDNA using RACE 36 and OZM3.f oligonucleotides. A portion of this reaction was re-amplified using OZM3.f and OZM2.r. Two amplification products were produced, one approximately 440 bp in length (s689-1) and the other approximately 490 bp (s689-2). The intensity of the s689-2 band was far greater than that of s689-1 (data not shown). Both fragments were purified, subcloned into the pBSIIKS(+) vector, and the nucleotide sequence was determined.

Sequencing revealed that both fragments coded for *ubc-2*. The s689-1 fragment sequence matched the wild type *ubc-2* coding region exactly (Figure 9). Five exon sequences of *ubc-2* were present, and all four introns had been correctly spliced from the pre-mRNA. Therefore, some mutant *let-70(s689)* message is correctly spliced.

The sequence of s689-2 contained the whole sequence of the fourth intron in addition to the five exons. The other intron sequences were absent. Thus, the *let-70(s689)* mutation is caused by mis-splicing of the fourth intron of *ubc-2*, such that the intron sequence remains part of the mature mRNA. Conceptual translation of the s689-2 sequence revealed an in-frame stop codon located immediately adjacent to the mutant splice donor site (Figure 9). This predicts that a truncated protein product lacking the carboxyl terminal 14 amino acids of UBC-2 may be translated in *let-70(s689)* mutant animals.

2.1 *smg*-mediated surveillance in *let-70(s689)* mutants

Since the *let-70(s689)* mutation is likely due to the presence of a premature stop codon in the mature mRNA caused by mis-splicing of the fourth intron, it is possible that *smg*-mediated mRNA surveillance may be involved in the manifestation of the mutation. Nonsense-mediated mRNA decay (NMD) or mRNA surveillance is a system present in many eukaryotes that is involved in the rapid degradation of mRNAs containing nonsense or frameshift mutations (Peltz *et al.*, 1994; Ruiz-Echevarria *et al.*, 1996). Nonsense-mutant mRNAs are degraded more rapidly than their wild type counterparts. Both *cis*-acting elements and *trans*-acting factors are involved in NMD. A complex of proteins is thought to assemble at stop codons during translation termination and scan downstream for the presence of *cis*-acting elements (Czaplinski *et al.*, 1998). If such elements are found, the mRNA is degraded. The mutant mRNA is decapped by decapping enzymes and degraded from the 5' end by exoribonuclease Xrnlp (Muhlrad and Parker, 1994).

In *C. elegans*, it is believed that the *smg* genes (*smg-1-smg-7*) constitute a mRNA surveillance system that protects cells from errors in mRNA synthesis or processing. The mRNA produced by nonsense alleles of many genes are unstable in a *smg(+)* background but have normal or near normal stability in *smg(-)* backgrounds (Cali and Anderson, 1998; Pulak and Anderson, 1993). In addition, there is an increase in the expression of mutant polypeptides when nonsense alleles are placed in a *smg(-)* background.

To determine whether *let-70(s689)* was affected by the NMD system, *let-70(s689) smg-*

```
  genomic ATGGCTCTCAAAAGA ATCCAGAAGGTAAGG TTTCAACACTTAACA ACAATAAAATTATTA TGTACGTTTCAGGAA CTCCAAGATCTCGGC
  N2 cDNA ATGGCTCTCAAAAGA ATCCAGAAG------ --------------- --------------- ------------GAA CTCCAAGATCTCGGC
s689 cDNA ATGGCTCTCAAAAGA ATCCAGAAG------ --------------- --------------- ------------GAA CTCCAAGATCTCGGC

  genomic CGTGATCCACCCGCA CAATGCTCCGCTGGA CCAGTTGGTGATGAT TTGTTCCATTGGCAA GCTACGATTATGGGC CCACCAGAGTCTCCC
  N2 cDNA CGTGATCCACCCGCA CAATGCTCCGCTGGA CCAGTTGGTGATGAT TTGTTCCATTGGCAA GCTACGATTATGGGC CCACCAGAGTCTCCC
s689 cDNA CGTGATCCACCCGCA CAATGCTCCGCTGGA CCAGTTGGTGATGAT TTGTTCCATTGGCAA GCTACGATTATGGGC CCACCAGAGTCTCCC

  genomic TATCAGGGAGGTGTC TTCTTCCTCACTATC CACTTCCCAACAGAC TATCCATTCAAACCA CCAAAGGTATTGATC GAAATTGGAAAAAAT
  N2 cDNA TATCAGGGAGGTGTC TTCTTCCTCACTATC CACTTCCCAACAGAC TATCCATTCAAACCA CCAAAGGT------- ---------------
s689 cDNA TATCAGGGAGGTGTC TTCTTCCTCACTATC CACTTCCCAACAGAC TATCCATTCAAACCA CCAAAGGT------- ---------------

  genomic AAATTTAATTTGTTT TCTTCAGGTTGCCTT CACCACTCGAATTTA TCATCCGAACATCAA TTCAAACGGAAGCAT CTGCCTTGACATTCT
  N2 cDNA --------------- ---------TGCCTT CACCACTCGAATTTA TCATCCGAACATCAA TTCAAACGGAAGCAT CTGCCTTGACATTCT
s689 cDNA --------------- ---------TGCCTT CACCACTCGAATTTA TCATCCGAACATCAA TTCAAACGGAAGCAT CTGCCTTGACATTCT

  genomic CCGTTCGCAGTGGTC GCCGGCTCTGACCAT TTCGAAAGGTTGATA TTATCATTATTGTTC GCGTTCTAACTTTTA ATTTTCAGTTCTGCT
  N2 cDNA CCGTTCGCAGTGGTC GCCGGCTCTGACCAT TTCGAAAG------- --------------- --------------- --------TTCTGCT
s689 CDNA CCGTTCGCAGTGGTC GCCGGCTCTGACCAT TTCGAAAG------- --------------- --------------- --------TTCTGCT

  genomic TTCGATCTGCTCGCT GCTCTGTGATCCAAA TCCGGATGATCCACT TGTGCCAGAGATTGC ACGCATCTACAAGAC GGATCGTGAAAGGTG
  N2 cDNA TTCGATCTGCTCGCT GCTCTGTGATCCAAA TCCGGATGATCCACT TGTGCCAGAGATTGC ACGCATCTACAAGAC GGATCGTGAAAG---
s689 cDNA TTCGATCTGCTCGCT GCTCTGTGATCCAAA TCCGGATGATCCACT TGTGCCAGAGATTGC ACGCATCTACAAGAC GGATCGTGAAAGATG

  genomic AATTTAGATTTTCTT TTAACTACTAAAAAT AATCGATTTTCAGGT ACAATCAATTGGCTA GAGAATGGACGCAAA AGTACGCTATGTGA
  N2 cDNA --------------- --------------- -------------GT ACAATCAATTGGCTA GAGAATGGACGCAAA AGTACGCTATGTGA
s689 cDNA AATTTAGATTTTCTT TTAACTACTAAAAAT AATCGATTTTCAGGT ACAATCAATTGGCTA GAGAATGGACGCAAA AGTACGCTATGTGA
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Figure 9. The fourth intron of *ubc-2* is not spliced from *let-70(s689)* pre-mRNA. Genomic and cDNA sequences of wild type *ubc-2* and cDNA sequence from *let-70(s689) ubc-2*. The mutation in *let-70(s689)* is underlined. Stop codons are indicated in bold. The intron sequence that is retained in *let-70(s689)* mRNA is shaded.

1(cc545) and *let-70(s689) smg-1(cc546)* double mutants were generated. Outcrossed *let-70(s689)/+* males were mated to *smg-1* mutant hermaphrodites. Nicotine-selected cross progeny were set to individual plates and the development of all twitcher progeny was examined. Twitcher progeny (*let-70/let-70*) were expected to be *smg-1(+)*, *smg-1(+)/smg-1*, or *smg-1/smg-1*. Approximately ¼ of the twitchers examined developed into egg-laying adults, while the remaining arrested at L3. It was presumed that the twitchers which developed into egg-laying adults were *let-70 smg-1* double mutants. In the putative double mutants, the intestinal and gonadal defects characteristic of *let-70* mutants were rescued (data not shown). A small number of eggs were laid, and a portion of these were fertilized. Embryos arrested just after gastrulation, at the pre-comma stage. This indicates that *let-70(s689)* is affected by *smg*-mediated mRNA surveillance, and that the mutant UBC-2 protein made in the *smg(-)* background can partially rescue the *let-70(s689)* mutation. This further implies that the mutant protein is partially functional when produced in *let-70 smg-1* double mutants.

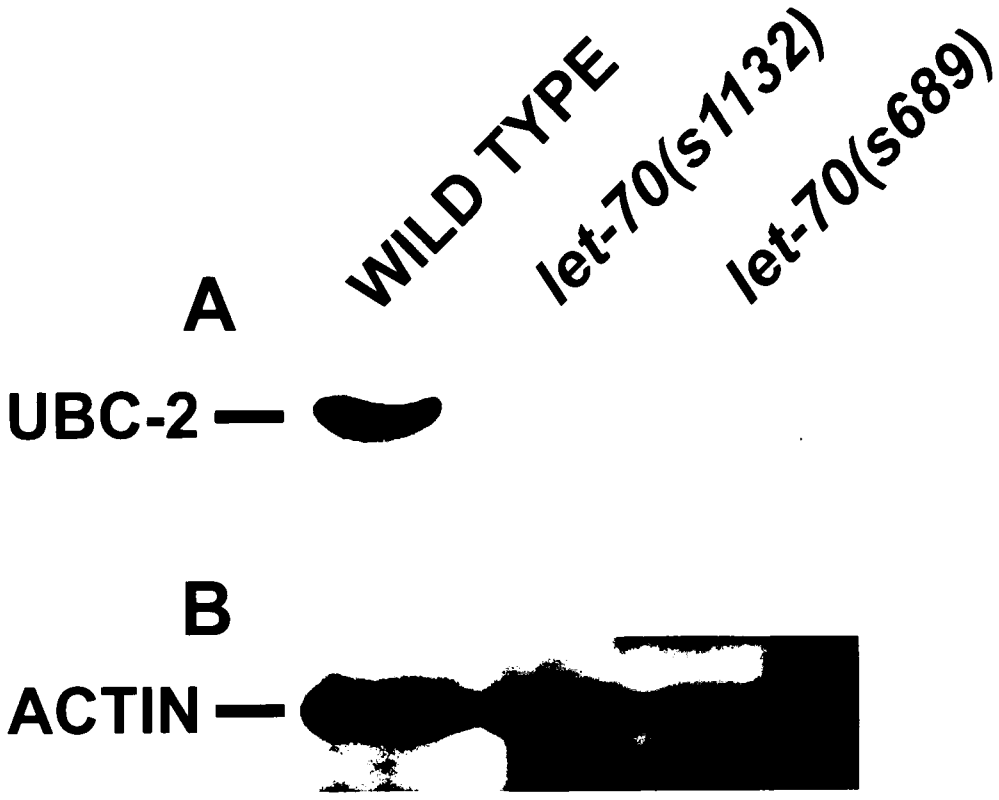
3. WESTERN ANALYSIS OF UBC-2 IN WILD TYPE AND let-70 MUTANTS

Western analysis of protein extracts was performed to determine whether UBC-2 was produced in *let-70(s1132)* and *let-70(s689)* animals. Individual *let-70* homozygotes were picked into Laemmli buffer and boiled to release proteins. Anti-UBC-2 polyclonal antibodies were used to detect UBC-2 in wild type and *let-70* mutant protein extracts. UBC-2 was seen in wild type samples at the expected size of 16.7 kDa (Figure 10). With a standard exposure, UBC-2 could not be detected in *let-70(s1132)* or *let-70(s689)* mutants; however, when the blot was overexposed, a faint band of UBC-2 was observed at 16.7 kDa in both the *let-70(s1132)* and *let-70(s689)* lanes, suggesting UBC-2 is present in the *let-70* mutants at a low concentration. Thus, both *let-70* mutant strains produce a small amount of UBC-2 protein that is the same size as wild type UBC-2. It should be noted that a truncated protein product was not observed in extracts prepared from *let-70(s689)* animals, even after long exposure.

4. RNA INTERFERENCE

Double-stranded RNA (dsRNA) can act as a signal for gene-specific silencing of expression in *C. elegans* (Fire *et al.*, 1998). Injection of dsRNA corresponding to the coding region of a specific gene results in potent and specific genetic interference of the corresponding gene products. The silencing effect is generally evident in the injected animal and its F1 progeny. In most cases, the phenotype produced by interference is extremely specific and

Figure 10. UBC-2 is extremely low or absent in *let-70(s1132)* or *let-70(s689)* animals. Protein extracts from wild type, *let-70(s1132)* and *let-70(s689)* worms were separated by SDS-PAGE electrophoresis, blotted as described in Materials and Methods, and probed with A) anti-UBC-2 polyclonal antibody or B) the monoclonal antibody C4, which recognizes actin.


WILD TYPE
let-70(s1132)
let-70(s689)
A
UBC-2
B
ACTIN

mimics loss-of-function mutations. This has proven useful for analyzing the putative null phenotype of genes where true null mutations do not exist (Fay *et al.*, 1999; Ashcroft *et al.*, 1999).

The molecular nature of the two *let-70* mutants suggests that neither is a true null allele. To identify the putative null phenotype of *let-70*, dsRNA interference was performed on *C. elegans*. Sense- and antisense-RNA directed to the coding region of *ubc-2* was prepared using bacteriophage RNA polymerase. The two strands were annealed and injected into young N2 adults. Injected animals were allowed to lay eggs for 15-20 hours (Brood A), transferred to a new plate for 10-12 hours (Brood B) then transferred once again (Brood C). The development of the F1 progeny from each brood was closely followed. Arrest stages were identified and the phenotype of arrested animals was determined.

4.1 RNA interference using GFP

The efficiency of dsRNA interference in *C. elegans* was first determined in a control experiment. Transgenic animals containing an *unc-54*::GFP construct were injected with dsRNA directed to the coding region of GFP, and the expression of GFP was examined in the progeny of injected animals. *unc-54* codes for myosin heavy chain A and is expressed in body wall and vulval muscles. Transgenic *unc-54*::GFP animals express GFP strongly in the four bands of body wall muscle that run from the nose to the tail, and in the vulval muscles. GFP expression can be visualized when animals are examined under 488 nm light. A number of individuals were injected and the F1 progeny were examined for GFP expression. From a total of 270 F1 roller progeny, 97% inhibition of GFP expression was observed in body wall and vulval muscles. Eight individuals did express *unc-54*::GFP. These animals were from Brood A and were among the oldest progeny, suggesting that eggshell formation had occurred prior to germline uptake of dsRNA. Thus, the block in *unc-54*::GFP protein expression using dsRNA was near 100% for progeny produced around 6 hours post-injection.

4.2 RNA interference using *ubc-2*

To identify the putative null phenotype of *ubc-2*, dsRNA interference of *ubc-2* was performed. Double-stranded RNA was synthesized to the complete coding sequence of *ubc-2*, injected into the gonad of young adult N2 animals and the development of the F1 progeny was monitored. Table 2 summarizes the results of the *ubc-2* RNA interference experiments. Of 285 F1 progeny, 146 (51%) arrested soon after gastrulation in the pre-comma stage, as

determined by DAPI staining. Of the animals that hatched, 90 (32% of the total) arrested as L3 larvae. The L3 arrested larvae had a phenotype similar to arrested *let-70* mutants: they were thin, with small irregularly shaped intestinal cells that lacked a microvillus and had very few gut granules (Figure 11). The intestinal cells generally remained attached to the basement membrane, resulting in an enlarged intestinal lumen. Some individuals were observed with intestinal cells that had detached from the basement membrane. About 17% of the animals developed into adults with no obvious mutant phenotype. All animals that developed into normal adults were from Brood A. It is likely that these individuals did not receive dsRNA from the injected parent before eggshell formation and thus, were not subject to RNA interference.

These results suggest that *let-70* null mutants are likely to require maternal UBC-2, and without it they are embryonic lethal. The L3 arrest phenotype appears to be caused by a reduction in UBC-2 levels. Alternatively, it may be the null phenotype of animals that revieve a maternal contribution of UBC-2. Both of the *let-70* mutations that have been isolated to date have L3 arrest phenotypes, indicating that *let-70(s1132)* and *let-70(s689)* are probably reduction-of-function mutants or genetic nulls.

To determine whether the *let-70* mutations function as null alleles, hemizygous animals were produced by placing *let-70(s1132)* and *let-70(s689)* over two deficiencies which uncover *let-70*, *mDf7* and *sDf80*. These crosses were performed by J. Schein in the laboratory of D.L. Baillie. The terminal phenotype of both hemizygous strains was L3 lethal, which supported the hypothesis that both could be a null allele of *let-70*.

Table 2. Effect of *ubc-2* dsRNA interference on *C. elegans* development

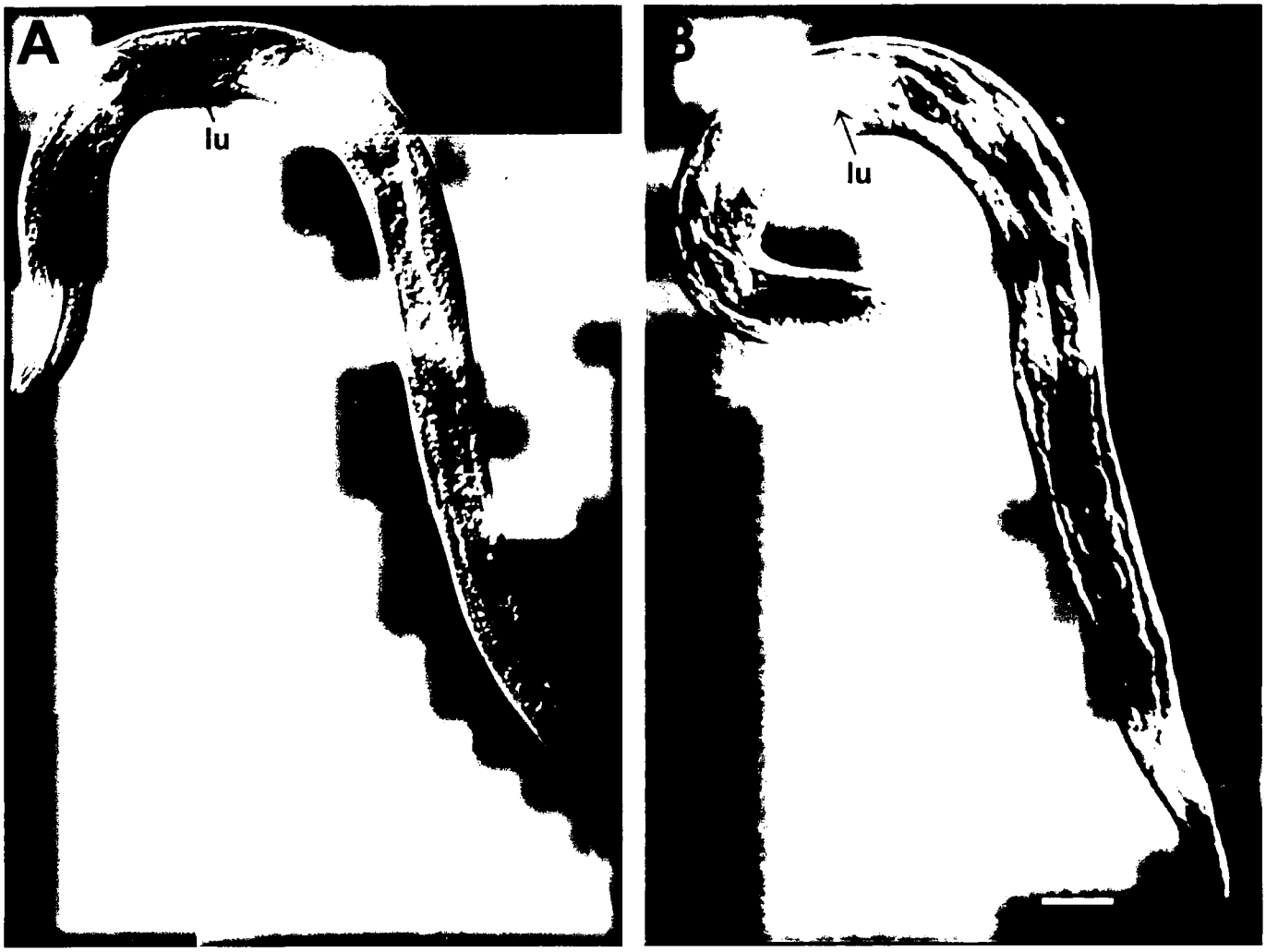
Total	Embryonic Lethal	L3 Lethal	Adult[1]
285	146	90	49

[1] all animals from Brood A

5. NON-COMPLEMENTATION SCREEN FOR NEW let-70 ALLELES

Information regarding the structure and function of a particular gene can be obtained from the molecular characterization of mutant alleles of the gene of interest. A null allele can be particularly useful in DNA-mediated transformation experiments, and in suppressor screens to isolate genes within a particular pathway. In an effort to isolate a null allele of *let-70*, a non-complementation screen was undertaken. A number of unidentified lethal mutants that mapped

Figure 11. The RNA interference phenotype is larval arrest. A) *let-70(s689)* L2 animal with an enlarged intestinal lumen (lu). B) L1-L2 progeny of a wild type worm injected with double stranded RNA directed to the coding region of *ubc-2*. Note the enlarged intestinal lumen anteriorly. Scale bar indicates 10 microns.


A
lu
B
lu

to within 1 map unit (mu) of *unc-22* were screened for complementation to *let-70(s689)*. Mutations that did not complement *let-70(s689)* were then tested for complementation to *let-70(s1132)*. The terminal phenotype of the mutation was determined by microscopic analysis and immunofluorescent staining with anti-UBC-2 antibodies and the monoclonal antibody DM 5.6, which stains myosin heavy chain A. The molecular nature of the non-complementing mutation was then determined by PCR amplification of *ubc-2* and surrounding sequences.

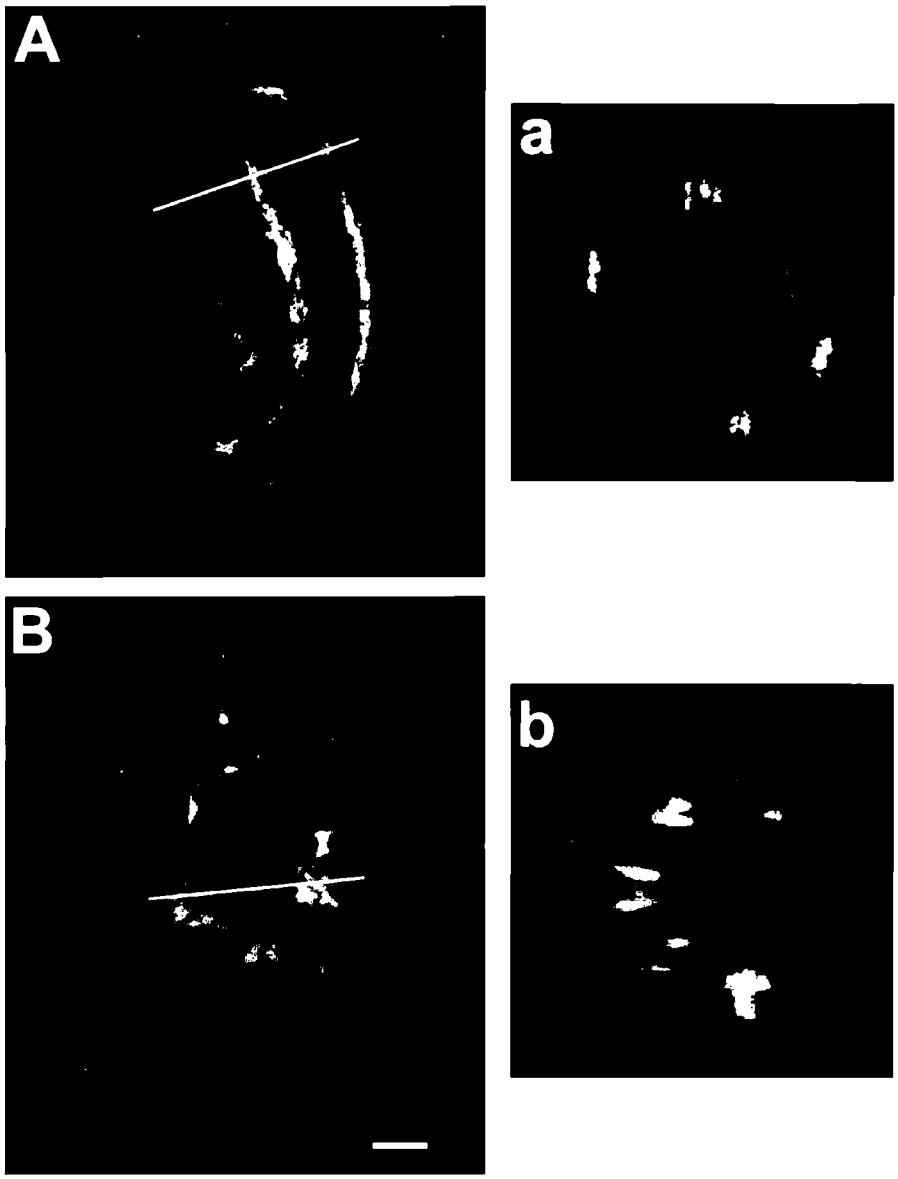
A total of 183 lethal mutants were screened for complementation to *let-70(s689)* as described in Section II.2.5. Only one strain, *let-x(s2293) unc-22 lev-1/nT1(IV);+/nT1(V)*, failed to complement the *let-70(s689)* mutation. In addition, *let-x(s2293)* also failed to complement *let-70(s1132)*. In both instances, *let-x(s2293)/let-70* animals were L3 lethal. The mutant *let-x(s2293)* strain was outcrossed seven times and rebalanced over *nT1* prior to further analysis.

5.1 Genetic characterization of *let-x(s2293)*

The terminal phenotype of *let-x(s2293)* homozygotes was embryonic lethality. Embryos persisted in the arrested state for 4-5 days before dying. To identify the embryonic arrest stage more specifically, *let-x(s2293)* embryos were examined by Nomarski optics, DAPI staining and immunofluorescent staining with anti-UBC-2 and mAb DM5.6. By DAPI staining, more than 500 nuclei were present in arrested embryos, although there was no indication of embryo elongation. This suggested that *let-x(s2293)* embryos arrested between 300 and 420 minutes after fertilization.

Immunostaining indicated that very little or no UBC-2 was present in *let-x(s2293)* mutant embryos (Figure 12). DM5.6 immunostaining showed that myosin had formed into distinct filamentous bands and had the appearance of myosin bands that are generally seen in embryos between 420 and 450 minutes after first cleavage. However, as shown in Figure 12, the body wall muscle bands were extremely disorganized. They did not form a continuous band from anterior to posterior. There were gaps in several places along the muscle band and the filaments had a thick, clumped appearance . The four sets of body wall muscle bands had formed but were disorganized and appeared to be concentrated along one surface of the animal rather than being equally spaced in four quadrants. Thus, the *let-x(s2293)* mutation affects the formation and the positioning or attachment of body wall muscle during embryonic development. Based on the appearance of the myosin filaments, *let-x(s2293)* embryos appear

Figure 12. *let-x(s2293)* arrested embryos do not express UBC-2 and display severe muscle developmental defects. Embryos were visualized by laser scanning confocal microscopy. Wild type (panels A and a) and *let-x(s2293)* (panels B and b) embryos were double-labelled with anti-UBC-2 (green, FITC) and DM5.6 (red, TRSC) which recognizes myosin heavy chain A. Panels A and B show the lateral view of a one and a half-fold embryo (~420 min.). In panel B, note the absence of UBC-2 staining and the highly disorganized muscle. The lines in panels A and B indicate the location where cross-sections of the TRSC channel were taken (panels a and b). In panel a, the four quadrants of myosin staining are associated with the bands of body wall muscle. In panel b, note the displacement of the muscle bands. Scale bar indicates 10 microns.


A
a
B
b

to arrest between 420 and 450 minutes (comma to 1 ½ fold stages), but do not undergo embryonic elongation.

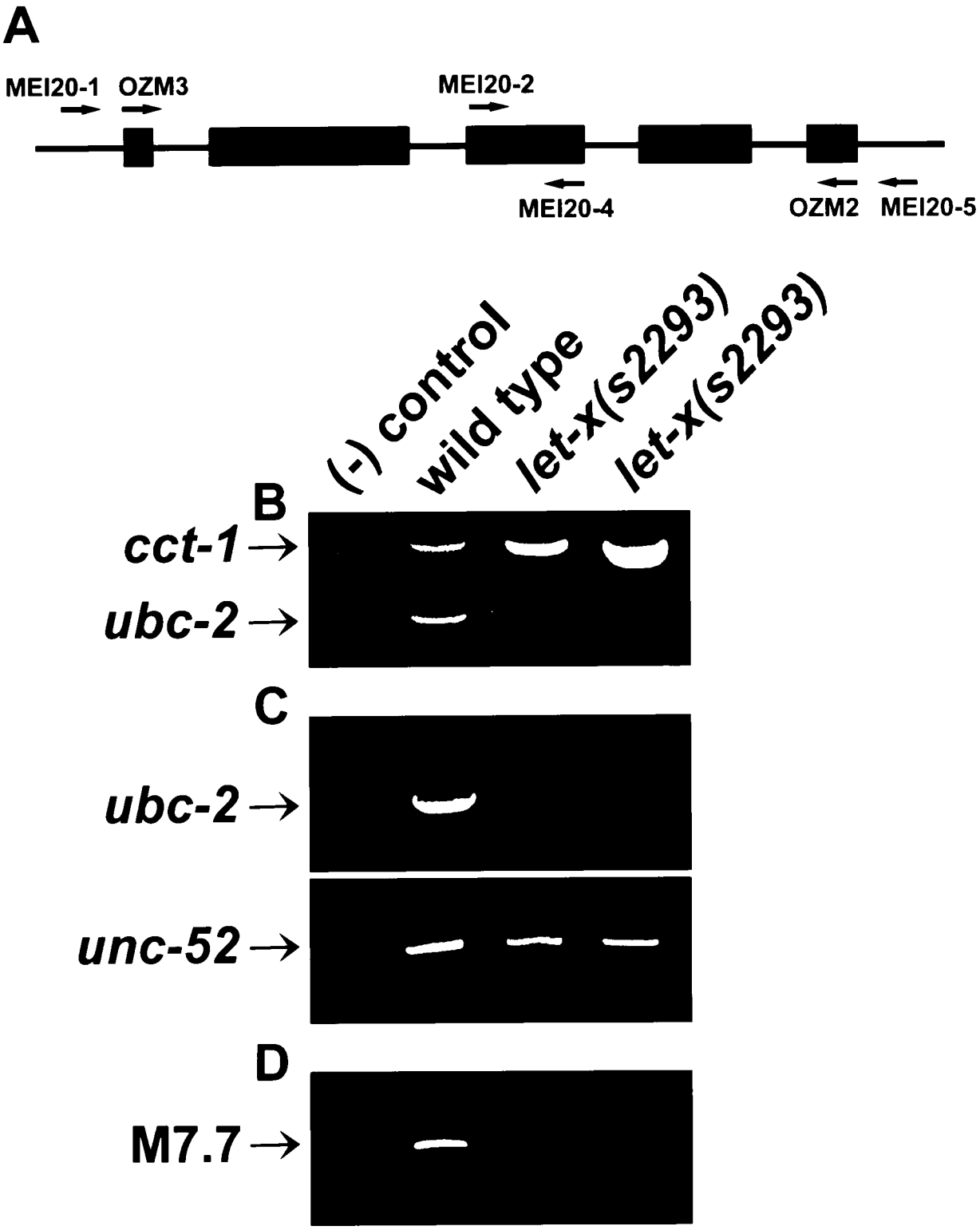
The effective lethal stage of *let-x(s2293)* animals was embryonic, and that of the *let-70* alleles was L3. Since *let-x(s2293)/let-70* animals were L3 lethal, it was possible that *let-x(s2293)* might be a null allele of *let-70*, while *let-70(s1132)* and *let-70(s689)* were reduction of function alleles. To examine the possibility that *let-x(s2293)* was a null allele of *let-70*, hemizygous animals were produced by placing *let-x(s2293)* over two deficiencies which uncover *let-70*, *mDf7* and *sDf80*. These crosses were performed by J. Schein in the laboratory of D.L. Baillie. The terminal phenotype of both hemizygous strains was embryonic lethal, which supported the hypothesis that *let-x(s2293)* could be a null allele of *let-70*.

As a control, *let-x(s2293)* was placed over the deficiency *nDf27*, which does not uncover *let-70*. If *let-x(s2293)* were an allele of *let-70*, then the mutation would not be uncovered by *nDf27* and hemizygous animals would be wild type. Interestingly, the terminal phenotype of this strain was also embryonic lethal, raising the possibility that *let-x(s2293)* was a large deletion encompassing the region from *let-70* to that uncovered by *nDf27*. Alternatively, a second mutation might exist in the *let-x(s2293)* strain that is located within the region uncovered by *nDf27*. It was possible that a second mutation was present and had not been removed by outcrossing the *let-x(s2293)* strain. The embryonic lethality of *let-x(s2293)* might then be due to the second site mutation, or a combination of both mutations.

5.2 The *let-x(s2293)* mutation is a large deletion

To determine the molecular nature of the *let-x(s2293)* mutation, PCR amplification of *ubc-2* was performed on single *let-x(s2293)* embryos. For this experiment, oligonucleotide primers OZM3.f and Mei 20-5.r were used to amplify *ubc-2*. The location of these primers on the *ubc-2* genomic sequence are shown in Figure 13A. As an internal positive control, the primers MIC12.f and MIC14.r, which amplify a portion of the *cct-1* gene, were included in the reaction. Attempts to amplify *ubc-2* from mutant embryos failed to produce a PCR product; however, the internal *cct-1* control product was consistently amplified (Figure 13B). The *let-x(s2293)* mutation affected the ability of at least one of the primer pairs to anneal to the corresponding genomic sequence. PCR reactions were then run using other primers in combination with OZM3.f and Mei 20-5.r. Table 3 lists all primer pair combinations tested for amplification of *ubc-2*, and the results obtained for wild type and *let-x(s2293)* arrested

Figure 13. *let-x(s2293)* is a large deletion that removes *ubc-2* and several other genes. PCR amplification of DNA from wild type and duplicate samples of *let-x(s2293)* embryos as described in Materials and Methods. For the (-) control, no DNA was added to the reaction. A) Schematic of genomic region surrounding ubc-2 gene. Coding regions are shown as boxes and non-coding regions as lines. Primers used for PCR reactions of *ubc-2* are indicated, with those that bind to the complementary strand located above the line and those that bind to the sense strand located below the line. B) *ubc-2* and *cct-1* were amplified from single embryos. Primers OZM3.f and Mei 20-5.r were used to amplify a 672 bp *ubc-2* fragment. As an internal control, a 1025 bp fragment of the *cct-1* gene was also amplified using primers MIC12.f and MIC14.r. C) A 3.6 kb fragment that includes *ubc-2* was amplified from a pool of 2-3 embryos using primers TS1.f and TS2.r. The *unc-52* control reaction was run on separate pools of embryos. Primers p23 and p150-3 were used to amplify a 4.7 kb fragment of *unc-52*. D) PCR amplification of a 743 bp fragment of M7.7 from single embryos using M7.7.f and M7.7.r primers. See Appendix C for primer sequences.


A
MEI20-1
OZM3
MEI20-2
MEI20-4
OZM2
MEI20-5
(-) control
wild type
let-x(s2293)
let-x(s2293)
B
cct-1
ubc-2
C
ubc-2
unc-52
D
M7.7

embryos. The location of each primer on the *ubc-2* genomic sequence is illustrated in Figure 13A. PCR products were obtained from wild type embryos using all primer pair combinations, while no *ubc-2*-directed PCR products were amplified from *let-x(s2293)* arrested embryos.

Since *ubc-2* could not be amplified in *let-x(s2293)* embryos, regardless of the oligonucleotides used, it was tempting to speculate that *ubc-2* was deleted in the *let-x(s2293)* strain. To test this possibility, long distance PCR was performed using oligonucleotides TS1.f and TS2.r, which were directed to sequences approximately 1500 bp upstream and downstream of *ubc-2*, respectively. While the 3.6 kb fragment was amplified in wild type embryos, no PCR product was obtained from *let-x(s2293)* arrested embryos (Figure 13C). However, a 4.7 kb fragment of the *unc-52* gene was produced from both wild type and mutant embryos. This information further supported the idea that *ubc-2* was deleted in *let-x(s2293)* mutant animals. It also suggested that the deletion extended into a region of flanking sequence, preventing at least one of the TS primers from annealing to its corresponding genomic sequence.

Table 3. Primer pair combinations used to amplify *ubc-2* in wild type and *let-x(s2293)* embryos

Primer combinations		PCR product obtained	
forward	reverse	wild type	*let-x(s2293)*
OZM3.f	MEI20-5.r	Yes	No
OZM3.f	OZM2.r	Yes	No
OZM3.f	MEI20-4.r	Yes	No
MEI20-1.f	MEI20-5.r	Yes	No
MEI20-1.f	OZM2.r	Yes	No
MEI20-1.f	MEI20-4.r	Yes	No
MEI20-2.f	MEI20-5.r	Yes	No
MEI20-2.f	OZM2.r	Yes	No

Since the TS1.f and TS2.r oligonucleotides were each located approximately 1.5 kb from the *ubc-2* genomic sequence, the deletion in *let-x(s2293)* appeared to be quite extensive, possibly encompassing several essential genes. PCR amplification of a serine-threonine kinase gene, M7.7, located approximately 10 kb upstream of *ubc-2* towards the region of *nDf27* was performed. As shown in Figure 13D, M7.7 was not amplified in *let-x(s2293)* arrested embryos. Therefore, the deletion in *let-70(s2293)* extends at least 10 kb upstream and includes *ubc-2* and

M7.7. Since *let-x(s2293)* is a large deletion spanning many genes, it cannot be considered an allele of *let-70*; thus *let-x(s2293)* was not included in any further analysis of *let-70* in this study.

6. IMMUNOFLUORESCENT STAINING OF WILD TYPE AND let-70 EMBRYOS, LARVAE AND ADULTS

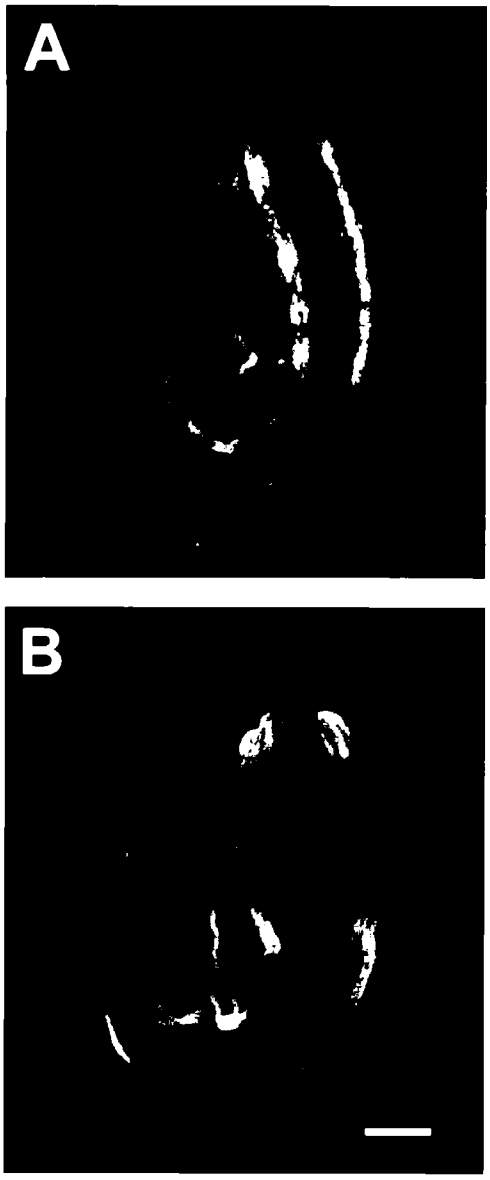
The expression pattern of UBC-2 during development in wild type, *unc-22* and *let-70* mutant animals was determined. The *let-70* mutant staining patterns were compared with those of *unc-22* mutants, since all *let-70* mutants were in an *unc-22* background. Embryos were prepared and immunostained as described in Section II.5.1. Larvae and adults were prepared by freeze fracture or by the method of Finney and Ruvkun (1990) as described in Section II.5.2. All animals were immunostained with a polyclonal antibody directed against UBC-2. To aid in the identification of cells and tissues, animals were counterstained with a variety of monoclonal antibodies (mAbs) directed against a number of specific tissues or cell types (see Section II.5.2 and Table 1).

The specificity of the UBC-2 polyclonal antibody was determined in wild type embryos. As shown in Figure 14, when anti-UBC-2 was pre-incubated with an excess of recombinant UBC-2 protein prior to immunostaining, virtually no staining of the embryos could be detected. In addition, when *C. elegans* protein extract was probed with anti-UBC-2 on a Western blot, only one band at 16.7 kDa was observed (data not shown). This demonstrated that anti-UBC-2 was specific for UBC-2 in *C. elegans*.

6.1 UBC-2 staining in embryos

Embryos were isolated and immunostained as described in Section II.5. To examine the UBC-2 staining pattern during early development, embryos were immunostained with anti-UBC-2 and counterstained with mAbs K76, C4 and anti-double stranded DNA, which stain P granules, actin and DNA, respectively. P granules are located in the P cell lineage during early cleavage; thus, K76 oriented the embryos by identification of the posterior end. The C4 antibody stained the actin cytoskeleton of cells and was useful for delineating the borders of individual cells, and anti-DNA localized the nucleus of each cell. These antibodies were utilized for examination of the UBC-2 staining pattern in early embryos up to the comma stage. UBC-2 staining was observed in wild type and *unc-22* embryos from fertilization and showed a tissue general UBC-2 staining pattern. UBC-2 staining did not localize to any specific cellular region; rather, it was fairly uniform in all cells throughout the embryo. The distribution and

Figure 14. The immunolocalization of anti-UBC-2 is UBC-2-specific. Laser scanning confocal microscopy of wild type embryos double-labelled with anti-UBC-2 (green, FITC) and DM5.6 (myosin heavy chain A; red, TRSC). Panels A and B show the lateral view of a one and a half-fold embryo (~420 min.). In panel B, anti-UBC-2 was pre-incubated with recombinant UBC-2 prior to immunostaining. Scale bar indicates 10 microns.


A
B

intensity of staining in both *let-70(s1132)* and *let-70(s689)* early embryos was similar to that observed in wild type and *unc-22* embryos (data not shown).

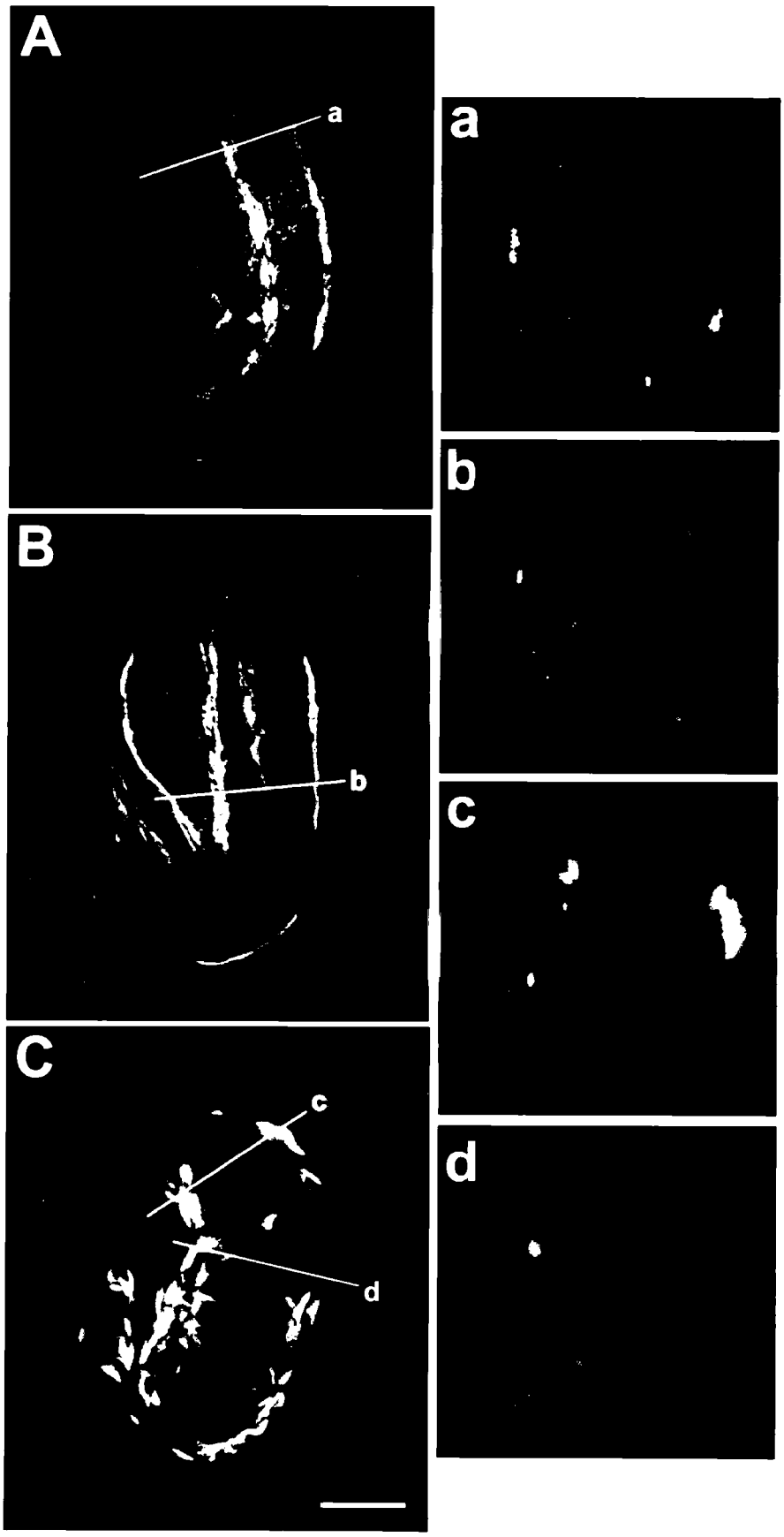
Embryos between 1.5 and 3-fold stage of development were stained with anti-UBC-2 and counterstained with mAbs DM 5.6, 1CB4 or C4. DM 5.6 recognizes myosin heavy chainA in body wall muscle and 1CB4 recognizes intestinal cells, sperm and several sensory neurons.

In wild type and *unc-22* embryos, UBC-2 stained almost all cells uniformly (Figure 15A). More intense UBC-2 staining was sometimes observed in the two gonad primordia cells, Z2 and Z3, suggesting that UBC-2 expression is enhanced in the gonad precursor cells of developing embryos (data not shown).

In mutant *let-70(s1132)* embryos, UBC-2 staining was ubiquitous, although there was a noticeable decrease in overall staining intensity (Figure 15B). An increase in the intensity of UBC-2 staining in the gonad primordia cells in a manner similar to that observed in wild type and *unc-22* embryos was often seen. Examination of body wall muscle and intestinal development using DM 5.6 and 1CB4 revealed no obvious developmental defects in *let-70(s1132)* mutant embryos. Thus, the embryonic development of *let-70(s1132)* embryos appeared to be similar to that of wild type and *unc-22* embryos.

In *let-70(s689)* mutant embryos, the intensity of UBC-2 staining varied from near wild type levels to virtually no UBC-2 staining. In general, however, the overall UBC-2 staining intensity was low (Figure 15C). Increased staining intensity of the gonad primordia cells was observed in many of the mutant embryos examined. DM 5.6 staining indicated that *let-70(s689)* mutant embryos developed body wall muscle defects. Myosin was not organized in ordered A-bands within the muscle cells and formed large aggregates, suggesting that muscle sarcomere assembly was defective. The four muscle quadrants were not continuous from anterior to posterior and there were regions where myosin A-bands were absent, which indicated that some muscle cells were positioned incorrectly or did not properly assemble the myosin A-bands (Figure 15c, d). In strongly affected embryos, the muscle bands were not properly located in the four quadrants, and one or more of the bands was displaced into another quadrant, or missing altogether. This displacement often affected only the dorsal bands of muscle. Thus, muscle cell attachment was affected in these mutants. The disorganized muscle phenotype was first observed in 1.5 fold embryos and continued throughout development. The muscle defects suggested that sarcomere assembly, muscle cell positioning and muscle cell attachment were all affected in *let-70(s689)* mutant embryos.

Figure 15. Immunolocalization of UBC-2 in wild type and *let-70* mutant embryos. Laser scanning confocal microscopy of embryos were double-labelled with anti-UBC-2 (green, FITC) and DM5.6 (myosin heavy chain A; red, TRSC). Panels A, B and C show the lateral view of a one and a half-fold embryo (~420 min.). A) Wild type embryo. a) TRSC channel image of a cross-section of wild type embryo taken at line a in panel A to show the four quadrants of myosin staining that are associated with the bands of body wall muscle. Dorsal is towards to top of the panel. B) *let-70(s1132)* embryo. Note the reduction in UBC-2 staining intensity. b) TRSC channel image of a cross-section of *let-70(s1132)* embryo taken at line b in panel B. C) *let-70(s689)* embryo. Note the reduction in UBC-2 staining intensity and the highly disorganized muscle. c) TRSC channel image of a cross section of *let-70(s689)* embryo taken at line c indicated in panel C. Note that the muscle cell in the right ventral quadrant is missing. d) Cross section of *let-70(s689)* embryo taken at line d indicated in panel C. Note that both the right dorsal and ventral muscle cells are missing. Scale bar indicates 10 microns.


A
a
B
b
C
c
d
a
b
c
d

6.2 UBC-2 staining in larvae and adults

Wild type and *unc-22* L1 larvae exhibited low levels of UBC-2 staining in almost all cells and tissues. The intensity of staining was slightly higher in the developing gonad. This pattern resembled that described for embryos. The low level of UBC-2 staining in all cells and tissues was observed throughout development. The specific staining patterns described below were observed as an increase in staining intensity above this overall background level.

In L2 larvae, a change in the cellular distribution of UBC-2 was observed. A dramatic increase in staining intensity was seen in the nucleoli of a number of cell types, including hypodermal, intestinal and body wall muscle cells (Figure 16B, C). The strong nucleolar expression of UBC-2 in these cell types continued throughout development. At L3, the nucleoli of several additional cell types exhibited increased UBC-2 expression, including those of the germ cells (Figure 16E, F). In the head of the animal, UBC-2 expression increased in the nucleoli of all pharyngeal muscle cells, pharyngeal neurons, the pharyngeal-intestinal valve cells, and several neurons located near the terminal bulb of the pharynx (Figure 17). These neurons may form part of the dorsal and/or ventral nerve cords. Interestingly, nucleolar staining of UBC-2 was conspicuously absent in many of the neurons that form part of the nerve ring (Figure 17B-D).

In summary, UBC-2 was expressed at a low background level in most cells and tissues in the animal throughout development. During larval development, UBC-2 levels increased in the nucleoli of a number of specific cell types. These cell types included hypodermal, intestinal, body wall muscle and germline. In the later stages of larval development, UBC-2 also became concentrated in the nucleoli of pharyngeal muscle cells, neurons associated with the pharynx, the pharyngeal-intestinal valve cells and several neurons near the terminal bulb of the pharynx.

Mutant *let-70* larvae were stained with anti-UBC-2 to determine the expression pattern of the mutant forms of UBC-2. The mutant larvae were counterstained with the mAbs DM 5.6 and 1CB4 to examine tissue development. In *let-70(s1132)* mutant larvae, UBC-2 levels were very low. The expression pattern, however, was similar to that of wild type (Figure 18A, B). The overall low level of background staining of UBC-2 was increased in gonad primordia cells and in the nucleoli of muscle, hypodermal, intestinal and germline cells. This pattern persisted despite the intestinal defects that developed in *let-70(s1132)* mutant larvae.

Figure 16. Immunolocalization of UBC-2 in wild type larvae. Laser scanning confocal microscopy of larvae double-labelled with anti-UBC-2 (green, FITC) and anti-double stranded DNA (red, TRSC). Panels A-C show a mid-body section of an L2 larva. Anterior is towards the top and ventral is left. A) TRSC channel image of L2 larva with representative intestine (int), hypodermis (hyp) and muscle (m) nuclei indicated. Note the anti-double-stranded DNA stains nuclei and often excludes nucleoli. B) FITC channel image of an L2 larva showing UBC-2 immunofluorescence. C) Both channels shown simultaneously. Note that the UBC-2 staining pattern aligns with the nucleoli. Panels D-F show a mid-body section of the developing gonad of an L4 larva. The region of the animal shown is a 4.4 micron thick slice through the gonad. The sections of the animal located above and below this slice have been removed. D) TRSC channel image of L4 larva showing the double-stranded DNA staining pattern. Anterior is left and ventral is towards the bottom. Representative mitotic germline (gl) and muscle (m) nuclei are indicated. E) FITC channel image of L4 larva showing UBC-2 staining pattern. F) Both channels shown simultaneously. Scale bar indicates 10 microns.

A
int
hyp
m
B
int
hyp
m
C
int
hyp
m
D
m
gl
E
m
gl
F
m
gl

Figure 17. Immunolocalization of UBC-2 in the head region of wild type animals. Laser scanning confocal microscopy of animals double-labelled with anti-UBC-2 (green, FITC) and anti-double stranded DNA (red, TRSC). A and E) The head region of wild type adults with both channels shown simultaneously. Anterior to left. B-D) Close-up of region within the box in panel A, showing part of the nerve ring (nr) and the terminal bulb of the pharynx. The region of the animal shown is a 2.6 micron thick slice through the head. The sections of the animal located above and below this slice have been removed. Representative pharynx (ph) and hypodermal (hyp) nuclei are indicated. Panel B shows the TRSC channel with the double-stranded DNA staining pattern. Note the nuclear staining which often excludes the nucleolus. Panel C shows the FITC channel with the UBC-2 staining pattern. Panel D shows both channels simultaneously. Note that the UBC-2 staining pattern aligns with the nucleoli of many cells, but does not stain nuclei or nucleoli of neurons in the nerve ring. F-G) Close-up of region within the box in panel E, showing isthmus and terminal bulb of the pharynx, pharyngeal-intestinal valve and part of the intestine. The region of the animal shown is a 2.6 micron thick slice through the head. The sections of the animal located above and below this slice have been removed. Representative pharynx (ph), pharyngeal-intestinal valve (pi), neuron (n) and intestine (int) nuclei are indicated. Panel F shows the TRSV channel with double-stranded DNA staining pattern. Panel G shows the UBC-2 staining pattern in the FITC channel. Panel H shows both channels simultaneously. Scale bar indicates 10 microns.

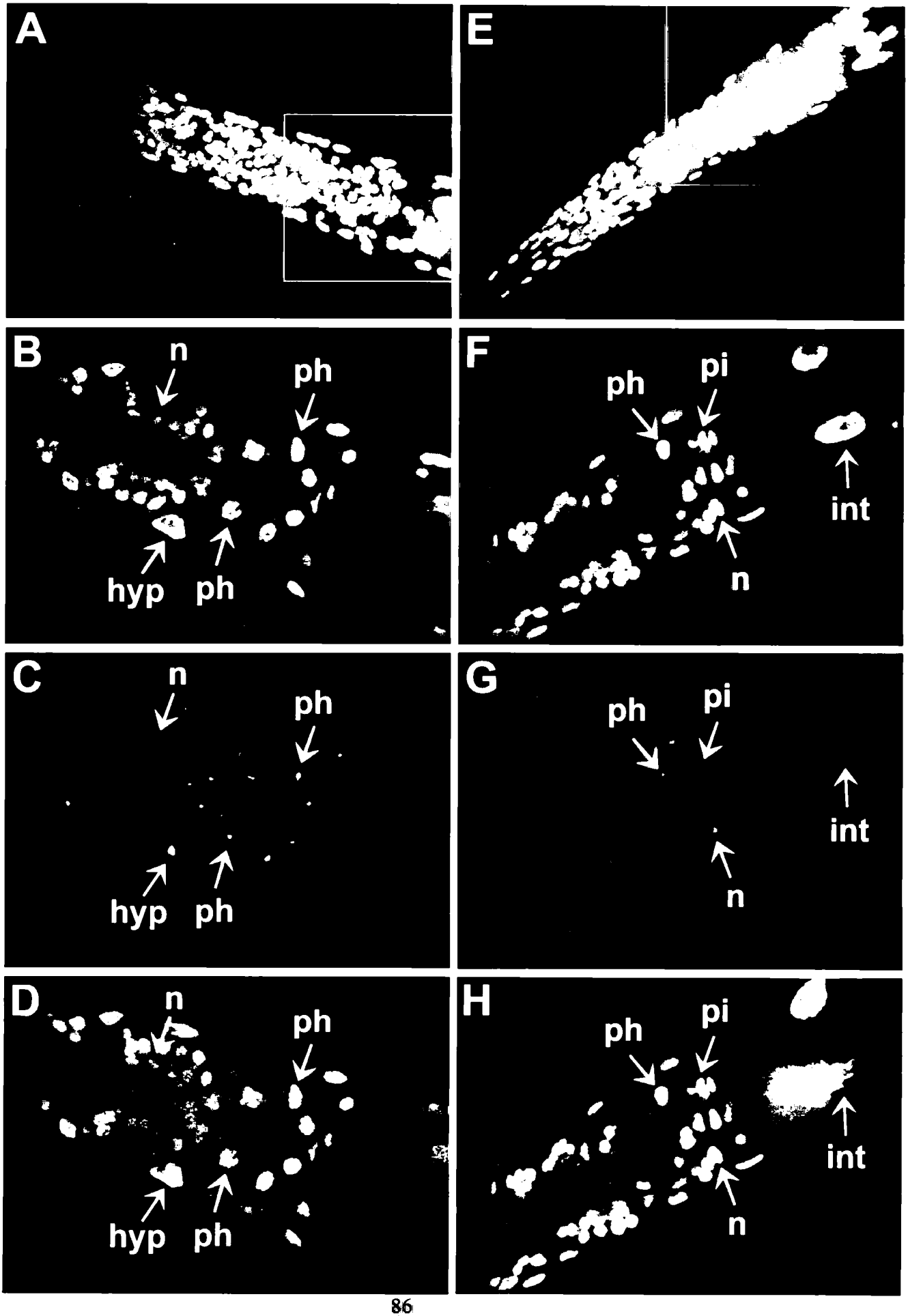

A
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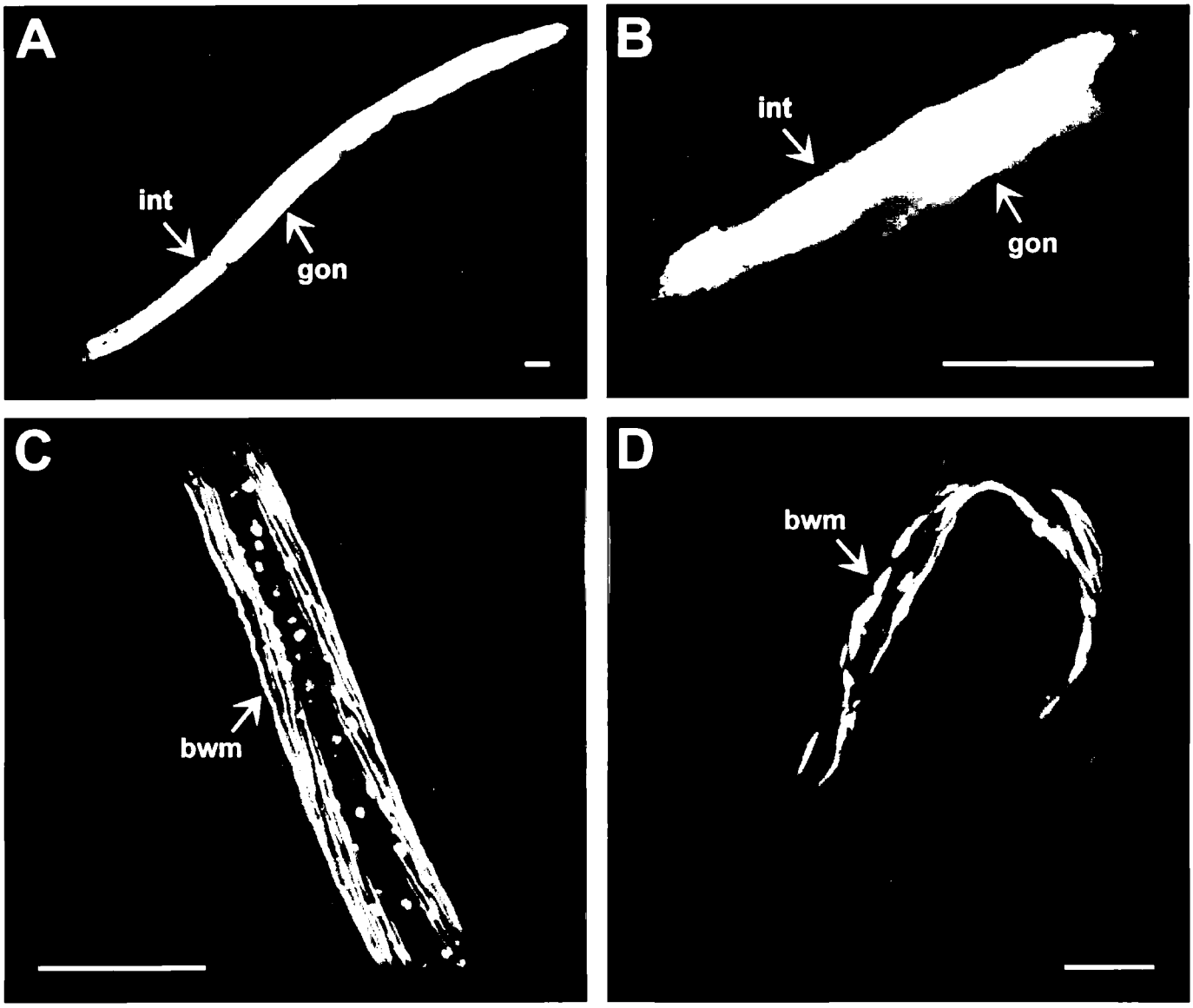
Figure 18. Immunolocalization of UBC-2 in *let-70* mutant animals. Laser scanning confocal microscopy of wild type (panel A) and *let-70(s1132)* (panel B) worms double-labelled with anti-UBC-2 (green, FITC) and 1CB4 (red, TRSC) which stains intestine cells, sperm and several sensory neurons. Anterior is left and dorsal is towards the top. Intestine (int) and gonad (gon) are indicated A) A wild type L3 larva. Note the strong UBC-2 staining in the gonad. B) *let-70(s1132)* L1 larva. Note the decreased UBC-2 staining intensity and the intestinal cell abnormalities. Panels C and D show wild type and *let-70(s689)* L2 larvae, respectively, double-labelled with anti-UBC-2 (green, FITC) and DM5.6 (red, TRSC) which stains myosin heavy chain A. A representative body wall muscle quadrant (bwm) is indicated. In C, anterior is towards the top and ventral is left. In D, anterior is left. Note the muscle displacement and disorganization. Scale bar indicates 10 microns.


A
int
gon
B
int
gon
C
bwm
D
bwm

In *let-70(s689)* mutant larvae, UBC-2 levels were virtually undetectable (Figure 18D). In addition, body wall muscle was extremely disorganized, in a manner similar to that observed in *let-70(s689)* mutant embryos. Myosin was not organized into ordered A-bands and formed large aggregates. In many larvae, one or more of the muscle quadrants were detached from the hypodermis. The detachment tended to occur mostly at mid-body, where the animals flex during locomotion. This strong muscle defect was seen only in L1-L3 mutant larvae. *let-70(s689)* mutants that developed beyond L3 did not exhibit such strong muscle assembly and attachment defects.

The adult staining pattern was examined solely in wild type adults. The UBC-2 staining pattern was similar to that seen in L3 larvae, with a low level of UBC-2 expression in most tissues and cells (data not shown). Increased expression was seen in the nucleoli of body wall muscle, pharyngeal muscle, intestine, hypodermis, germline, pharyngeal neurons and several neurons in the ventral nerve cord.

7. CONSTRUCTION OF TRANSGENIC ubc-2::GFP ANIMALS

The expression of *ubc-2* during development in live animals was examined using transgenic lines carrying *ubc-2*::GFP fusion constructs. The *ubc-2*::GFP fusions were constructed by inserting the GFP coding region into the second exon of *ubc-2* in the plasmid pZM13. The latter contained 7 kb of sequence upstream and 6 kb downstream of *ubc-2*. When expressed as a transgenic array, pZM13 was capable of fully rescuing homozygous mutant *let-70(s689)* animals (see Section III.8.2). Two fusion constructs were made. In pTS1.1, the NLS of SV40 was inserted upstream of the GFP coding sequence. The NLS should localize the fusion protein to the nuclei of cells that express it. The pTS2.2 construct lacked an NLS. Injection mixtures contained random genomic DNA, the marker plasmid pRF4 and the *ubc-2*::GFP fusion construct of interest. Random genomic DNA was included in the injection mixtures to allow both somatic and germline expression of the transgenic array. N2 animals were injected and stable transgenic lines were established (Appendix C). Transgenic individuals from each line were examined for GFP expression, and the cells and tissues in which *ubc-2*::GFP was expressed were identified.

7.1 Expression of *ubc-2*::GFP in transgenic lines carrying pTS2.2

Transgenic animals carrying pTS2.2 expressed *ubc-2*::GFP in many cells and tissues. A number of animals expressed the transgene in a ubiquitous pattern similar to that observed in

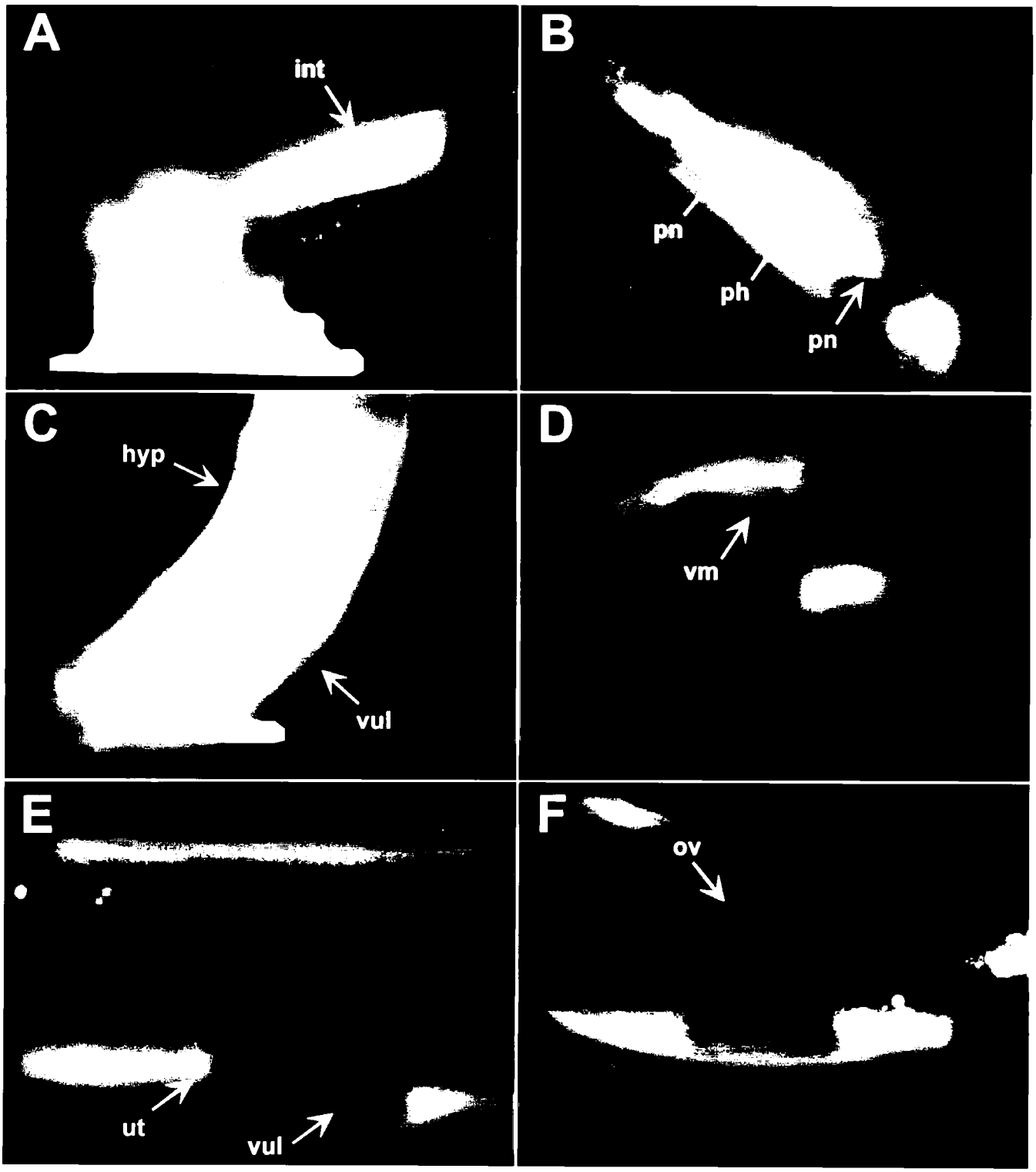
embryos and larvae immunostained with anti-UBC-2. This pattern has been previously described in Section III.6.

Many animals expressed the transgene only in specific cells and tissues in a mosaic pattern. Mosaicism in transgenics results from the loss of the extrachromosomal array in cells during development, such that the array is expressed only in a subset of cells. For the analysis of UBC-2 expression, mosaic animals proved to be very useful. In many mosaics, background expression of *ubc-2*::GFP was absent, and allowed visualization of *ubc-2*::GFP expression in cells that had previously been obscured by background expression levels. Mosaic *ubc-2*::GFP expression was seen in a number of cell types that had been previously identified by immunostaining, such as body wall muscle, pharyngeal muscle, intestine and hypodermis (Figure 19A, B, and C). Expression was observed in the cytoplasm of these cells and there was usually an increase in the intensity of expression in the nucleus. Interestingly, the whole nucleus, rather than just the nucleolus, exhibited increased *ubc-2*::GFP expression.

Several cells and tissues that had not previously been shown to express UBC-2 were found to express *ubc-2*::GFP. Expression was seen in the developing vulva of L3 and L4 larvae, the vulval muscles, the uterus and the somatic gonad (Figure 19C, D, E and F). Expression was also observed in the spermatheca and anal sphincter muscle (not shown). The *ubc-2*::GFP expression patterns in these tissues were observed in a number of transgenic individuals.

Several neurons also expressed *ubc-2*::GFP. The cell bodies and processes of several I-class pharyngeal neurons expressed *ubc-2*::GFP (Figure 19B). Several other neurons in the head region expressed *ubc-2*::GFP in both the nuclei and processes (Figure 20). The nuclei of these neurons were located near the terminal bulb of the pharynx. One process extended anteriorly, while two processes extended posteriorly, with one travelling along the ventral nerve cord. Based on their location, these neurons appeared to be AVM and SDQL. Both of these neurons are descendents of the Q neuroblast lineage, with AVM being involved in mechanosensation. In addition, one neuron with a cell body located in the tail region and a process that ran anteriorly along the body expressed *ubc-2*::GFP (not shown). It is possible that this is the sensory neuron PQR, another Q neuroblast descendant; however, the twist in the body of the animals caused by the *rol-6* mutation prevented unequivocal identification of many neurons. In summary, the ventral nerve cord, at least two neurons that extend along the body of the animal and at least one tail neuron expressed UBC-2 in their nuclei and in the neural processes. Many

Figure 19. UBC-2::GFP fusion protein is expressed in a number of tissues in *C. elegans*. Transgenic worms carrying pTS2.2 extrachromosomal arrays were visualized for expression of the UBC-2::GFP fusion protein. A) Adult worm showing the terminal bulb of the pharynx and the anterior portion of the intestine. Anterior is left in all panels. Note the strong UBC-2::GFP expression in cytoplasm and nucleus of the intestine cell (int). The yellow spots are a subset of gut granules that autofluoresce under 488 nm light. B) Head of an adult worm. Note the strong UBC-2::GFP expression in the processes of the pharyngeal neurons (pn) and in the nucleus of the pharyngeal muscle cells (ph). C) Mid-body of L3 larva showing strong UBC-2::GFP expression in hypodermal cell (hyp) and in the developing vulva (vul). D) Ventral surface of adult mid-body. Note UBC-2::GFP expression in the vulva muscles (vm). E) Mid-body of adult with UBC-2::GFP expression in uterus (ut) and vulva of gonad. F) Adult animal showing oviduct (ov) with oocytes inside. Note UBC-2::GFP expression in the somatic oviduct tissue. Scale bar indicates 10 microns.


A
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B
pn
ph
pn
C
hyp
vul
D
vm
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ut
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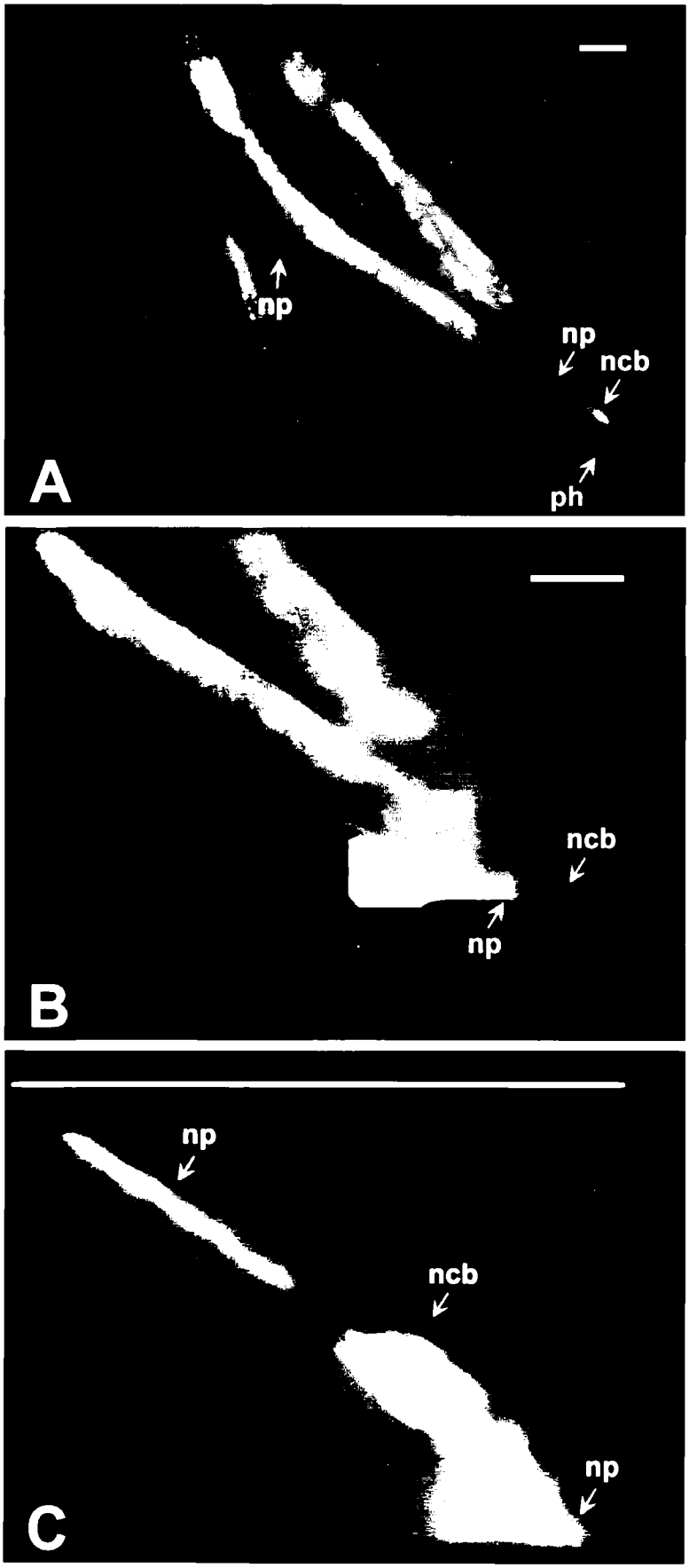
Figure 20. UBC-2::GFP is expressed in several neurons. Transgenic worms carrying pTS2.2 extrachromosomal arrays were visualized under 488 nm light for expression of the UBC-2::GFP fusion protein. A) L4 larva expressing UBC-2::GFP in neuron cell bodies (ncb) and processes (np). Anterior is right in all panels. B) Close-up of upper body at a different focal plane to show the connection of the neural processes to the cell bodies near the pharynx. C) Close-up of neuron cell body showing processes extending anteriorly and posteriorly. Scale bar indicates 10 microns.


np
np
ncb
ph
A
ncb
np
B
np
ncb
np
C

of these neurons appear to be descendants of the Q neuroblast lineage.

An interesting pattern of *ubc-2*::GFP expression was observed in the body wall muscle. When transgenic animals were observed under low magnification, *ubc-2*::GFP expression was seen in all body wall muscle cells (Figure 21A). Expression was localized to the nucleus and was also observed in a punctate pattern that matched the striations of the muscle sarcomere (Figure 21B, C). The punctate *ubc-2*::GFP expression pattern within the sarcomere appeared to localize to dense bodies or M-lines.

7.2 Expression of *ubc-2*::GFP in transgenic lines carrying pTS1.1

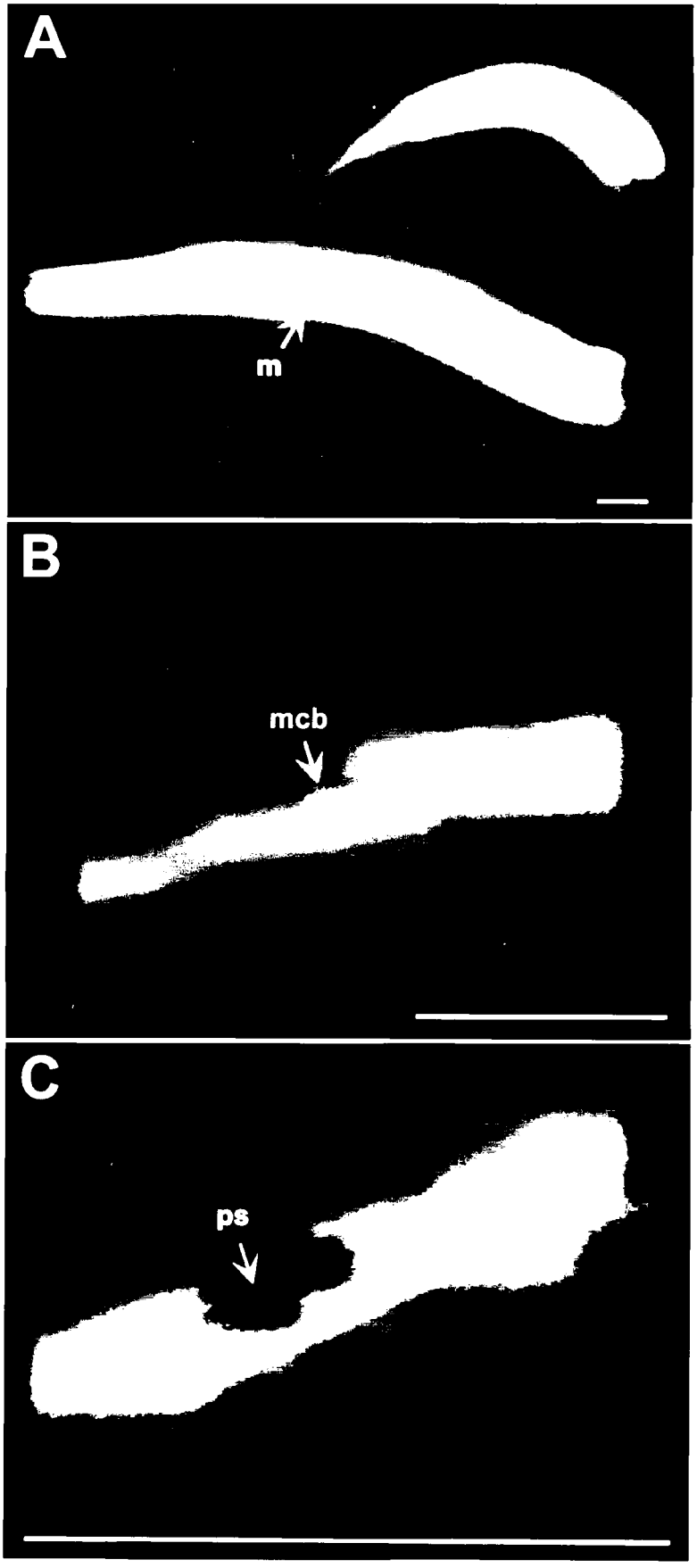
The expression of *ubc-2*::GFP in transgenics carrying pTS1.1 was extremely poor. Due to the mosaic nature of the extrachromosomal array, only a small number of nuclei were observed to express the transgene in individual animals. Cells that expressed *ubc-2*::GFP in the nucleus included hypodermis, intestine and muscle (data not shown). However, it was often difficult to identify the nuclei that expressed *ubc-2*::GFP because there was no counterstain available to aid in cell identification.

8. DNA-MEDIATED TRANSFORMATION RESCUE OF let-70

A number of transgenic lines were constructed and tested for their ability to rescue the *let-70* mutation. The results are summarized in Table 4. One construct, pZMI.1, included 1.4 kb of sequence upstream of *ubc-2* and 2.5 kb of downstream sequence. Attempts to rescue *let-70* mutants with pZMI.1 were unsuccessful. This construct was utilized in most of the earlier transgenic *ubc-2::lacZ* expression studies (Zhen, 1995; Zhen *et al.*, 1996).

A larger construct, pZM13, which included approximately 7 kb of upstream and 6 kb of downstream sequence was also made (M. Zhen, unpubl.), and the transgenic line PC125ubEx110 was established with this construct. When PC125ubEx110 transgenics were crossed to *let-70* mutants, the resulting twitcher/roller progeny were partially rescued (Figure 17B, E). Transgenics developed past the L3 lethal stage and became adults. Interestingly, the phenotype of these partially rescued animals was similar to that of *let-70(s689)* homozygotes that developed into early adults. The intestinal defects were rescued and all transgenic animals had large, healthy-looking intestinal cells with many gut granules. However, gonadal development was abnormal and the animals were sterile. The type and extent of germline abnormalities varied. In some animals, the gonad itself failed to develop properly. The distal tips of the gonad arms were bulbous. One arm often did not reflex during outgrowth, or would

Figure 21. UBC-2::GFP is expressed in the muscle nucleus and in the sarcomere. Transgenic worms carrying pTS2.2 extrachromosomal arrays were visualized under 488 nm light for expression of the UBC-2::GFP fusion protein. A) An L4 larva expressing UBC-2::GFP in the body wall muscle bands (m). The yellow spots are a subset of gut granules that autofluoresce under 488 nm light. Anterior is left. B) Three individual muscle cells expressing UBC-2::GFP in the cell nucleus (mcb) and in a striated pattern along the length of the muscle cells. C) Close-up of muscle cells showing the punctate staining pattern (ps) in the muscle striations. Scale bar indicates 10 microns.


A
m
B
mcb
C
ps

bend several times during development. Oocytes formed in some animals, but failed to reach the uterus and were not fertilized. Thus, an oocyte, whether fertilized or not, was never observed inside the uterus of these animals. The uterus itself was a mass of undifferentiated cells with an almost tumorous appearance.

Table 4. Summary of DNA-mediated transformation rescue experiments

Plasmid Construct	Mutant Background	Genomic DNA	Rescue	Upstream Sequence	Downstream Sequence
pZMI.1	*s1132*	No	No	1.4 kb	2.5 kb
	s689				
pZM13	*s1132*	No	Partial[1]	7 kb	6 kb
	s689				
M7	*s1132*	No	Partial[1]	21 kb	1.5 kb
	s689				
pZM13	*s1132*	Yes	MEL[2]	7 kb	6 kb
	s689	Yes	FULL		
G47J11	*s1132*	Yes	No	8 kb	15 kb
	s689				

[1]Rescue to sterile adult; no eggs laid
[2]Maternal effect lethal; embryos arrest at gastrulation

Attempts were made to rescue *let-70* mutants using cosmid M7, which contained 21 kb up- and 1.5 kb downstream of *ubc-2* (J. Schein, unpubl.). It was thought that the inclusion of additional upstream sequence might permit the full expression of UBC-2. Transgenic lines containing M7 as an extrachromosomal array were established and placed in a *let-70* background. Twitcher animals were only partially rescued by the introduction of the M7 transgenic array and exhibited a sterile adult phenotype similar to that of mutant animals carrying the pZM13 array.

All previously mentioned transgenic animals carrying *ubc-2* constructs had been generated using conventional transformation techniques in which the plasmid of interest was co-injected with a marker plasmid (Fire, 1986). Such transgenes are heritably transmitted as extrachromosomal arrays with efficient expression in most somatic tissues (Chalfie *et al.*, 1994; Fire *et al.*, 1990). Interestingly, many laboratories have reported the inability to observe any reporter transgene expression in the germline. This led to the general theory that transgenes are recognized differently by the germline and the soma, and are somehow repressed in the

postembryonic germline. Immunofluorescent staining of *C. elegans* with anti-UBC-2 showed that UBC-2 was strongly expressed in the germline (see Section III.6). As discussed above, *let-70* mutants carrying UBC-2-containing extrachromosomal arrays were partially rescued in a manner that suggested that somatic defects were rescued while germline defects were not. Together, this information indicated that germline expression of UBC-2 might be required for development. Recent work by Kelly *et al.* (1997) has shown that the inclusion of random genomic fragments of nematode DNA in the injection mixture results in robust expression of transgenes in both germline and somatic tissue. It is believed that addition of genomic DNA increases the genetic complexity of the extrachromosomal array, thus preventing silencing of the array in the germline.

To determine if germline expression of *let-70* was required for development, wild type animals were injected with pZM13 and N2 genomic DNA fragments (see Section II.6.3) to generate transgenic lines. Each transgenic line was then crossed with both alleles of *let-70*, and the progeny were examined for the presence of twitcher/roller animals. These animals were presumably homozygous *let-70* animals carrying the pZM13 transgenic array. The development of these animals was then monitored.

8.1 N2 genomic DNA fragments do not rescue *let-70*

Control injections were performed in which wild type individuals were injected with a mixture of pRF4 and N2 genomic DNA fragments alone (cx-control). Several independent stable lines were established. Each line was placed in *let-70(s689)* and *let-70(s1132)* mutant backgrounds, and the development of twitcher/roller progeny was followed. Homozygous *let-70* mutants that contained the cx-control array arrested at L3. The arrest phenotype of these animals was similar to that observed in *let-70* mutant strains. They were thin with small intestine cells that lacked microvilli and gut granules. Thus, extrachromosomal arrays containing N2 genomic DNA fragments alone were not sufficient to rescue *let-70* mutant strains.

8.2 Germline and somatic expression of pZM13 can rescue *let-70(s689)*

N2 genomic DNA fragments were mixed with pZM13 and pRF4 to make a complex pZM13 mixture (cx-pZM13), injected into N2 animals, and six independent lines were generated (Appendix C). When placed in a *let-70(s689)* background, homozygous mutants carrying cx-pZM13 were fully rescued. Full rescue was obtained with all six transgenic lines

(Figure 22C, F). Intestinal cells were nearly wild type in appearance and a fully functional gonad was produced. However, the rescued *let-70(s689)* animals exhibited a slow growth phenotype and were slightly egg-laying defective such that embryos were retained in the uterus longer than normal before being released. Brood size was fairly small, ranging from 15 to 150 eggs, with a mixture of fertilized and unfertilized embryos. Approximately 30% of the eggs that were laid hatched into viable progeny. These phenotypes may be caused by mosaic expression of the transgene, since it was not integrated into the genome.

The complete rescue of the *let-70(s689)* mutation with cx-pZM13 indicated that germline expression of *let-70* was required for *C. elegans* development. Since the genomic DNA alone was unable to rescue *let-70* mutants, it appears that combined somatic and germline expression of pZM13 is necessary for rescue of the *let-70(s689)* mutation. Thus, cx-pZM13 contains all of the information required for the proper spatial and temporal expression of *ubc-2*.

8.3 Germline and somatic expression of pZM13 does not rescue *let-70(s1132)*

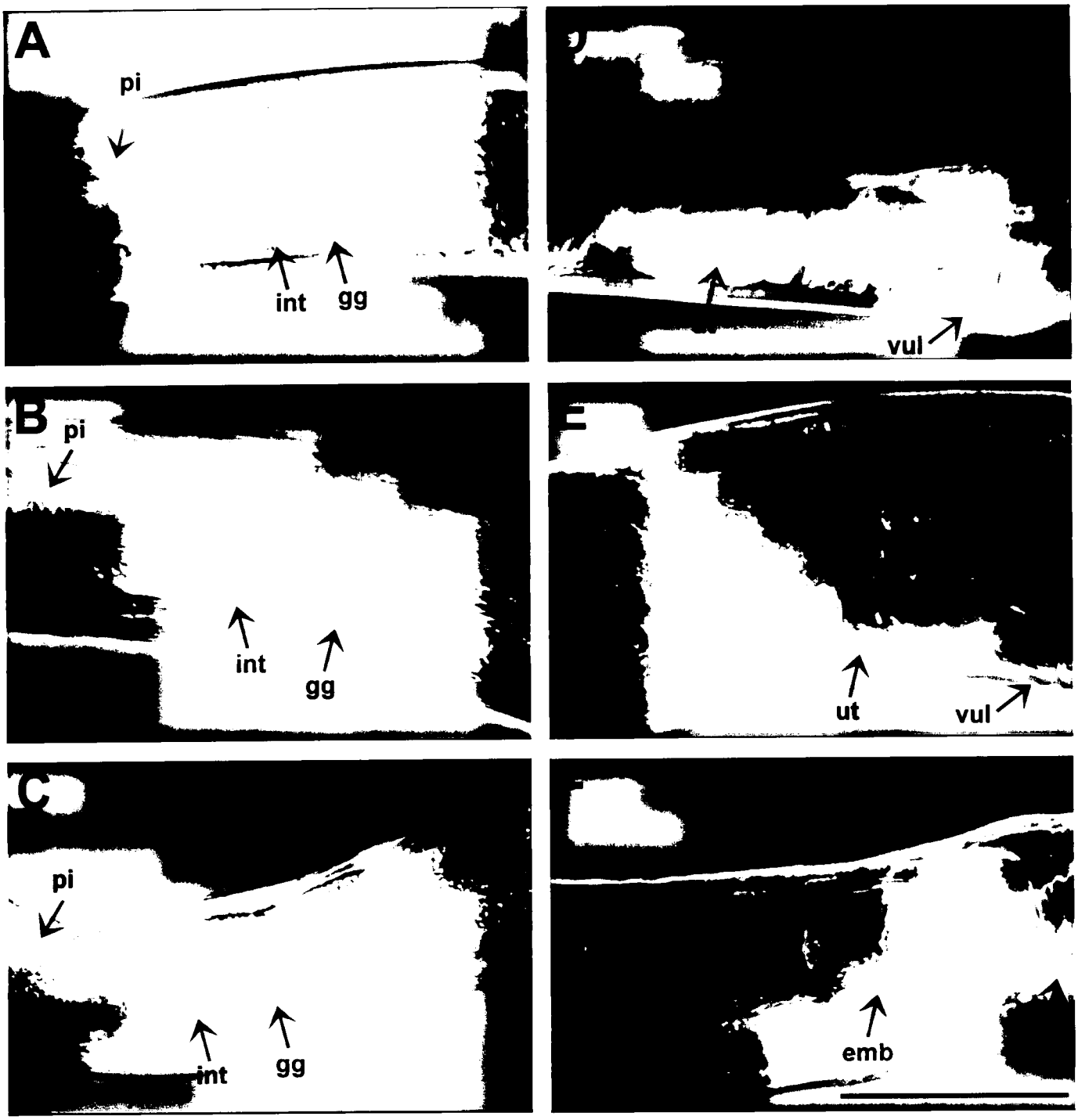
Homozygous *let-70(s1132)* mutants were not completely rescued by the complex pZM13 array. Transgenic mutants developed beyond the L3 lethal stage and became egg-laying adults. The gonad developed normally, and hermaphrodites laid around 100 fertilized eggs. However, all embryos arrested at gastrulation or soon thereafter. No viable progeny were produced by *let-70(s1132)* animals that carried the cx-pZM13 array. Therefore, the same extrachromosomal array that was capable of fully rescuing *let-70(s689)* mutants was incapable of rescuing *let-70(s1132)* mutants. This suggested that a feature unique to *let-70(s1132)* affected the ability of cx-pZM13 to rescue the strain.

9. DNA-MEDIATED TRANSFORMATION RESCUE WITH A TEMPERATURE-SENSITIVE ubc-2 ALLELE

The ability to control the developmental expression of a gene has greatly contributed to our understanding of gene function. In genetics, temperature-sensitive mutations have proven to be one of the most useful methods for controlling gene expression. In yeast, a number of temperature-sensitive E2 mutations have been isolated (Ellison *et al.*, 1991; Goebl *et al.*, 1988). It was therefore of interest to construct a temperature-sensitive *ubc-2* mutant that would permit one to examine the temporal requirements for *ubc-2* expression in *C. elegans*.

The full rescue of the *let-70(s689)* mutation indicated that, when expressed in somatic and germline tissue, pZM13 contained all of the *let-70* control elements required for proper

Figure 22. *let-70(s689)* is partially rescued by somatic expression of *ubc-2* transgenic arrays and is fully rescued by somatic and germline expression of *ubc-2*. Worms were visualized under Nomarski optics. A and D) Wild type animal. Panel A shows the terminal bulb of the pharynx and the upper intestine. Anterior is left. The pharyngeal-intestinal valve (pi), a representative intestine cell (int) and gut granules (gg) are indicated. Panel D shows the gonad with uterus (ut) and vulva (vul) indicated. B and E) Somatic expression of the pZM13 transgenic array in a *let-70(s689)* mutant background. The strain is PC125ubEx110. Partial rescue is evident. Panel B shows rescue of the intestinal defects. Panel E shows that gonad development is defective. Note the granular appearance of the uterus. C and F) Germline and somatic expression of pZM13 transgenic array in a *let-70(s689)* mutant background. The strain is PC167ubEx137. Full rescue was obtained. In panel C, the intestinal defects have been rescued. Note the wild type appearance of the intestinal cells with numerous gut granules. Panel F shows the ventral surface of a rescued animal. The uterus contains two developing embryos (emb). Note that one embryo is in comma stage. Scale bar indicates 50 microns.


A
pi
int
gg
vul
B
pi
int
gg
ut
vul
C
pi
int
gg
emb

expression of UBC-2 during development. Mei Zhen had previously constructed a putative temperature-sensitive allele of *ubc-2*, pZM13.34, which contained a P61S alteration (see Section II.6.2). In yeast, the permissive temperature for this allele was 30°C while the non-permissive temperature was 39°C (Ellison *et al.*, 1991). Earlier attempts to rescue *let-70* mutants with this putative temperature-sensitive allele at 15°C and 25°C had been unsuccessful. However, the transgenic lines had been generated by conventional techniques, which indicated that pZM13.34 would probably not have been expressed in the germline. Rescue experiments with the putative temperature-sensitive *let-70* allele, pZM13.34, were therefore repeated using transformation techniques that allowed both germline and somatic expression of the transgene. N2 genomic DNA fragments were mixed with pRF4 and pZM13.34 to make the complex mixtures cx-pZM13.34, injected into N2 animals and stable transgenic lines were established.

Transgenic lines containing complex pZM13.34 extrachromosomal arrays were crossed to *let-70* animals and roller progeny that twitched in 1% nicotine were set to individual plates. The development of twitcher/roller animals at the putative permissive (15°C) and non-permissive (25°C) temperatures was monitored.

When transgenic lines carrying the cx-pZM13.34 extrachromosomal arrays were placed in a *let-70(s1132)* background, twitcher/roller animals developed into sterile adults at both 15°C and 25°C. The phenotype was similar to that previously described for partially rescued animals (see Section III.8.1). The intestinal defect was rescued but oocytes did not move into the uterus, and the uterus consisted of a mass of undifferentiated cells. The partially rescued phenotype suggested that cx-pZM13.34 was able to rescue the somatic defects of *let-70(s1132)*, but could not rescue the germline defects, despite the expectation that cx-pZM13.34 would be expressed in the germline. In addition, it did not appear that cx-pZM13.34 functioned in a temperature-dependent manner, since similar phenotypes were observed at both temperatures.

The cx-pZM13.34 array was also placed in a *let-70(s689)* background. At 15°C, transgenic twitcher/rollers developed into egg-laying adults. A large number of fertilized and unfertilized eggs were laid; however, embryos arrested at gastrulation and no progeny hatched. This suggested that cx-pZM13.34 in a *let-70(s689)* background at 15°C was a maternal effect lethal. Interestingly, twitcher/roller animals maintained at 25°C were fully rescued. The animals had a slow growth phenotype, laid few eggs and only a small portion of those hatched and developed into adults. The phenotype was very similar to that of *let-70(s689)* mutants that were rescued by somatic and germline expression of cx-pZM13 (see Section III.8.2), although

the mutants rescued with cx-pZM13.34 appeared less healthy. It is possible that integration of the extrachromosomal array into the genome of *let-70(s689)* animals might further increase the survival of this strain.

In summary, it appears that cx-pZM13.34 is a cold-sensitive allele of *let-70* in *C. elegans*, and that the permissive temperature is 25°C while the non-permissive temperature is 15°C.

10. C. briggsae FOSMID IDENTIFICATION, SEQUENCING AND COMPARISON TO C. elegans

The evolutionary conservation of genes and proteins between closely related species has been useful for the identification of functionally important domains and structures within a gene or protein family (Pilgrim *et al.*, 1995; Snutch and Baillie, 1983). In addition, the comparison of flanking sequences has identified important regions that control the temporal and spatial expression of specific proteins. For *C. elegans*, DNA and polypeptide sequences are often compared with sequences from a closely related nematode species, *C. briggsae*. While both nematode species are almost identical in development and morphology (Nigon and Dougherty, 1949), it has been estimated that the two species diverged 23-40 million years ago (Emmons *et al.*, 1979; Heschl and Baillie, 1990; Kennedy *et al.*, 1993). Comparison of sequences between the two species has demonstrated that synteny is conserved, often over large sequence tracts (Thacker *et al.*, 1999), and for polycistronic transcription units (Page, 1999). However, DNA sequence conservation between the two species is confined to protein-coding sequences and short flanking sequences. Conserved protein-coding sequences have identified domains and structures that are important in protein function and conserved 5' flanking sequences have identified cis-acting elements that are involved in the regulation of gene expression (Heschl and Baillie, 1990; Krause *et al.*, 1994; Kuwabara, 1996; Lee *et al.*, 1992).

10.1 Identification and sequencing of *C. briggsae* fosmid G47J11

In this study, the evolutionary conservation of *ubc-2* and flanking sequences was examined between *C. elegans* and *C. briggsae*. To identify the *C. briggsae* genomic clone containing the *ubc-2* homolog, a *C. briggsae* gridded fosmid filter was hybridized with a ^{32}P-labelled *ubc-2* genomic DNA fragment. The *ubc-2* fragment hybridized to several fosmids on the filter, all of which mapped to a single contig, indicating that the *ubc-2* genomic fragment had hybridized to a single copy gene in *C. briggsae*. Contig mapping of the fosmids was

carried out at the Genome Sequencing Center, St. Louis, MO. Fosmids located within the contig were obtained from the Genome Sequencing Center. PCR amplification of the fosmids using oligonucleotide primers OZM2.r and OZM3.f identified those containing the *C. briggsae* homolog of *ubc-2* (*Cb-ubc-2*), and the fosmid G47J11 was submitted to the Genome Sequencing Center for sequencing.

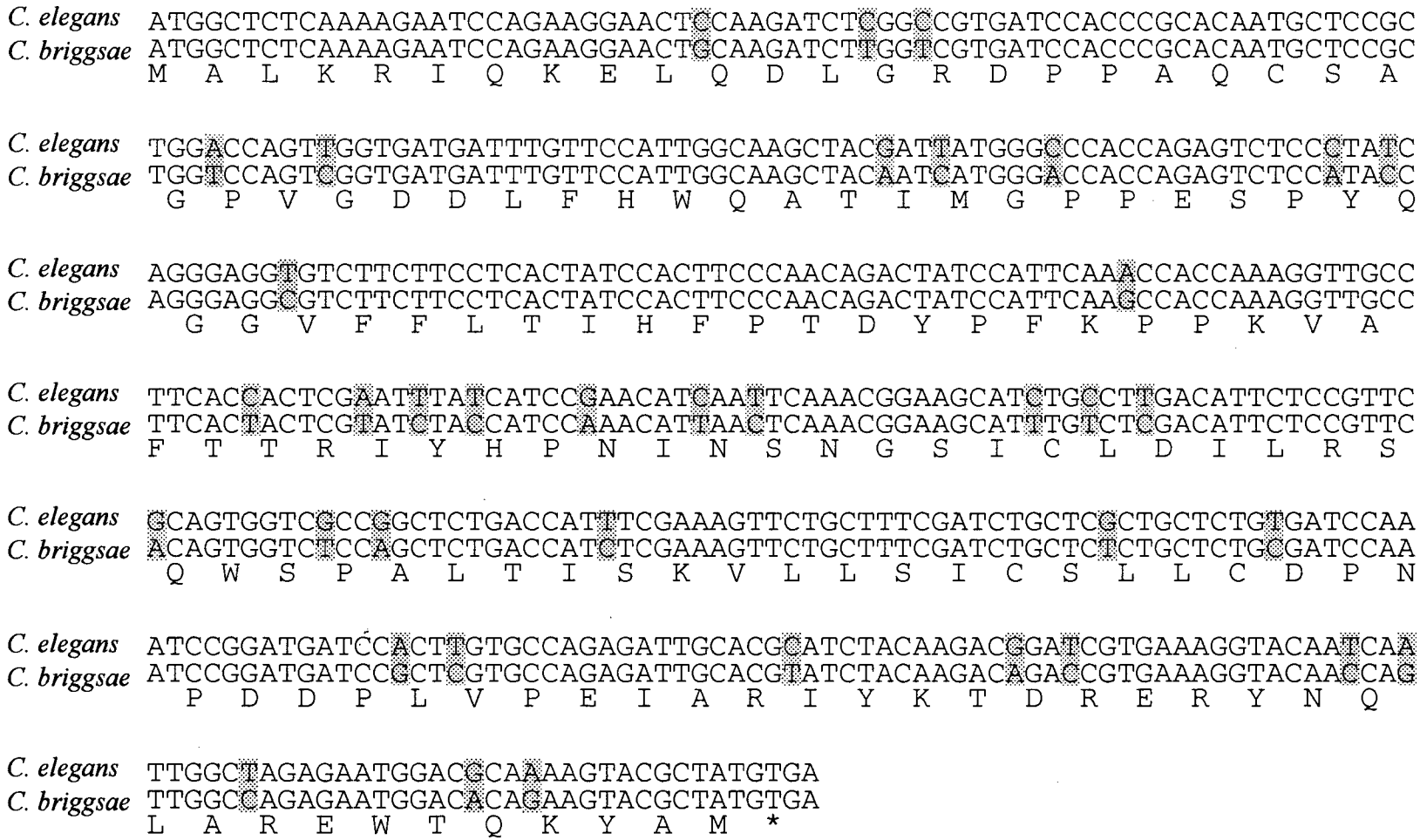
10.2 Sequencing and comparison of *Cb-ubc-2* with *Ce-ubc-2*

While the fosmid sequencing was in progress, the *Cb-ubc-2* homolog was subcloned and sequenced. *Cb-ubc-2* was amplified by PCR using primers OZM2.r and OZM3.f. High fidelity Taq polymerase (Boehringer Mannheim) was used in the reaction to reduce amplification errors. The amplification product was sub-cloned into pBSIIKS(+) and sequenced. The sequence of *Cb-ubc-2* was determined and compared with *C. elegans ubc-2* (*Ce-ubc-2*; Figure 23). Comparison of the two gene sequences indicated that they were 79.7% identical and the coding sequences were 91.4% identical. When the coding sequence was conceptually translated, all of the nucleotide changes between *Ce-ubc-2* and *Cb-ubc-2* occurred in the third position of codons, and indicated that the coding sequences were 100% conserved. Thus, the polypeptide sequences of *Ce-ubc-2* and *Cb-ubc-2* were 100% identical. The high degree of sequence conservation in *ubc-2* between the two species suggests that selective pressure on *ubc-2* has been strong.

10.3 *Cb-ubc-2* does not rescue *let-70* mutants

Many *C. briggsae* genes have exhibited functional conservation, as demonstrated by rescue of *C. elegans* mutant phenotypes by DNA-mediated transformation (Krause *et al.*, 1994; Kuwabara, 1996; Maduro and Pilgrim, 1996; Thacker *et al.*, 1999). To determine the functional conservation of *Cb-ubc-2*, the *C. briggsae* fosmid G47J11 was transformed into *let-70* mutants. G47J11 was co-injected with random genomic DNA fragments and the marker plasmid pRF4 into N2 animals, and stable transgenic lines were established. The cx-G47J11 transgenic arrays were placed in a *let-70(s1132)* and a *let-70(s689)* background, and the survival of twitcher/roller progeny was examined. Mutant animals from both *let-70* strains that contained cx-G47J11 extrachromosomal arrays arrested at L3; the arrest phenotype was similar to that seen in *let-70* homozygous mutants (Table 4). Therefore, the 23 kb *C. briggsae* fragment containing the *ubc-2* homolog was unable to rescue the *let-70* lethality in *C. elegans*.

Figure 23. ***C. elegans*** **and** ***C. briggsae ubc-2*** **polypeptide sequences are absolutely conserved.** Nucleotide coding sequence of *C. elegans* and *C. briggsae ubc-2*. Sequence differences between species are shaded. The protein sequence is indicated below the nucleotide sequence in single letter code. Stop codons are indicated with an asterisk.


C. elegans ATGGCTCTCAAAAGAATCCAGAAGGAACTCCAAGATCTCGGCCGTGATCCACCCGCACAATGCTCCGC
C. briggsae ATGGCTCTCAAAAGAATCCAGAAGGAACTGCAAGATCTTGGTCGTGATCCACCCGCACAATGCTCCGC
M A L K R I Q K E L Q D L G R D P P A Q C S A
C. elegans TGGACCAGTTGGTGATGATTTGTTCCATTGGCAAGCTACGATTATGGGCCCACCAGAGTCTCCCTATC
C. briggsae TGGTCCAGTCGGTGATGATTTGTTCCATTGGCAAGCTACAATCATGGGACCACCAGAGTCTCCATACC
G P V G D D L F H W Q A T I M G P P E S P Y Q
C. elegans AGGGAGGTGTCTTCTTCCTCACTATCCACTTCCCAACAGACTATCCATTCAAACCACCAAAGGTTGCC
C. briggsae AGGGAGGCGTCTTCTTCCTCACTATCCACTTCCCAACAGACTATCCATTCAAGCCACCAAAGGTTGCC
G G V F F L T I H F P T D Y P F K P P K V A
C. elegans TTCACCACTCGAATTTATCATCCGAACATCAATTCAAACGGAAGCATCTGCCTTGACATTCTCCGTTC
C. briggsae TTCACTACTCGTATCTACCATCCAAACATTAACTCAAACGGAAGCATTTGTCTCGACATTCTCCGTTC
F T T R I Y H P N I N S N G S I C L D I L R S
C. elegans GCAGTGGTCGCCGGCTCTGACCATTTCGAAAGTTCTGCTTTCGATCTGCTCGCTGCTCTGTGATCCAA
C. briggsae ACAGTGGTCTCCAGCTCTGACCATCTCGAAAGTTCTGCTTTCGATCTGCTCTCTGCTCTGCGATCCAA
Q W S P A L T I S K V L L S I C S L L C D P N
C. elegans ATCCGGATGATCCACTTGTGCCAGAGATTGCACGCATCTACAAGACGGATCGTGAAAGGTACAATCAA
C. briggsae ATCCGGATGATCCGCTCGTGCCAGAGATTGCACGTATCTACAAGACAGACCGTGAAAGGTACAACCAG
P D D P L V P E I A R I Y K T D R E R Y N Q
C. elegans TTGGCTAGAGAATGGACGCAAAAGTACGCTATGTGA
C. briggsae TTGGCCAGAGAATGGACACAGAAGTACGCTATGTGA
L A R E W T Q K Y A M *

10.4 Organization and sequencing of regions flanking *ubc-2*

To generate a contiguous *C. elegans* sequence for comparison, the reverse complement of cosmid sequence M7 was joined to the sequence of the yeast artificial chromosome (YAC), Y5F2. A portion of this DNA sequence (called M7+Y5F2), consisting of 10 kb upstream and 20 kb downstream of *ubc-2*, was utilized for comparison to the complete *C. briggsae* fosmid sequence, G47J11. The M7+Y5F2 sequence was analyzed using Genefinder (P. Green and L. Hillier, unpubl.) which predicts the locations of putative genes on DNA sequences. Within this 31.5 kb region, twelve genes were predicted. The locations and direction of transcription of these genes is illustrated in Figure 24A. A cluster of five genes is located upstream of *ubc-2* (M7.3, M7.7, M7.6, M7.4 and M7.2). The predicted gene M7.3 has similarity to the KH domain family of RNA binding proteins. M7.6 and M7.4 have no known homologs in other species. These three genes are transcribed in the same direction, and are located quite close to one another. The ATG start codon of M7.6 is located 22 nucleotides (nt) downstream of the predicted polyadenylation signal for M7.3, and the ATG start codon for M7.4 is 257 nt downstream of the predicted polyadenylation signal for M7.6. These features suggest that these three predicted genes form a polycistronic unit. M7.7, a predicted serine/threonine kinase, is transcribed from the complementary strand. The coding sequence of M7.7 is located within several introns of M7.3. M7.2, a predicted kinesin light chain gene is located nearest to *ubc-2* and is transcribed off the complementary strand.

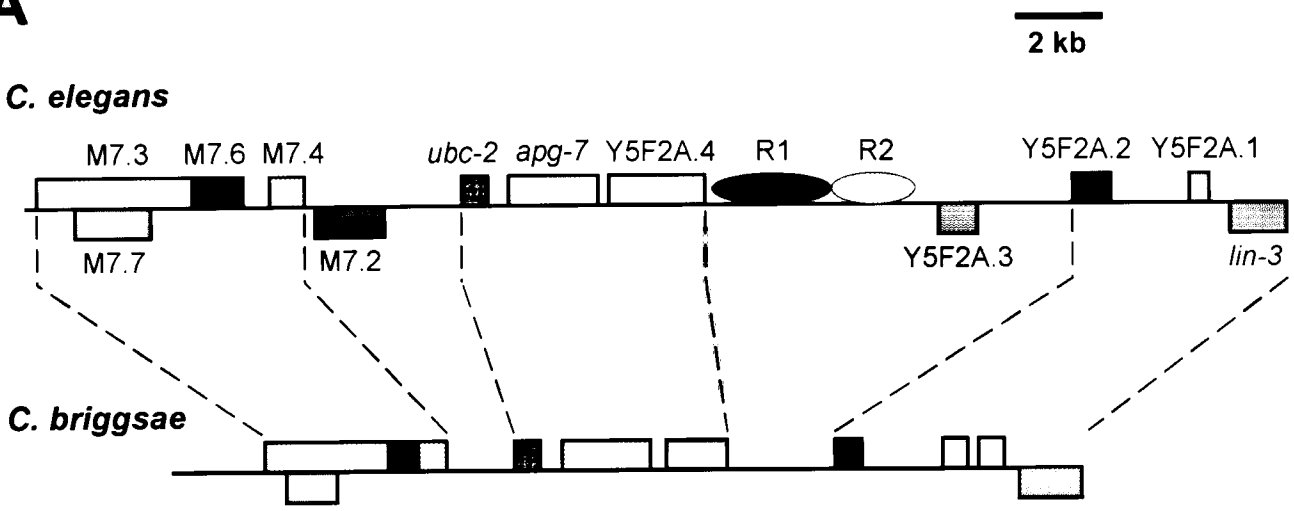
Downstream of the gene cluster that includes *ubc-2* lies a 3.6 kb repetitive sequence, R1. The repeat unit is 40 nucleotides long, and is fairly well conserved throughout its length. Immediately downstream of R1 lies a second repetitive sequence of 1.4 kb, R2, which is composed of a repeat unit 9 bp long that is also fairly well conserved. Four genes are predicted downstream of these repetitive sequences. Y5F2A.3 is transcribed from the complementary strand and has no known homology in other species. Y5F2A.1 and Y5F2A.2 are predicted transthyretin-like genes. Downstream of these genes lies *lin-3*, which is transcribed from the complementary strand.

10.5 *ubc-2* is part of a polycistronic unit

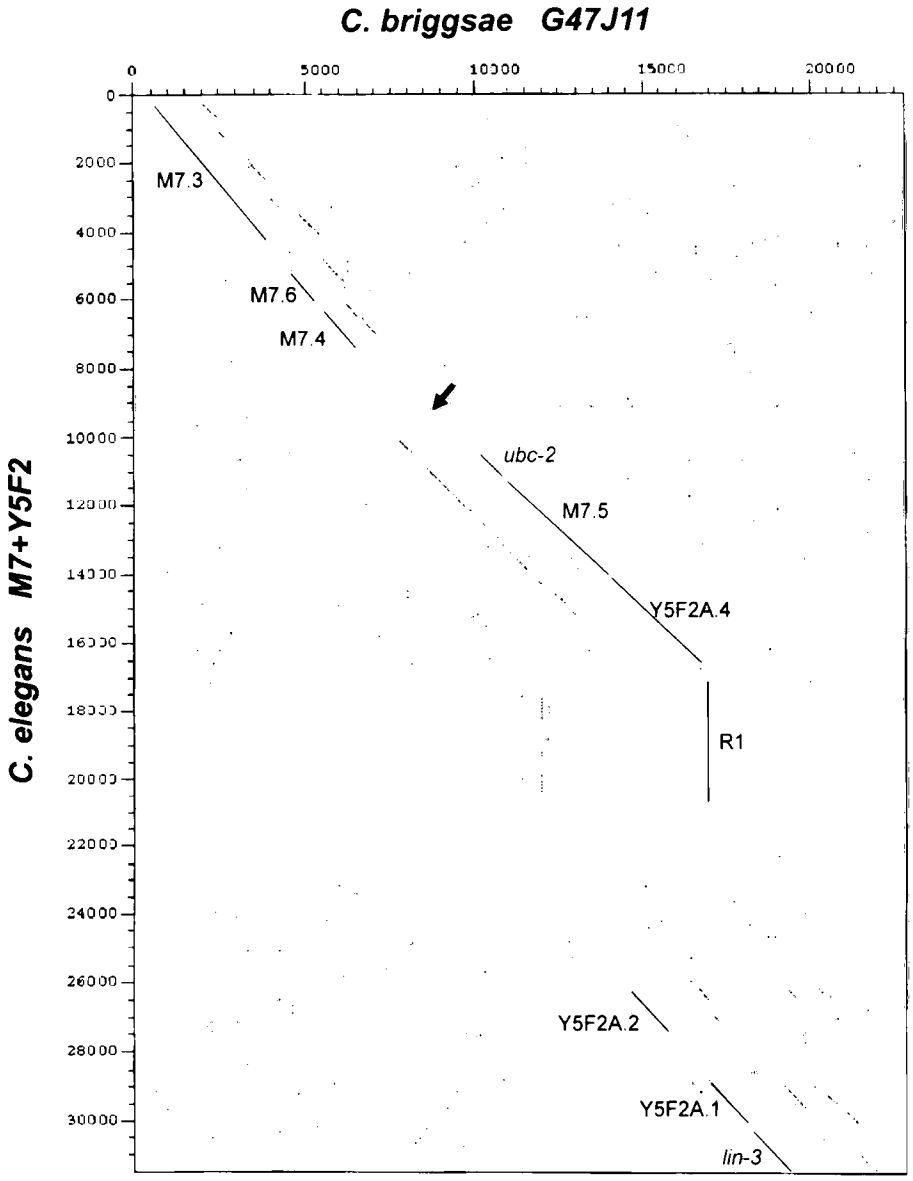
Two genes, M7.5 and Y5F2A.4, form a gene cluster with *ubc-2*. BLAST (basic local alignment search tool) searches predict that M7.5 has homology to a yeast hypothetical protein, YHX1 and a yeast gene called APG7. Comparison of YHX1 and APG7 sequences indicated

Figure 24. Genomic organization of the *C. elegans* and *C. briggsae ubc-2* region. A) Schematic representation of the *ubc-2* region from *C. elegans* M7+Y5F2 (top) and *C. briggsae* G47J11 (bottom). Genes predicted by Genefinder are indicated as boxes and are labelled, non-coding regions as lines and repeat sequences as ovals. Genes located above the line are transcribed left to right, and genes located below the line are transcribed in the opposite direction. Homologous genes are pattern coded. Note that the genomic region in *C. briggsae* is lacking the genes M7.2 and Y5F2A.3 and the repeats R1 and R2. In addition, the Y5F2A.1 homolog has been duplicated. Scale bar indicates 2 kb. B) Dot matrix comparison of *C. elegans* M7+Y5F2 and *C. briggsae* G47J11 sequences showing syntenic conservation. M7+Y5F2 sequences (vertical axis) are plotted against G47J11 (horizontal axis). Regions of similarity between the two sequences show as diagonal lines. The relative positions of genes are indicated by solid lines with the accompanying gene name. The arrow indicates a possible control region.

A


2 kb
C. elegans
M7.3
M7.6
M7.4
ubc-2
apg-7
Y5F2A.4
R1
R2
Y5F2A.2
Y5F2A.1
M7.7
M7.2
Y5F2A.3
lin-3
C. briggsae

B

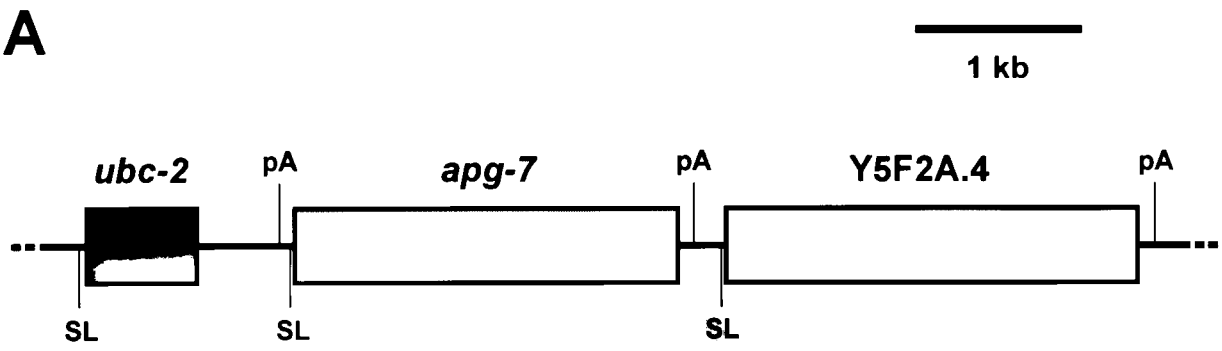

C. briggsae G47J11
C. elegans M7+Y5F2
0
5000
10000
15000
20000
2000
4000
6000
8000
10000
12000
14000
16000
18000
20000
22000
24000
26000
28000
30000
M7.3
M7.6
M7.4
ubc-2
M7.5
Y5F2A.4
R1
Y5F2A.2
Y5F2A.1
lin-3

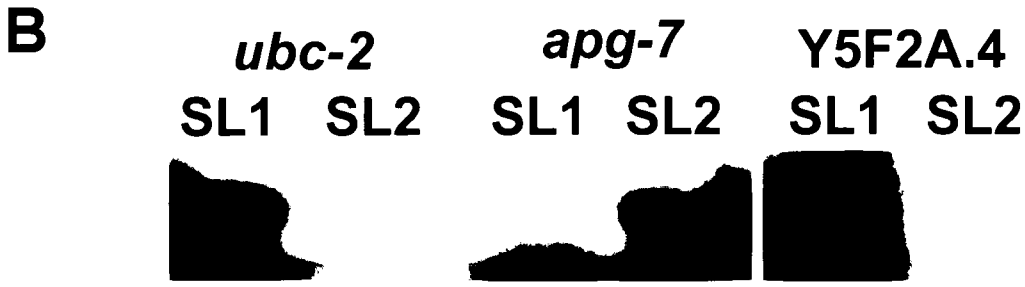
that they are the same gene. Thus, M7.5 is a *C. elegans* homolog of APG7, and will henceforth be called *apg-7*. Y5F2A.4 is a predicted zinc finger protein. Analysis of the organization of the three genes, *ubc-2*, *apg-7* and Y5F2A.4 suggested that they may be co-transcribed as a polycistronic unit. All three genes are closely spaced and transcribed in the same orientation. The predicted ATG start codon of *apg-7* is located 40 nt downstream of the *ubc-2* polyadenylation signal (Figure 25A). Immediately upstream of the *apg-7* predicted ATG start codon is a perfect *trans*-splice donor site. The predicted ATG start codon of Y5F2A.4 is located 120 nt downstream of the predicted *apg-7* polyadenylation signal. Again, a *trans*-splice donor site is located upstream of the Y5F2A.4 ATG start. The genomic organization of these genes suggests that they form an operon such that transcription of all three genes is driven by a single promoter/enhancer region located 5' of the *ubc-2* gene.

10.6 *Trans*-splicing of genes in the polycistronic unit

The genomic organization of the three genes *ubc-2*, *apg-7* and Y5F2A.4 strongly suggested that they formed a polycistronic transcriptional unit. As described in Section I.6.6, genes within operons can often be identified by the leader sequence that is *trans*-spliced to the mRNA during processing. In general, monocistronic genes and genes that lie near the promoters of polycistronic groups accept the SL1 leader, while downstream genes may either be exclusively SL2-spliced, or may utilize a combination of SL1- and SL2-splicing. Previous analysis of *ubc-2* cDNA indicated that *ubc-2* was SL1-spliced (Zhen *et al.*, 1996). To determine if *ubc-2*, *apg-7* and Y5F2A.4 were SL1- and/or SL2-spliced, Southern analysis of cDNA PCR products was performed. Total RNA was isolated from a mixed stage population of *C. elegans* and first strand cDNA was synthesized. PCR amplification was performed using primers specific for SL1 or SL2 and a primer specific for the gene of interest. For *ubc-2*, OZM2.r was used in combination with each of the SL primers. For *apg-7* and Y5F2A.4, the YHX1-B1.r and Zinc1.r primers were used, respectively. The amplified samples were loaded onto agarose gels, separated by electrophoresis and transferred to a nylon membrane. The membrane was hybridized with ^{32}P-labelled pZM13, which contains a single copy of each gene. The results of the Southern blot are shown in Figure 25B. As can be seen, *ubc-2* was exclusively SL1-spliced. The *apg-7* gene was both SL1- and SL2-spliced, although it appeared that it was preferentially SL2-spliced. Y5F2A.4 appeared to be mostly SL1-spliced.

Figure 25. *ubc-2* is part of a polycistronic unit that includes *apg-7* and a zinc finger gene, and is exclusively SL1-spliced. A) Schematic representation of the *ubc-2*-containing polycistronic unit. Genes are indicated as boxes and non-coding regions as lines. Predicted splice leader sequences (SL) and polyadenylation sites (pA) are indicated. Scale bar indicates 1 kb. B) *ubc-2* and Y5F2A.4 are exclusively SL1-spliced while *apg-7* is SL1- and SL2-spliced. First strand cDNA was PCR amplified, blotted and probed with radiolabelled pZM13. The PCR primers used were SL1 (spliced leader 1) or SL2 (spliced leader 2) in combination with OZM2 (for *ubc-2*), YHX1-B (for *apg-7*) and zinc1 (for Y5F2A.4). See Appendix C for PCR primer sequences.


A
1 kb
ubc-2
pA
apg-7
pA
Y5F2A.4
pA
SL
SL
SL


B
ubc-2
apg-7
Y5F2A.4
SL1 SL2
SL1 SL2
SL1 SL2

10.7 Syntenic conservation in the *ubc-2* region between *C. elegans* and *C. briggsae*

The extent of genomic conservation between *C. elegans* and *C. briggsae* throughout the *ubc-2* region was determined. DNA sequence comparisons between the 31.5 kb M7+Y5F2 sequence of *C. elegans* and the 23 kb G47J11 fosmid sequence of *C. briggsae* were performed using the Dotter program (Sonnhammer and Durbin, 1996), which compares every nucleotide from one sequence to every nucleotide on the other sequence to create a dot-matrix plot of similarity and a sequence alignment display. This program provides a powerful means for identifying homologies between two sets of sequences. Figure 24B shows that syntenic conservation was fairly strong over this large stretch of DNA with some interesting exceptions. Many of the genes were conserved in location and orientation, and exhibited a high degree of sequence similarity. Intergenic sequences were reduced in length in *C. briggsae* throughout the region. This has also been observed with other genes such as *unc-119* and *bli-4* (Maduro and Pilgrim, 1996; Thacker *et al.*, 1999).

The structure of the gene cluster containing the *C. briggsae* homologs of M7.3, M7.7, M7.6 and M7.4 was conserved. In *C. briggsae*, however, the intergenic regions were much shorter, which brought the predicted genes much closer to one another. Within this cluster, the stop codon of a gene was generally located within 100 bp of the start codon of another gene. In many cases, however, it was difficult to recognize the polyadenylation signal within the short sequence span between the genes. The placement and orientation of the M7.7 homolog (the putative serine-threonine kinase) within the introns of the M7.3 gene was conserved.

An interesting exception to the synteny of this region was the apparent deletion of sequences within the region of the M7.2 gene in *C. briggsae*. M7.2 codes for a kinesin light chain in *C. elegans* and is missing in the corresponding region in *C. briggsae*. As a consequence of this deletion, *Cb*-M7.4 is in close proximity to the *Cb-ubc-2* gene. The ATG start codon of *Cb-ubc-2* is 1458 nt downstream of the putative polyadenylation site of *Cb*-M7.4, while in *C. elegans*, that same intergenic region is around 3.6 kb and includes the M7.2 gene.

Comparison of the *ubc-2* region in *C. briggsae* indicated that the genomic arrangement of *Cb-ubc-2*, *Cb-apg-7* and *Cb*-Y5F2A.4 genes followed the same organization as in *C. elegans*. The predicted ATG start of *Cb-apg-7* is located 65 nt downstream of the *Cb-ubc-2* polyadenylation signal, and a *trans*-splice consensus sequence is located immediately upstream of the ATG start. Interestingly, the sequence of the intergenic region between *Cb-ubc-2* and

Cb-apg-7 is highly conserved. The predicted ATG start of *Cb*-Y5F2A.4 is 265 nt downstream of the putative *Cb-apg-7* polyadenylation site. In a reversal of the trend, the intergenic sequence lengths within this polycistronic unit are longer in *C. briggsae* than they are in *C. elegans*. Alignment of the *Ce-apg-7* and *Cb-apg-7* sequences indicates 65.3% identity in genomic sequence. The putative coding sequences were 65.4% identical. The introns of *apg-7* were fairly short, and thus did not strongly affect the identities between the genomic and the coding sequences. The polypeptide sequence was 62.5% identical. Polypeptide sequence conservation of *apg-7* between the two nematode species was much lower than that observed for *ubc-2*, which was 100%. Sequence similarity was even less with the Y5F2A.4 homologs. Genomic sequences were 46.2 % identical, coding sequences were 63.6 % , while polypeptide sequence identity was 55.4 %.

Upstream of the *ubc-2* polycistronic unit there is a short region of similarity between the two species which may include promoter/enhancer control elements of the operon (Figure 24B). A TATA box was predicted in *C. elegans* within this region 170 bp upstream of the ATG start codon. However, this element was not conserved in *C. briggsae*. In fact, no TATA elements were found within the 2 kb region upstream of *Cb-ubc-2*.

A 9 kb sequence segment downstream of the *ubc-2* polycistronic unit was deleted in *C. briggsae*. This segment included the large repetitive sequence and the Y5F2A.3 gene. The location of the repetitive element is indicated in Figure 24B as several long vertical lines (marked R1), indicating that some sequences within the repetitive element are present in the *C. briggsae* genomic sequence. An examination of the genomic sequence revealed that the sequences present in *C. briggsae* with similarity to the repeat element were mostly poly-T tracts, which are part of the *C. elegans* repeat element, R1.

Portions of the Y5F2A.2 transthyretin-like gene sequence, including its location and orientation, were conserved in *C. briggsae*. Interestingly, the Y5F2A.1 gene homolog has been partially duplicated in *C. briggsae* and the duplication is located immediately downstream of the first gene copy, with the same orientation. Downstream of the *Cb*-Y5F2A.1 gene, the *Cb-lin-3* gene is also conserved in sequence, location and orientation.

In summary, synteny has been conserved in some regions of the genomic sequence flanking *ubc-2* in *C. elegans* and *C. briggsae*. The *ubc-2* polycistronic unit exhibits conservation in gene structure, orientation and in parts of the sequence. Some interesting exceptions to the syntenic conservation have been identified. Genomic sequences immediately

surrounding the *ubc-2* polycistronic unit have been deleted in *C. briggsae*. The deletion removes approximately 2.5 kb upstream of *ubc-2* and includes the kinesin light chain gene, M7.2. Another deletion removes approximately 9 kb downstream of the *ubc-2* polycistronic unit. The sequences absent in *C. briggsae* include the 3.5 kb repeat sequence and the putative transthyretin-like gene, Y5F2A.3. In addition, there has been a partial duplication of the Y5F2A.1 transthyretin-like gene in *C. briggsae*. Thus, the regions immediately surrounding the *ubc-2* polycistronic unit have not been conserved between the two nematode species, although the unit itself has been conserved, as have the sequences flanking the deleted regions.

IV. DISCUSSION

1. SUMMARY

In this study, a detailed analysis of the *let-70* mutant phenotype was undertaken. The principle phenotype was L3 lethal, although some *let-70(s689)* animals developed into sterile adults. Defects were observed in muscle positioning, attachment and sarcomere assembly. Intestinal cell attachment and maturation were also affected. Within the reproductive system, the somatic gonad and vulva failed to develop properly, and oocytes did not mature. Through dsRNA interference and analysis of a cold-sensitive allele of *let-70*, pZM13.34, a maternal requirement for UBC-2 was identified.

The *let-70(s689)* allele is a mutation in the splice donor site of the fourth intron that is subject to *smg*-mediated mRNA surveillance. The *s2293* allele is a large deletion that spans many essential genes, including *ubc-2*.

ubc-2 forms part of a polycistronic unit with two other genes, *apg-7* and Y5F2A.4. The amino acid composition of *C. elegans* UBC-2 is 100% identical to *C. briggsae* UBC-2. When genomic sequences surrounding the *ubc-2* gene were compared between the two species, a high degree of synteny was observed; however, some interesting differences were seen in the sequences surrounding the *ubc-2* operon.

2. COMPARATIVE GENOMIC ANALYSIS OF ubc-2

Comparative genomic analysis was used to delineate the structural organization of *ubc-2* and the sequences surrounding this gene. The polypeptide sequence of UBC-2 is absolutely conserved between *C. elegans* and *C. briggsae*. While 100% polypeptide sequence conservation has been noted for specific protein domains between the two species (Kuwabara and Shah, 1994), such high conservation has, to our knowledge, not been previously documented for complete polypeptide sequences. In general, the highest degree of polypeptide sequence conservation between homologous genes in *C. elegans* and *C. briggsae* ranges from 90-96% for genes such as UbL, *unc-119* and *bli-4* (Jones and Candido, 1993; Maduro and Pilgrim, 1996; Thacker *et al.*, 1999). This indicates that selective pressure on *ubc-2* is extremely high. The absolute conservation of UBC-2, coupled with its lethality when mutated, also indicates that UBC-2 performs an important function that is sensitive to amino acid alterations. Since UBC-2 is a small protein, its function may depend upon the entire structure of the protein such that polypeptide sequence alterations are not tolerated. The other genes that

are believed to form a polycistronic unit with *ubc-2* are not as well conserved, indicating that *ubc-2* is the only gene within the operon that is subject to such strong selective pressure.

The genomic sequences surrounding *ubc-2* indicate that *ubc-2* may be the first gene of an operon that contains two other genes, *apg-7* and the putative zinc finger protein, Y5F2A.4. The genes are in close proximity to one another, and a *trans*-splice donor site is located immediately upstream of the ATG start codon of all three genes. Both *ubc-2* and Y5F2A.4 appear to be only SL1-spliced while *apg-7* is both SL1- and SL2-spliced. In *C. elegans* operons, the first gene is usually SL1-spliced while all other downstream genes are either exclusively SL2-spliced or are spliced by a mixture of SL1 and SL2 (Spieth *et al.*, 1993; Zorio *et al.*, 1994). Based on this information, *ubc-2* and *apg-7* are likely to form an operon, although it is not clear whether Y5F2A.4 is also part of this operon.

A new form of operon has been identified in which the mRNA of the downstream gene is *trans*-spliced to SL1 rather than SL2 (Hengartner and Horvitz, 1994). In this type of operon, the intercistronic sequence is lacking, such that the polyadenylation site of the upstream gene is adjacent to the *trans*-splice site of the downstream gene. Although this particular situation does not exist between *apg-7* and Y5F2A.4, it serves to indicate that SL2-splicing of downstream genes is not an absolute requirement for operons. Thus, Y5F2A.4 might be part of the *ubc-2/apg-7* operon, and may utilize a novel splicing mechanism.

Many *C. elegans* operons are made up of genes with related functions (Clark *et al.*, 1994; Huang *et al.*, 1994; Page, 1999; Treinin *et al.*, 1998). Thus, it is interesting to note that the *apg-7* gene is part of a system similar to ubiquitin-mediated degradation. In yeast, Apg7 is a ubiquitin-activating enzyme-like protein that is involved in autophagy (Mizushima *et al.*, 1998), the bulk delivery of cytoplasmic material to the lysosome for degradation (Dunn, 1994). In a process similar to ubiquitination, Apg12 (a ubiquitin-like molecule) is activated by binding to Apg7 via a high-energy thioester bond. Apg12 is transferred through Apg10 (an E2-like molecule) and is conjugated to Apg5 via an isopeptide bond. Homologs of this system have been found in *Drosophila*, humans, and now in *C. elegans*, indicating that this conjugation system is conserved (Mizushima *et al.*, 1998). UBC-2 and APG-7 probably do not function within the same pathway, although the activities of their respective pathways are similar. It is more likely that these genes are required at approximately the same time during development and thus utilize a common promoter.

Synteny is conserved over a large sequence tract surrounding the *ubc-2* gene in *C. elegans* and *C. briggsae*. There are, however, several regions where synteny is not maintained. In particular, sequences immediately upstream and downstream of the *ubc-2* operon are different between the two species. The upstream kinesin light chain gene, and a downstream 9 kb segment including a repetitive sequence and the Y5F2A.3 gene, are absent in *C. briggsae*. Whether these sequences have been translocated or deleted is unknown. However, the *Cb*-Y5F2A.1 gene has been partially duplicated, which suggests that a translocation event has taken place in *C. briggsae*. It is tempting to speculate that the absence of the 9 kb segment might be associated with this event. It is of interest to note that while the sequences immediately surrounding the *ubc-2* operon have been altered, the operon itself and more distant sequences have not changed dramatically between the two species. These alterations indicate that relatively large scale changes have occurred that affect the synteny of *C. elegans* and *C. briggsae* sequences. The effect of these sequence alterations on gene expression in this region is unknown.

The inability of the *C. briggsae* fosmid G47J11 to rescue *C. elegans let-70* mutants was surprising. Considering the absolute conservation of UBC-2 between the two species, *Cb-ubc-2* should be able to rescue *C. elegans let-70* mutants if expressed in the proper spatial and temporal pattern. Thus, it appears that *Cb-ubc-2* was not properly expressed in *C. elegans* mutants. Several attempts were made to generate transgenic animals with functional *Cb-ubc-2* by injecting a number of different constructs at varying DNA concentrations. While roller progeny were produced in most cases, indicating that pRF4 was present and functional, G47J11 may not have been incorporated at a concentration that was conducive to proper *ubc-2* expression.

It is possible that the fosmid sequence has undergone a rearrangement leading to the apparent deletions observed in the regions surrounding the *ubc-2* operon. A comparison of this region between the fosmid and *C. briggsae* genomic DNA using Southern analysis would help to determine if such a rearrangement had occurred.

G47J11 contains 8 kb up- and 15 kb downstream of *Cb-ubc-2*, while the *C. elegans* plasmid pZM13, which can fully rescue *let-70(s689)* mutants, contains 7 kb up- and 6 kb downstream of *ubc-2*. If G47J11 is missing a key regulatory element for *ubc-2* expression, it is probably located upstream, since the fosmid contains a much longer downstream sequence than pZM13. Interestingly, a TATA element was located approximately 170 bp upstream of the *Ce-*

ubc-2 ATG start codon, while one could not be found upstream of *Cb-ubc-2*. As noted above, the sequences upstream of the *ubc-2* operon in these two species have diverged. In *C. elegans*, a kinesin light chain gene is located upstream of *Ce-ubc-2* while in *C. briggsae*, this gene is missing. The M7.4 homolog is closer to *Cb-ubc-2*, with the ATG start codon of *Cb-ubc-2* only 1458 nt downstream of the putative polyadenylation site of M7.4 (see Figure 24). This raises the possibility that the *Cb-ubc-2* operon is linked to the putative upstream operon such that all of the genes are transcribed under a common promoter. If this has occurred, it raises the question of how such a change in the organization of *ubc-2* regulation could occur between *C. elegans* and *C. briggsae* without affecting the essential UBC-2 function during the intermediate stages.

The 5' non-coding region of the putative upstream operon containing the genes M7.3, M7.6 and M7.4 is not present in G47J11. If *Cb-ubc-2* is a downstream gene within this operon, then G47J11 would not contain the *Cb-ubc-2* promoter and therefore could not express *Cb-ubc-2*. A *C. briggsae* fosmid with a longer sequence upstream of *Cb-ubc-2* has been placed in the sequencing queue at the Genome Sequencing Center in St. Louis. If this fosmid contains the promoter region for the putative operon, it will be interesting to determine whether it can rescue *let-70* mutants.

3. let-70(s689) IS SUBJECT TO smg-MEDIATED mRNA SURVEILLANCE

Sequencing of the *ubc-2* cDNA generated from *let-70(s689)* mutant animals shows that the fourth intron of *ubc-2* is generally not removed when the pre-mRNA is processed. Conceptual translation of the mutant cDNA reveals an in-frame termination codon within the fourth intron, suggesting that a truncated protein product could be produced in *let-70(s689)* mutants. However, a truncated UBC-2 product is not detected in extracts from *let-70(s689)* mutants, indicating that if made, it is highly unstable. Alternatively, the *s689* mRNA might be subject to nonsense-mediated mRNA decay (NMD) such that the *s689* transcript is degraded prior to translation, resulting in very little protein product.

The partial rescue of *let-70(s689) smg-1* double mutants indicates that the mutant mRNA transcript is subject to NMD. Removal of the mRNA surveillance system is sufficient to rescue *let-70 smg-1* double mutants, but cannot rescue the progeny of these mutants, causing maternal effect lethality. Two possible explanations can be envisioned for the partial rescue. Both hypotheses assume that the *smg-1* mutation prevents proper NMD function, and that

mutant *ubc-2* transcripts fail to be efficiently degraded. The first possibility is that a truncated UBC-2 protein is produced that is partially active and capable of rescuing some of the *let-70* defects. The truncated protein would lack 14 amino acids from the carboxyl terminus,implying that these residues are not required to rescue somatic and germline defects, but are necessary for early embryonic function.

The second possibility is based on the observation that a small amount of correctly spliced *ubc-2* mRNA is produced in *let-70(s689)* mutants, indicating that the *C. elegans* spliceosome can occasionally utilize the mutated splice donor site, remove the fourth intron, and produce correctly spliced *ubc-2* mRNA. In *let-70(s689)* animals, all signals necessary for proper splicing of the fourth intron are present with the exception of the 5' splice donor site. Perhaps the spliceosome recognizes the splicing signals that are present and, while searching for a 5' splice donor site, occasionally utilizes the mutated site. If this occurs in *let-70(s689)* mutants, the amount of properly spliced *ubc-2* transcript would depend on the frequency with which the spliceosome uses the mutant 5' splice donor site. The probability of mutant site usage would vary between individuals, and might explain why the *let-70(s689)* mutant phenotype is so highly variable. If a correctly spliced transcript were produced fairly often, then the individual might produce enough wild type UBC-2 to survive to the adult stage. An obvious extension of this scenario is that some *let-70(s689)* mutant animals might produce enough UBC-2 to generate viable offspring. In fact, viable offspring were sometimes observed, although it was not determined whether these individuals were surviving mutants or revertants resulting from recombination events occurring between *let-70* and *unc-22*.

In *let-70 smg-1* double mutants, improper utilization of the mutant 5' splice donor site by the spliceosome might also occur. Since the mutant mRNA is likely to be more stable in the *smg-1* background, a greater number of correctly spliced *ubc-2* transcripts might be generated than in *let-70(s689)* single mutants. Thus, more wild type UBC-2 may be produced in *let-70 smg-1* double mutants, perhaps in sufficient quantities to rescue the somatic and germline defects. However, this level generally appears to be insufficient for embryogenesis. This scenario would also predict that some individuals should produce enough UBC-2 for maternal rescue as well. While this was not observed, an examination of a larger number of progeny from *let-70 smg-1* double mutants might reveal rare full rescue of *let-70 smg-1* double mutants.

4. let-70(s1132) ANIMALS PRODUCE AN UNSTABLE FORM OF UBC-2

Western analysis indicates that the mutant form of UBC-2 produced in *let-70(s1132)* animals is unstable. The histidine to tyrosine mutation affects the conserved HPN tripeptide that is necessary for maintaining tertiary structure of UBCs in vertebrates (see Section I.3.2; R. W. Bohnsack, pers. comm.). Thus, it appears that the instability of UBC-2 in *let-70(s1132)* animals is caused by misfolding of the mutant protein. If this protein is inactive and quickly degraded, *s1132* may represent a genetic null allele of *let-70*, indicating that the *let-70(s1132)* mutant phenotype is due to loss of UBC-2.

5. UBC-2 STRUCTURE: INSIGHTS FROM A TEMPERATURE-SENSITIVE ALLELE

The P61S allele of *let-70*, pZM13.34, is cold-sensitive when expressed in a *let-70(s689)* background. At 25°C, *let-70(s689)* transgenics are fully rescued, indicating that the pZM13.34 protein is functional in both germline and somatic tissue. However, at 15°C, transgenic animals expressing pZM13.34 exhibited maternal effect lethality, indicating that the temperature-dependent structural change in pZM13.34 protein abolishes maternal UBC-2 function, but not somatic function. Thus, UBC-2 activity within the early embryo is distinct from its other functions during development, and may involve different regions of the protein. A number of *C. elegans* proteins perform discrete functions during development. For example, GLP-1 is involved in early cell fate decisions during embryogenesis and is also important in signalling from the distal tip cell to promote germline proliferation (Schedl, 1997; Schnabel and Priess, 1997).

Many protein-protein associations occur through hydrophobic interactions, the strength of which are based mainly on entropy changes, and thus decrease with temperature. The cold sensitivity of the pZM13.34 allele suggests that a protein-protein interaction is lost at 15°C that may be driven by hydrophobic effects. The P61S mutation may destabilize UBC-2-protein interactions such that they are lost at lower temperature. The association may involve a specific E3-binding or substrate-binding site on UBC-2. In addition, it is known that E2s will self-associate to form dimers and multimers (Ptak *et al.*, 1994; Leggett and Candido, 1997). Thus, the ability of the pZM13.34 protein to bind to itself or other protein partners may be impaired at lower temperature.

While pZM13.34 is capable of rescuing *let-70(s689)* mutants at 25°C, it is unable to rescue *let-70(s1132)* mutants at any temperature. Interestingly, however, the intestinal defects

were rescued, while the somatic gonad defects and sterility were not. Body wall muscle defects were not assessed in these animals. Despite the probable presence of a second site mutation in *let-70(s1132)* animals, the rescue of intestinal, but not gonadal, defects by the pZM13.34 protein raises the possibility that UBC-2 activity during intestinal development might be distinct from its function during gonad formation. The protein binding partners may change during development and in different tissues. If UBC-2 self-associates to become active, pZM13.34 and *s1132* proteins might interact with each other in transgenic animals, producing a complex that functions improperly and interferes with gonadal development.

The cold-sensitive allele of *let-70* shows that UBC-2 has distinct functions during *C. elegans* development that can be separated into embryonic, somatic and germline activities. Since the pZM13.34 allele interferes with some of these functions, it may prove useful in identifying UBC-2 activity at specific times during development. By utilizing the maternal effect lethal phenotype of *let-70(s689)* mutants carrying the pZM13.34 transgene, genetic screens could be designed to identify suppressors of *let-70* embryonic lethality. Similarly, genetic screens could be designed to identify suppressors of *let-70* somatic and germline defects. UBC-2 probably interacts with a number of E3s and/or substrates at various times during development, and such screens may help to identify and isolate these proteins.

6. BOTH MATERNAL AND POST-EMBRYONIC CONTRIBUTIONS OF UBC-2 ARE REQUIRED

As described by Fire *et al* (1998), RNA interference in *C. elegans* is believed to silence expression in a gene-specific manner. Phenotypes produced by interference mimic loss-of-function mutations and have been used to determine the putative null state of many genes (Fitzgerald and Schwarzbauer, 1998; Fay *et al.*, 1999; Ashcroft *et al.*, 1999). RNA interference of *ubc-2* produced two phenotypes: embryonic lethal and L3 lethal. The embryonic lethality supports the previously described *let-70(s689) smg-1* double mutant and temperature-sensitive allele data which indicates a maternal requirement for UBC-2. Post-embryonically, the *let-70* null phenotype appears to be L3 lethality. This suggests that *let-70(s1132)* is a null allele, a possibility that is supported by the instability of UBC-2 in mutant animals and the observation that hemizygous *let-70(s1132)* animals are also L3 lethal. The L3 arrest may result from the inability of *let-70* mutants to degrade a specific substrate protein at a critical time.

Alternatively, defects caused by UBC-2 dysfunction may accumulate such that by L3, the animals are unable to develop any further.

The similarity of the UBC domain within the E2 family of enzymes raises the possibility that the phenotypes observed by RNA interference may be caused by the silencing of many E2s. Two lines of evidence suggest that this is not the case. First, when *ubc-2* is hybridized to *C. elegans* genomic DNA, only one gene product is detected (Zhen, 1996). Second, both the embryonic and L3 arrest phenotypes were observed in *let-70* mutants and in transgenic animals containing different alleles of *let-70*, suggesting that these phenotypes are the result of specific loss or reduction in UBC-2 function.

7. UBC-2 IS REQUIRED IN BOTH SOMATIC AND GERMLINE TISSUE

The plasmid pZM13 is capable of fully rescuing *let-70(s689)* when expressed in both somatic and germline tissue. According to Kelly *et al.* (1997), somatic and germline expression of extrachromosomal arrays can be obtained in *C. elegans* when random genomic DNA fragments are included in the injection mixture. It is believed that the additional DNA increases the genetic complexity of the array, thus preventing its silencing in the germline. This appears to be true for UBC-2, since injection of pZM13 alone gives partial rescue of *let-70(s689)* mutants, while addition of random genomic DNA in the injection mixture results in full rescue. Thus, pZM13 appears to contain all of the sequences necessary for the proper spatial and temporal expression of UBC-2.

Although somatic and germline expression of pZM13 can rescue *let-70(s689)*, pZM13 is incapable of rescuing *let-70(s1132)*, indicating that a fundamental difference exists between the two mutant strains. The simplest explanation is that *let-70(s1132)* has a second site mutation that affects the rescuing ability of pZM13. Although this strain was outcrossed to N2 animals at least ten times, a second mutation may yet exist that is close to *let-70* or lies between *let-70* and *unc-22*. The second mutation, if present, appears to be required embryonically since expression of pZM13 was able to rescue the F1 but not the F2 progeny of transgenic *let-70(s1132)* animals. Alternatively, the mutant UBC-2 protein produced in *let-70(s1132)* animals may interfere with the function of the wild type protein through E2 self-association. This is a less likely scenario since *s1132* does not act as a dominant negative allele; rather, it is recessive, and heterozygotes are phenotypically wild type.

8. THE POSSIBLE ROLE OF UBC-2 IN C. elegans SIGNALLING PATHWAYS

The pleiotropic phenotype of *let-70* provides insight into the role of UBC-2 during *C. elegans* development. The phenotype includes defects in intestinal cell maturation and attachment to the hypodermis, the positioning, assembly and attachment of body wall muscle cells, gonad and vulva development, and the formation, maturation and migration of oocytes. Both somatic and germline expression of UBC-2 are required to rescue *let-70* mutants. A maternal effect lethal (MEL) phenotype was observed in the progeny of animals injected with dsRNA directed to *ubc-2*; in the progeny of rescued *let-70(s689) smg-1* double mutants; and in transgenic *let-70(s689)* mutants carrying pZM13.34 when raised at the non-permissive temperature. Embryonic expression of UBC-2 was ubiquitous, and during larval development, expression became concentrated in the nucleoli of many cells, in the dense bodies or M-lines of body wall muscle and in certain neurons.

In general, UBC-2 plays a role in three facets of *C. elegans* development: embryonic, somatic and germline. The embryonic activity of UBC-2 may be related to early cell fate decisions. The migration, attachment and maturation of most cells are somatic functions, while oocyte maturation is likely due to UBC-2 activity in the germline.

UBC-2 may function in development in a number of ways. UBC-2 may be required independently during the processes of cell attachment, muscle cell positioning, sarcomere assembly, gonad and vulval development and oocyte maturation. A reduction or loss of UBC-2 activity could affect each pathway separately, resulting in the pleiotropic phenotype that is observed in *let-70* mutant animals. However, many of these developmental programs utilize the same or similar signalling pathways, some of which are known to be regulated by ubiquitin-mediated protein degradation. Thus, it is possible that the *let-70* phenotype is caused by a decrease in UBC-2 activity within a small number of signalling pathways that ultimately affect many developmental processes. The phenotypes observed in *let-70* mutants suggests that these pathways might include *C. elegans* Wnt/Wg, NF-κB, integrin-mediated and MAPK signalling cascades. In addition, UBC-2 may play a role in cell cycle progression within the germline.

8.1 Integrin-mediated and MAPK signalling pathways

In *let-70* mutants, the detachment phenotype observed in body wall muscle and intestinal cells indicates that UBC-2 is involved in cell attachment. During development, body wall muscle cells, intestinal cells and the somatic gonad form attachments to the hypodermis

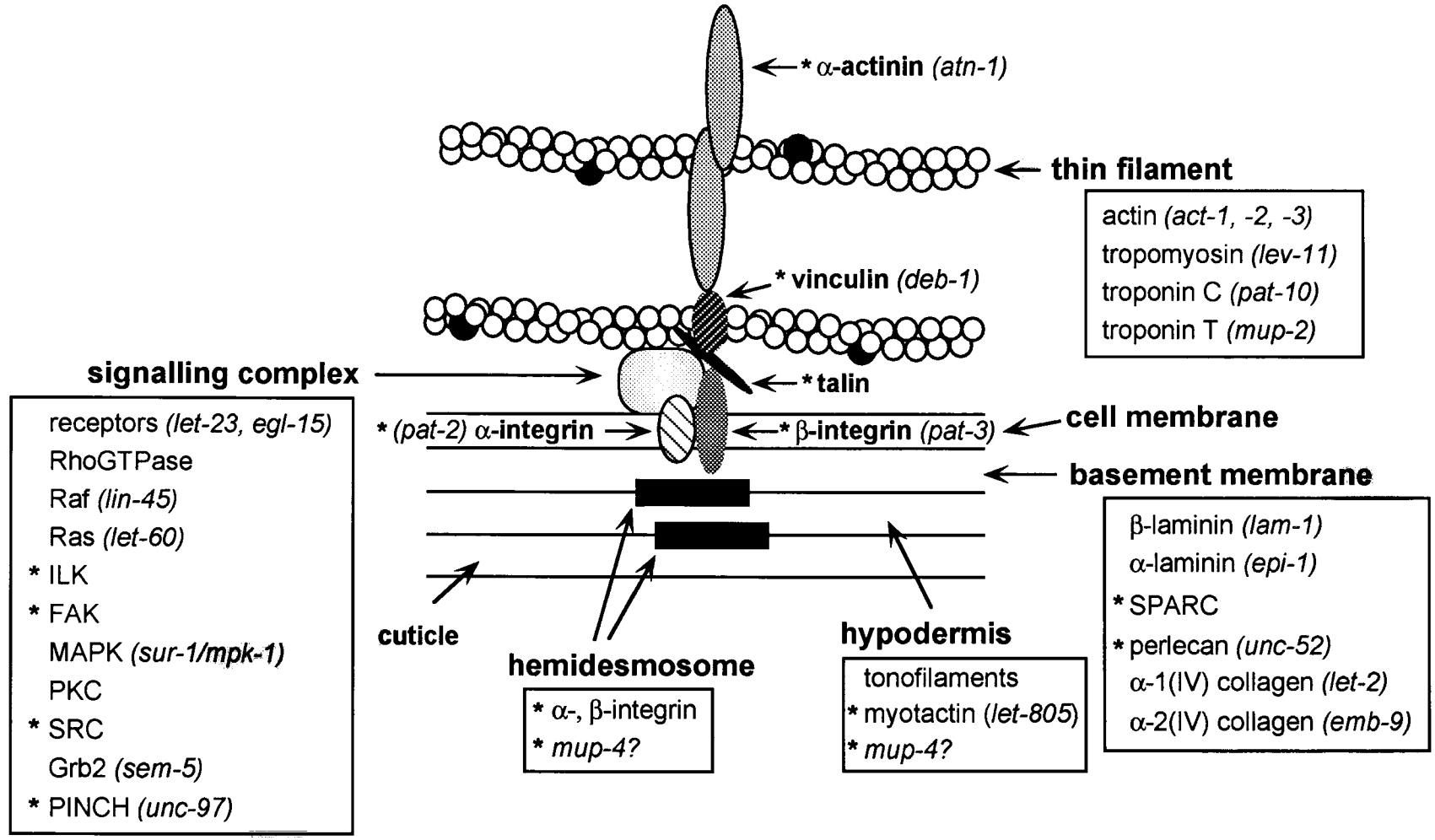
through the basement membrane (also called extracellular membrane; ECM). In *C. elegans*, the process of body wall muscle attachment to the ECM has been intensively studied (see Section I.6.4), and it is believed that other cell-ECM attachments are formed by a similar process. In muscle, cell-ECM attachments utilize integrins (Gettner *et al.*, 1995), suggesting that UBC-2 may play a role in anchoring cells to the ECM via integrin receptors.

During muscle development, cell positioning, cell attachment and sarcomere assembly are integrated processes. Many signalling pathways and structural components in muscle, basement membrane and hypodermis function co-operatively to mediate the formation of body wall muscle (see Section I.6.4). Briefly, myoblast cells migrate from a lateral position to the dorsal and ventral quadrants. Muscle, hypodermal and basement membrane components localize at sites called dense bodies, where integrin-based attachment structures are formed and the muscle sarcomere is assembled.

Mutations in many *C. elegans* genes involved in cell attachment result in mutant muscle phenotypes that resemble that of *let-70*. Examples include *deb-1* (vinculin; Barstead and Waterston, 1989), *pat-3* (β-integrin; Williams and Waterston, 1994; Gettner *et al.*, 1995), *pat-2* (α-integrin; Williams and Waterston, 1994), *unc-52* (perlecan; Rogalski *et al.*, 1993), *mua-3* (a novel transmembrane protein; E.A. Bucher, pers. comm.), *mua-1* (a transcription factor; J. Plenefish, pers. comm.), *mup-2* (troponin T; Myers *et al.*, 1996) and *mup-4* (a novel transmembrane protein; E.A. Bucher, pers. comm.). The null allele of these genes is usually Pat (paralyzed arrest at embryonic two-fold stage; Williams and Waterston, 1994) or 3-fold arrest. Since the presumed null phenotype of *let-70* is arrest at pre-comma stage, UBC-2 probably functions upstream of the genes listed above. Thus, UBC-2 likely plays a role in events that lead to the localization of muscle and hypodermal structures, and the formation of cell adhesions.

C. elegans dense bodies are similar to vertebrate focal adhesion complexes (FACs; Burridge *et al.*, 1997). As shown in Figure 26, both consist of transmembrane integrin receptors associated with multimeric protein complexes that function to anchor cytoskeletal structural proteins to the ECM and serve as recipients and generators of signalling information (Boudreau and Jones, 1999; Schoenwaelder and Burridge, 1999). The formation and activity of FACs are regulated by many extracellular and intracellular inputs. At least 20 proteins can be recruited to the integrin/ECM binding site, including Rho GTPases, growth factor receptors, Raf, Ras, focal adhesion kinase (FAK), integrin-linked kinase (ILK), protein kinase C (PKC)

Figure 26. Model of proposed *C. elegans* adhesion complex components. Schematic representation of the adhesion complex in *C. elegans* with proposed components indicated. Known *C. elegans* mutations are shown in brackets. Components known to localize to *C. elegans* dense bodies are marked with an asterisk. Components marked with a question mark have not been unequivocally localized. See text for explanation of abbreviations.


* α-actinin (atn-1)
thin filament
actin (act-1, -2, -3)
tropomyosin (lev-11)
troponin C (pat-10)
troponin T (mup-2)
* vinculin (deb-1)
* talin
signalling complex
receptors (let-23, egl-15)
RhoGTPase
Raf (lin-45)
Ras (let-60)
* ILK
* FAK
MAPK (sur-1/mpk-1)
PKC
* SRC
Grb2 (sem-5)
* PINCH (unc-97)
* (pat-2) α-integrin
* β-integrin (pat-3)
cell membrane
basement membrane
β-laminin (lam-1)
α-laminin (epi-1)
* SPARC
* perlecan (unc-52)
α-1(IV) collagen (let-2)
α-2(IV) collagen (emb-9)
cuticle
hemidesmosome
* α-, β-integrin
* mup-4?
hypodermis
tonofilaments
* myotactin (let-805)
* mup-4?

and MAP kinases (MAPK; Miyamoto *et al.*, 1995). In *C. elegans*, FAK and ILK homologs have recently been identified and are found to be associated with dense bodies (R.J. Barstead, pers. comm. and D.G. Moerman, per. comm., respectively), indicating that the dense body is a site for signal transduction as well as cellular adhesion and muscle formation.

Many integrin-dependent and growth factor signalling pathways lead to the modulation of Ras-mediated MAPK signal transduction cascades, which affect cell migration, adhesion, growth and differentiation (Mainiero *et al.*, 1995; Marshall, 1995; Wang *et al.*, 1998). In multicellular organisms, many MAPK signalling pathways are regulated by ubiquitin-mediated degradation of proteins such as the growth factor receptor PDGFRα (Miyake *et al.*, 1998) and PKC (Lu *et al.*, 1998). The transcription factors c-Fos and c-Jun activate genes in response to a number of inducers that act through the MAPK signal transduction pathway. As described in Section I.5.3, the expression of c-Fos and c-Jun are regulated by UbcH5B-dependent degradation. In *Drosophila*, D-Jun activation affects cell fate decisions and cellular differentiation during *Drosophila* eye development (Isaksson *et al.*, 1997).

These studies implicate members of the UBC4 branch of E2s in regulating MAPK signalling pathways that are associated with FACs and affect cellular differentiation. Homologous MAPK pathways in *C. elegans* may therefore be associated with dense bodies, and affect cell growth and differentiation. If the E2 involved in this pathway is also conserved, then UBC-2 may mediate the ubiquitination of substrates in MAPK signalling pathways.

In *C. elegans*, the migration and differentiation of Q neuroblasts, sex myoblasts, distal tip cells and vulval cells are, in part, regulated by integrin-mediated signalling pathways that involve interactions between the migrating cell and the basement membrane (Hedgecock *et al.*, 1987; Baum *et al.*, 1999; Gettner *et al.*, 1995). Many of these cell types express UBC-2, and their development is defective in *let-70* mutants, which suggests that UBC-2 may play a role in integrin-dependent and MAPK signalling pathways.

In transgenic animals carrying the *ubc-2*::GFP transgenic array, several neurons that express the fusion protein appear to be descendants of the Q neuroblasts. Thus, UBC-2 may be involved in Q neuroblast migration and differentiation.

Sex myoblasts are descendants of the M lineage and develop into vulval muscle cells. Once sex myoblasts form, they migrate to a position above the gonad, differentiate and attach to the body wall, uterus and vulva. The migration of sex myoblasts is integrin-dependent, and is regulated by Ras-mediated MAPK signalling via the fibroblast growth factor (FGF) homolog

(Chen and Stern, 1998; Gettner *et al.*, 1995; Clark *et al.*, 1992; Stern *et al.*, 1993; Sundaram and Han, 1995). Transgenic nematodes express UBC-2::GFP in the vulval muscle cells, which provides supporting evidence of a role for UBC-2 activity in integrin-dependent and MAPK signalling pathways that regulate sex myoblast migration and differentiation.

Ras-mediated MAPK signalling is also involved in vulval cell fate decisions and morphogenesis. In *C. elegans* larvae, specific cells become competent to respond to LIN-3, an epidermal growth factor (EGF) homolog that activates the Ras-mediated MAPK pathway and signals vulval morphogenesis. Since vulval development is defective in *let-70* mutants, ubiquitin-mediated degradation may play a role in regulating the signalling pathways that specify vulval cell fate and differentiation.

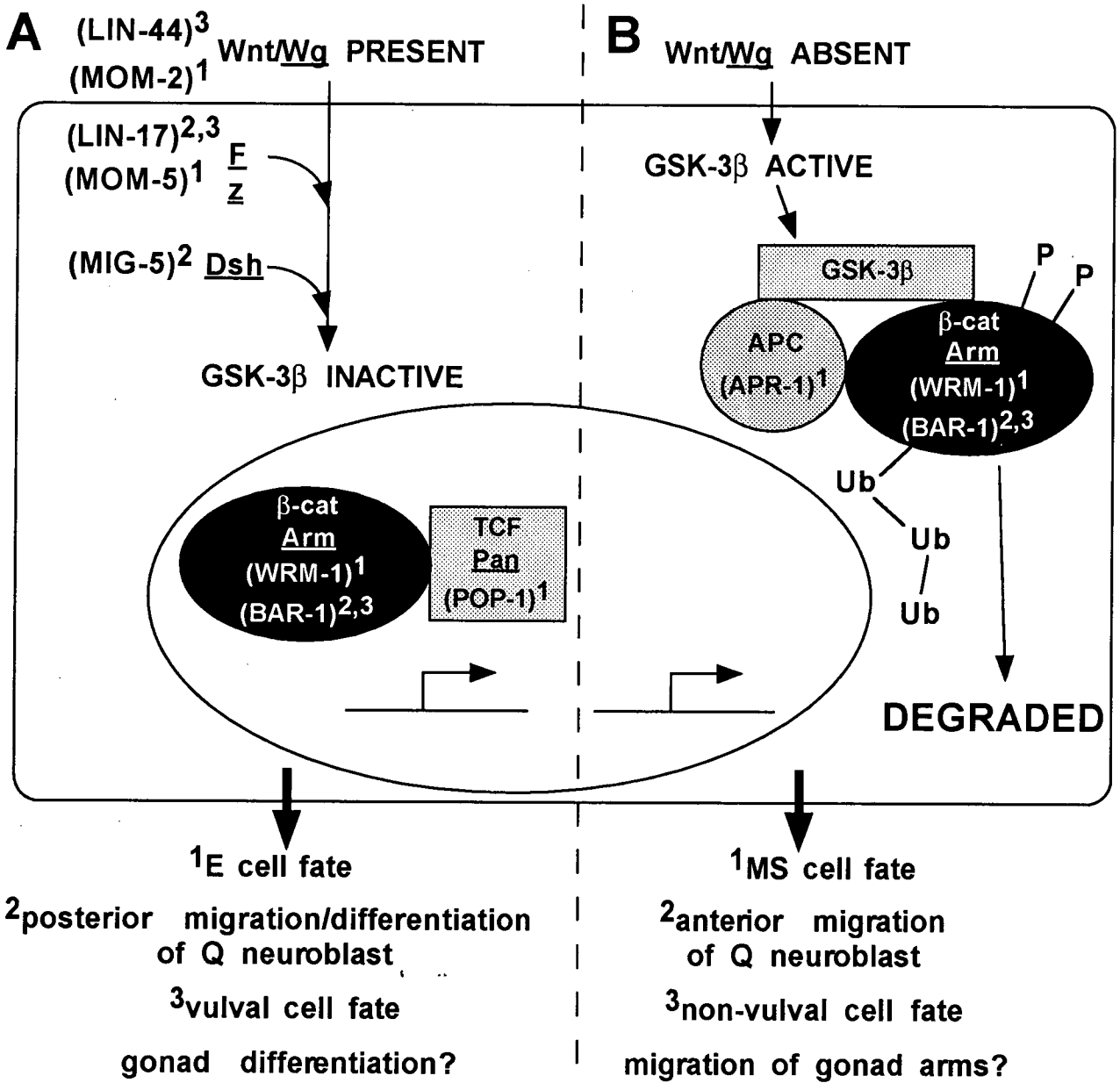
During gonadogenesis, distal tip cells undergo a complex stereotyped migration in which they follow the basement membrane to form U-shaped structures that differentiate into the somatic gonad. Migration of the distal tip cells is controlled by the metalloprotease, GON-1, which restructures the basement membrane as migration proceeds (Blelloch and Kimble, 1999). Integrin-mediated signalling at the basement membrane is likely to be involved in controlling distal tip cell migrations as well (Hedgecock *et al.*, 1987; Antebi *et al.*, 1997). During gonad formation in *let-70* mutants, distal tip cell migration is not strongly affected; however, their ability to change direction is affected. Thus, it appears that UBC-2-mediated ubiquitination and degradation of proteins may be important to cue directional changes to the distal tip cells, a process that may also involve integrin-mediated pathways.

UBC-2 is also required during somatic gonad differentiation. The spermatheca and uterus are unable to differentiate in *let-70* mutants, resulting in the formation of a large mass of undetermined cells. Myoepithelial cell formation may also be affected, since oocytes are not transported through the oviduct in *let-70* mutants. Thus, UBC-2 is required to cue the leader cells to change direction during migration, and for differentiation of the somatic gonad once migration is complete. These two processes might be controlled by a single activity of UBC-2, or they may be regulated by independent processes that each require UBC-2.

8.2 The Wnt/Wg pathway

The Wnt/Wg pathway, which is conserved between *C. elegans*, *Drosophila* and vertebrates (Rocheleau *et al.*, 1997; Thorpe *et al.*, 1997), is regulated by ubiquitin-mediated

Figure 27. Model for the action of Wnt/Wg signalling pathway components. Vertebrate, *Drosophila* (underlined) and *C. elegans* (italicized) homologs are indicated. For *C. elegans*, components that function during E and MS cell fate determination[1], Q neuroblast migration and differentiation[2], and vulval cell fate determination[3] are indicated. A) Signalling from Wnt inactivates GSK-3β, which allows the translocation of β-catenin, its binding to TCF, and the transcription of genes involved in mesoderm differentiation. In *C. elegans*, Wnt homolog signalling results in the developmental programs indicated. B) In the absence of Wnt signalling, GSK-3β forms a complex with β-catenin and APC, resulting in the phosphorylation, ubiquitination and subsequent degradation of β-catenin. In *C. elegans*, lack of Wnt signalling results in the developmental programs indicated.


A
(LIN-44)3
(MOM-2)1
Wnt/Wg PRESENT
(LIN-17)2,3
(MOM-5)1
F
z
(MIG-5)2
Dsh
GSK-3β INACTIVE
β-cat
Arm
(WRM-1)1
(BAR-1)2,3
TCF
Pan
(POP-1)1
B
Wnt/Wg ABSENT
GSK-3β ACTIVE
GSK-3β
P
P
APC
(APR-1)1
β-cat
Arm
(WRM-1)1
(BAR-1)2,3
Ub
Ub
Ub
DEGRADED
1E cell fate
2posterior migration/differentiation of Q neuroblast
3vulval cell fate
gonad differentiation?
1MS cell fate
2anterior migration of Q neuroblast
3non-vulval cell fate
migration of gonad arms?

degradation (see Section I.5.6; Figure 27). In *C. elegans*, Wnt/Wg is involved in early cell fate decisions, gonad and vulval development, and Q neuroblast migration (Lin *et al.*, 1995; Sawa *et al.*, 1996; Sternberg and Horvitz, 1988; Guo, 1995; Harris *et al.*, 1996). In *Drosophila* it plays a role in segment polarity and in vertebrates it is involved in axis formation. Since Wnt/Wg in *C. elegans* controls a number of developmental processes that are defective in *let-70* mutants, and the pathway is regulated by ubiquitin-mediated proteolysis, it is possible that UBC-2 might be involved in Wnt/Wg regulation in *C. elegans*.

As described in Section I.5.6, signals from Wnt/Wg activate a signal transduction pathway that culminates in the accumulation of β-catenin/Armadillo (β-cat/Arm), which translocates to the nucleus and promotes the transcription of genes involved in mesoderm formation (Figure 27). β-cat/Arm levels are regulated by ubiquitin-mediated proteolysis, such that in the absence of Wnt/Wg signalling, β-cat/Arm is ubiquitinated and degraded (reviewed in Willert and Nusse, 1998). In *C. elegans*, a number of genes involved in Wnt/Wg signalling have been identified, and several homologs exist for many of the components of the pathway. For example, two β-cat/Arm homologs are found in *C. elegans*, WRM-1 and BAR-1, and each homolog is utilized in a separate developmental pathway (Lin *et al.*, 1995; Guo, 1995; Sawa *et al.*, 1996).

In the four cell embryo of *C. elegans*, Wnt/Wg signalling is required for the specification of E and MS cell fates (Figure 27). The *C. elegans* homolog of Wnt/Wg, MOM-2, signals the P2 blastomere to activate MOM-5 (Fz receptor homolog) in the EMS cell, leading to the accumulation of WRM-1. High levels of WRM-1 downregulate POP-1 (a Tcf/LEF-1 family member) post-transcriptionally. When the EMS cell divides, the posterior daughter contains low levels of POP-1 and takes on the E cell fate, while the anterior cell has higher POP-1 levels and becomes the MS blastomere (Lin *et al.*, 1995). Thus, WRM-1 and POP-1 regulation via the Wnt/Wg signalling pathway affects cell fate decisions and is important in endoderm (E) and mesoderm (MS) formation in *C. elegans*.

The conservation of the Wnt/Wg pathway between species suggests that WRM-1 levels are regulated by the ubiquitin system, possibly mediated by UBC-2. In UBC-2 mutants, one would expect that WRM-1 levels would accumulate throughout the EMS blastomere regardless of Wnt/Wg signalling. This would result in high POP-1 levels in the daughter cells, causing both cells to take on an MS fate. This could be confirmed by examining early four cell stage

let-70 embryos for POP-1 levels and, in later stage embryos, examining for the presence of intestinal markers such as *ges-1* (Edgar and McGhee, 1986; Kennedy *et al.*, 1993).

WRM-1 is a strong candidate substrate for UBC-2 ubiquitination, and the E3 involved in WRM-1 degradation is likely to be an $SCF^{\beta\text{-TrCP/Slimb}}$ homolog. If *C. elegans* UBC-2, WRM-1 and homologs of the $SCF^{\beta\text{-TrCP/Slimb}}$ complex were biochemically purified or recombinantly expressed, an *in vitro* assay could determine if UBC-2 can utilize the $SCF^{\beta\text{-TrCP/Slimb}}$ complex to ubiquitinate WRM-1.

The Wnt/Wg signalling pathway also functions early in vulval development to regulate the competence of hypodermal cells to take on a vulval cell fate (Eisenmann *et al.*, 1998). The genes involved in Wnt/Wg signalling during vulval development differ from those utilized during embryogenesis, although the pathway itself is unchanged (Figure 27). The genes include *lin-44* (the *Wnt/Wg* homolog; Herman *et al.*, 1995), *lin-17* (*Fz*), *bar-1* (*β-cat*; Eisenmann *et al.*, 1998), *mom-1* and *mom-3*. As a result of LIN-44 signalling, certain cells respond to the LIN-3 inductive signal and adopt a vulval cell fate. LIN-3, an EGF homolog, activates the Ras-mediated MAPK pathway and signals vulval morphogenesis. Thus, both MAPK and Wnt/Wg pathways are involved in regulating vulval morphogenesis.

Since UBC-2 activity is necessary for proper vulval development, it is likely to be involved in regulation of the Wnt/Wg and/or MAPK pathways that signal vulval cell fate decisions. In the Wnt/Wg pathway, UBC-2 probably functions to affect BAR-1 levels, in a manner similar to that described for WRM-1. If it does, one would expect BAR-1 to accumulate in *let-70* mutants, which might prevent some vulval precursor cells from adopting a vulval cell fate. Since vulval morphogenesis follows a stereotyped pattern, the cell fates of vulval precursor cells could be determined in *let-70* mutants, and might help to identify whether vulval cell fate decisions are affected by a decrease in UBC-2 expression.

The Wnt/Wg pathway has been implicated in gonad development (Sawa *et al.*, 1996; Sternberg and Horvitz, 1988), and may be involved in the migration of leader cells and/or the development of the somatic gonad. It is also known to be involved in Q neuroblast migration (Figure 27; Guo, 1995; Harris *et al.*, 1996; Maloof *et al.*, 1999). A role for UBC-2 in the regulation of Wnt/Wg pathways is supported by the observations that gonad development is defective in *let-70* mutants, and several neurons that express UBC-2::GFP fusions in transgenic animals appear to be descendants of the Q neuroblasts. Involvement of UBC-2 in the

ubiquitination and degradation of all β-cat homologs in *C. elegans*, including BAR-1 and WRM-1, would provide an explanation for many of the *let-70* phenotypes that are observed.

8.3 NF-κB-mediated signalling

In *Drosophila*, Dorsal (NF-κB) signalling is involved in mesodermal patterning through regulation of the transcription factor, Twist (Cripps *et al.*, 1998). In mammalian systems, NF-κB is activated through two mechanisms: ubiquitin-mediated processing of the inactive NF-κB p105 precursor into an active p50 subunit, and the ubiquitin-mediated degradation of its inhibitor, IκBα. Both of these processes are mediated by a member of the UBC4 branch of E2s (see Sections I.5.4 and I.5.5; Alkalay *et al.*, 1995; Baldi *et al.*, 1996). Thus, myogenesis in *Drosophila* is regulated by ubiquitin-dependent mechanisms that are likely mediated by an E2 of the UBC4 branch.

HLH-8, a *C. elegans* Twist homolog, is directly involved in activating gene expression in post-embryonic mesoderm (Harfe *et al.*, 1998). The *hlh-8* gene is inactive in early embryos, and turned on only in mesodermal cells of the M lineage to affect non-striated muscle cell fate decisions. M divisions in early larval development yield body wall muscle, coelomocytes and sex myoblasts, which later develop into vulval muscle cells (Sulston and Horvitz, 1977; Harfe *et al.*, 1998).

If the NF-κB/Dorsal signalling pathway is conserved between *C. elegans*, *Drosophila* and mammals, then UBC-2 might be involved in the activation of HLH-8 and the specification of mesodermal cell fates. Indeed, transgenic animals express UBC-2::GFP in the vulval muscles, indicating that UBC-2 is expressed in some descendants of the M lineage. In *let-70* animals, where UBC-2 expression is low, *hlh-8* activation may be defective, thus altering M lineage cell fate decisions. HLH-8 levels could be assessed in *let-70* mutants using immunofluorescent staining with anti-HLH-8 antibodies. In addition, an examination of M lineage cell fates in *let-70* mutants may help to determine whether UBC-2 is involved in this process.

Candidate substrates for UBC-2 ubiquitination are the *C. elegans* homologs of the NF-κB p105 precursor or IκB, and the E3 involved in IκB degradation is likely to be an $SCF^{\beta\text{-}TrCP}$ homolog. Interestingly, in mammals, the E2/E3 complex involved in IκB degradation is the same as that utilized in β-cat degradation, namely UbcH5 and $SCF^{\beta\text{-}TrCP}$. Perhaps this pathway is conserved in *C. elegans*. An *in vitro* assay, similar to that described in Section IV.7.2, might

determine if UBC-2 can utilize the $SCF^{\beta\text{-}TrCP}$ complex to ubiquitinate IκB. Alternatively, a genetic approach could be used. Genes for components of the NF-κB/IκB pathways could be knocked out in *C. elegans* to generate mutant strains. Epistasis analysis might help determine the role of UBC-2 in these pathways.

8.4 Transcriptional regulation during myogenesis

The *hlh-1* gene, the homolog of vertebrate MyoD, is important in *C. elegans* myogenesis (Krause *et al.*, 1990), and is a likely candidate gene for ubiquitin-dependent proteolysis during early development. MyoD in vertebrates is a tissue-specific transcription factor that is involved in the regulation of skeletal muscle development (Davis *et al.*, 1987; Weintraub, 1993). It is a short-lived protein that is degraded by the ubiquitin-dependent proteolysis system both *in vivo* and *in vitro* (Abu Hatoum *et al.*, 1998). The ubiquitin-conjugating enzyme $E2_{14K}$ mediates the ubiquitination of MyoD. There has been debate regarding the homology of many mammalian E2s, due in part to the high degree of sequence similarity which exists among them. Until recently, $E2_{14K}$ was believed to be the ortholog of yeast UBC4/5. Currently, however, it is considered to be the ortholog of yeast RAD6 (Wing *et al.*, 1992), which in *C. elegans* is *ubc-1* (Leggett *et al.*, 1995). Thus, it is difficult to predict which *C. elegans* E2 may be responsible for *hlh-1* ubiquitination. There is little doubt, however, that *hlh-1* is a strong candidate substrate for ubiquitin-dependent proteolysis. It remains to be determined which E2 mediates this process in *C. elegans*.

8.5 Cell cycle regulation

In addition to somatic gonad defects, aberrant germline development is observed in adult *let-70(s689)* mutants. The number of germ cells produced is lower than normal, suggesting that UBC-2 may be involved in mitotic and/or meiotic progression, such that reduced levels of UBC-2 lead to a reduction in germ cell proliferation. Church *et al* (1995) showed that the MAPK signalling cascade is required for meiotic cell cycle progression in *C. elegans*. UBC-2 may be involved in regulating meiosis through modulation of MAPK signalling. A role for UBC-2 in cell cycle progression is also supported by the finding that in *Drosophila* the UBC-2 homolog, *UbcD1*, is required for proper telomere behavior during mitosis and meiosis (Cenci *et al.*, 1997). In addition, a rat homolog called Ubc4-testis, is expressed solely in rat testis and is involved in the formation and maturation of spermatocytes (Wing *et al.*, 1996; S. Wing, pers. comm.). In yeast, ubiquitin-mediated degradation is

necessary for cell cycle progression at the G1/S transition and to promote anaphase (see Section I.5.2). While it is known that Cdc34 is involved in G1/S phase progression, the E2 that interacts with the APC/C is unknown; however, it is believed to be Ubc4 (Feldman *et al.*, 1997; King *et al.*, 1995). Thus, in *C. elegans*, UBC-2 may interact with the APC/C to ubiquitinate substrates such as A and B-type cyclins and inhibitor proteins, thus promoting sister chromatid separation and exit from telophase in mitotic and meiotic germ cells. A detailed examination of the *let-70* mutant gonad using DAPI and immunostaining with anti-dsDNA antibodies might reveal defects in cell division due to a reduction in UBC-2 levels. Biochemical analysis of the *C. elegans* APC/C might reveal that UBC-2 interacts with the complex to promote the ubiquitination of substrate proteins.

In *let-70(s689)* mutants, oocyte development in hermaphrodites and spermatocyte formation in males occurs prematurely within the gonad. There are several possible explanations for this. The mutation might affect the formation of the myoepithelial sheath surrounding the oviduct in hermaphrodites and the vas deferens in males such that they fail to contract properly to transport germ cells. In hermaphrodites, the spermatheca and uterus are improperly formed and may be unable to receive germ cells. Thus, oocytes would remain in the oviduct and may even back up into the ovary.

Germline defects similar to those described for *let-70(s689)* mutants are seen in the progeny of animals injected with dsRNA directed to the basement membrane protein SPARC (osteonectin; Fitzgerald and Schwarzbauer, 1998). In addition to the germline defects, RNAi(ost) mutants fail to produce gut granules, a defect that is also seen in *let-70* mutants. Thus, it appears that the basement membrane is involved in processes that direct gut granule formation and oocyte maturation. Perhaps UBC-2 activity at or near the basement membrane is also associated with gut granule formation in the intestine. A subset of gut granules are known to be yolk proteins that are transported to developing oocytes. Yolk proteins are required for the maturation of oocytes. Thus, the inability of *let-70(s689)* oocytes to mature may simply be due to a lack of yolk proteins. However, males, which do not normally produce yolk proteins, are also affected. Thus, the premature development of the spermatocytes, and possibly the oocytes, may be due to an additional function of UBC-2 that is independent of yolk protein production.

9. UBC-2 IN THE NUCLEOLUS

Immunofluorescent staining of wild type *C. elegans* with anti-UBC-2 shows that UBC-2 is concentrated in the nucleolus of a number of cell types, including intestine, muscle, hypodermis, some neurons and the germline. In *let-70* mutants, conspicuous nucleolar expression of UBC-2 was not observed, and this may be relevant to the defects observed in these tissues. An examination of some of the functions of the nucleolus may provide insights into the site of action of UBC-2 in this organelle.

The major function of nucleolus is rRNA transcription, rRNA processing and ribosome assembly. It is also involved in the processing of some mRNAs, small nuclear RNAs, tRNAs, and telomerase RNA (reviewed in Pederson, 1998; Scheer and Hock, 1999). Ribosome assembly requires the constant influx of approximately 80 ribosomal proteins from the cytoplasm to the nucleolus, and the export of completed ribosomal subunits in the opposite direction. Signalling pathways that utilize fibroblast growth factor, nerve growth factor and protein kinase C are involved in ribosome assembly and RNA processing (Bonnet *et al.*, 1996; Zhou *et al.*, 1997). While there is no evidence that ubiquitin degradation plays a role in ribosome assembly or RNA processing, it may be involved in signalling pathways that lead to the activation or maintenance of these processes.

The nucleolus also functions in the silencing of tandemly repeated rDNA (Bryk *et al.*, 1997; Smith and Boeke, 1997). In yeast, gene silencing occurs at telomeres, the silent mating type loci (HM) and rDNA repeats in nucleolar genes (Gottschling *et al.*, 1990; Rine and Herskowitz, 1987). Silencing at the telomeres and HM loci involves the silent information regulators Sir2, Sir3, and Sir4. With the exception of Sir2, rDNA silencing is regulated by a different set of proteins. In the nucleolus, Sir2 associates with Net1 (also called Cfi1; Visintin *et al.*, 1999) and Cdc14 to form the RENT complex (regulator of nucleolar silencing and telophase; Straight *et al.*, 1999), which interacts with other proteins to facilitate gene silencing, suppress recombination, maintain nucleolar integrity, and regulate exit from mitosis and entry into meiosis.

There is some evidence that the ubiquitin-conjugating enzyme, Rad6 is required for silencing at telomere and HM loci (Huang *et al.*, 1997) and also plays a role in rDNA repeat silencing (Bryk *et al.*, 1997). Rad6, known to be involved in DNA repair, is also believed to play a role in the organization of chromatin structure (Bailly *et al.*, 1997; Siede, 1988; Sung *et al.*, 1988). Hence, Rad6 may facilitate the formation of nucleosomal structures which are

resistant to transcription and recombination at silenced loci such as telomeres, the HM loci and rDNA repeat. Moazed and Johnson (1996) found that deletion of the deubiquitinating enzyme, Ubp3, results in increased silencing at telomeres and the HM loci. It has been suggested that the activation of the gene silencing complex is regulated by the ubiquitin-dependent degradation of an inhibitor protein, and removal of ubiquitin from such an inhibitor by Ubp3 would prevent its degradation, thus leading to inactivation of the silencing complex.

If this system is conserved in *C. elegans*, then the Rad6 homolog, UBC-1 would likely mediate regulation of the silencing complex. It is possible that both UBC-2 and UBC-1 function to mediate gene silencing in *C. elegans*. Alternatively, UBC-2 may have taken over the role of regulating gene silencing in the nucleolus of *C. elegans*. UBC-2 could also be involved in a process within the nucleolus that is unrelated to gene silencing.

The nucleolus also has a regulatory function. As described earlier, Net1, Sir2 and Cdc14 are associated with the rDNA genes during interphase and function in gene silencing. During anaphase, the RENT complex disperses, and Sir2 and Cdc14 diffuse throughout nucleus and cytoplasm, while Net1 remains in nucleolus (Straight *et al.*, 1999; Visintin *et al.*, 1999). The release of Cdc14 from nucleolar sequestration is necessary for exit from mitosis. Mitotic exit is regulated by ubiquitin-dependent degradation of B-type cyclins and the inhibitor Sic1 (see Section I.5.2.2), thus providing a connection between ubiquitin-mediated proteolysis and the nucleolus. It is believed that the RENT complex sequesters Cdc14 in the nucleolus to prevent Cdc14 activity in the nucleus at inappropriate times during the cell cycle. The physical segregation of interacting proteins would be an effective method for the regulation of many pathways.

There are several examples of transient nucleolar localization as a regulatory mechanism. The *Drosophila* RNA helicase gene *pitchoune*, which is essential for larval development and is a potential target of the transcription factor Myc, is nucleolar localized (Zaffran *et al.*, 1998). Stimulation of mammalian cells with EGF results in the translocation of the zinc finger protein ZPR1 from cytoplasm to nucleus, and its accumulation in the nucleolus (Galcheva-Gargova *et al.*, 1998). The tumor suppressor protein $p19^{Arf}$ is located in the nucleolus. It activates the tumor suppressor p53 by sequestering the p53 inhibitor, Mdm2 in the nucleolus (Weber *et al.*, 1999). Mdm2 is a ubiquitin ligase that has been shown to function *in vivo* and *in vitro* with UbcH5 to ubiquitinate p53, leading to its destruction via the 26S

proteasome (see Section I.5.7; Honda *et al.*, 1997). Thus, a possible role for the nucleolus lies in sequestering proteins, thereby preventing their activity within the nucleus or cytoplasm.

Ubiquitin-dependent proteolysis appears to play an important role in the process of nucleolar sequestration. It may be involved in maintaining the activity of protein complexes within the nucleolus at specific times during the cell cycle through destruction of an inhibitor protein, in a manner similar to that presented for regulation of the gene silencing complex. Indeed, UbcH5-mediated degradation of the inhibitor IκB allows NF-κB to translocate into the nucleus where it activates gene expression (Alkalay *et al.*, 1995). Degradation of inhibitors may be a general mechanism for UBC4 branch-mediated degradation processes.

It is possible that certain E2s (for example, *C. elegans* UBC-2) are themselves sequestered in the nucleolus in a manner similar to that of the E3, Mdm2. This would physically separate the E2 from its target substrate, thus preventing inappropriate ubiquitination. E2s could be specifically targeted to the nucleolus, or they might be transported into the nucleolus passively while associated with an E3. The E2s might remain within the nucleolus until required in the nucleus or cytoplasm for activity.

In general, the expression pattern of the UBC-2::GFP fusion protein faithfully mimics that of wild type UBC-2. There is, however, an interesting exception. Unlike the wild type UBC-2 expression pattern, UBC-2::GFP expression was not confined to the nucleolus, but was spread throughout the nucleus. To construct the *ubc-2*::GFP fusion, the gene coding for GFP was inserted into the second exon of *ubc-2*. The resultant UBC-2::GFP fusion protein contains only the amino-terminus of UBC-2 fused to GFP. The expression of the UBC-2::GFP fusion protein throughout the nucleus suggests that the nucleolar localization of UBC-2 occurs post-translationally. The carboxyl region of *ubc-2* may contain a nucleolar localization signal, or UBC-2 may be targeted to the nucleolus through interaction with other proteins. These interactions appear to require the carboxyl-terminus of UBC-2. It is interesting to note that the localization of UBC-2::GFP to the nucleus and to the sarcomere of body wall muscle still occurs in the fusion protein. The concentration of UBC-2 to these regions must utilize a different signal from that which localizes UBC-2 to the nucleolus. This localization may occur prior to translation, or may be due to internal signals or protein interactions within the amino-terminus of UBC-2. The construction of a fully functional UBC-2::GFP construct would be useful for determining the signals responsible for UBC-2 localization.

10. CONCLUSIONS AND PROSPECTS

In summary, UBC-2 is involved in many aspects of *C. elegans* development. Using a combination of genetic and molecular techniques to examine the expression of UBC-2 in wild type and mutant animals, it has been possible to identify some of the requirements for UBC-2 during eukaryotic development. A maternal contribution of UBC-2 is necessary during embryogenesis. In addition, it is essential post-embryonically for the proper development of most tissues within the nematode, including the germline. UBC-2 appears to function in the regulation of signalling pathways that include one or more of the Wnt/Wg, NF-κB, integrin-mediated and MAPK cascades.

The cold-sensitive allele of *let-70*, pZM13.34, may prove useful for further analysis of the role of UBC-2 in *C. elegans* development. When integrated into the *let-70(s689)* background, it will be possible to determine the temperature-sensitive period of *let-70*. In addition, pZM13.34 could be utilized in genetic screens to identify suppressors of *let-70*. Since UBC-2 appears to have distinct functions during embryonic, somatic and germline development, the screens could be designed to isolate suppressors that are unique for each developmental process. An alternative method for isolating proteins that interact with UBC-2, the yeast two hybrid screen, has been initiated in the laboratory.

The present study has provided insight into the identity of components of UBC-2-mediated protein degradation, including several E3s and target proteins. Possible E3s are homologs of the APC/C involved in cell cycle progression and the $SCF^{\beta\text{-}TrCP}$ involved in Wnt/Wg and IκB signalling. Some likely target proteins include WRM-1 and BAR-1, MyoD, the NF-kB p105 precursor and IkB homolog. Experiments could be designed to determine if any of these E3s or putative substrates are downstream effectors of UBC-2. With completion of the genome sequencing project, components of the E3 complexes could be identified, isolated and *in vitro* ubiquitination assays utilized to determine whether UBC-2 interacts with the E3s to ubiquitinate specific target proteins. In addition, yeast two hybrid analysis could be performed using specific proteins that are believed to interact directly with UBC-2.

Mutations in many of the genes in these pathways already exist. Recently, the relative ease with which *C. elegans* gene knockouts can be obtained makes it possible to mutate every gene within a pathway. Thus, a complete genetic analysis of the signalling pathways could ultimately be performed to determine the genetic interactions of UBC-2 with other components

of the pathways. Genetic analysis would also provide valuable information regarding spatial and temporal requirements for the pathways.

Through a combination of biochemical and genetic analysis, the role of UBC-2 and ubiquitin-mediated protein degradation in the development of multicellular organisms can be further elucidated.

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VI. APPENDICES

APPENDIX A. C. elegans STRAINS UTILIZED IN STUDY

Strain	Reference
N2	-
AF16	-
BC2020 *let-70(s1132) unc-22(s7) unc-31(e169)/nT1(IV);+/nT1(V)*[1]	(Clark *et al.*, 1988)
BC5643 *let-70(s1132) unc-22(s7) unc-31(e169)/nT1(IV);+/nT1(V)*	This study
BC1093 *let-70(s689) unc-22(s7)/nT1(IV);+/nT1(V)*[1]	(Rogalski and Baillie, 1985)
PC156 *let-70(s689) unc-22(s7)/nT1(IV);+/nT1(V)*	This study
DR00789 *dpy-13(e184sd)/nT1(IV);unc-42(e270)/nT1(V)*[1]	-
BC3835 *let-x(s2293) unc-22(s7) lev-1(x22)/nT1(IV);+/nT1(V)*[1]	(Marra, 1994)
PC157 *let-x(s2293) unc-22(s7) lev-1(x22)/nT1(IV);+/nT1(V)*	This study
PD8119 *smg-1(cc545)*[2]	(Hodgkin *et al.*, 1989)
PD8120 *smg-1(cc546)*[2]	(Hodgkin *et al.*, 1989)

[1]Kindly provided by Dr. D. Baillie, Simon Fraser University, Vancouver
[2]Obtained from *Caenorhabditis* Genetics Center (CGC)

APPENDIX B. TRANSGENIC STRAINS UTILIZED IN THE PRESENT STUDY

Strain	Genotype	Selection	Construct	Random DNA
PC115	UbEx100	pRF4	pZMI.1	NO
PC125	UbEx110	pRF4	pZM13	NO

APPENDIX C. TRANSGENIC STRAINS PRODUCED IN THE PRESENT STUDY

Strain	Genotype	Selection	Construct	Random DNA
PC167	UbEx137	pRF4	pZM13	YES
PC168	UbEx138	pRF4	pZM13	YES

PC169	UbEx139	pRF4	pZM13	YES
PC170	UbEx140	pRF4	pZM13	YES
PC175	UbEx143	pRF4	NONE	YES
PC176	UbEx144	pRF4	NONE	YES
PC178	UbEx146	pRF4	pZM13.34	YES
PC179	UbEx147	pRF4	pZM13.34	YES
PC180	UbEx148	pRF4	pTS2.2	YES
PC181	UbEx149	pRF4	pTS1.1	YES
PC182	UbEx150	pRF4	pTS1.1	YES
PC183	UbEx151	pRF4	pTS2.2	YES
PC184	UbEx152	pRF4	*Cb*-G47J11	YES

APPENDIX D. LIST OF OLIGONUCLEOTIDE SEQUENCES

primer name[1]	**Sequence 5' to 3'**[2]
OZM3.f	GAGGAT*CCATGG*CTCTCAAAAGAATCCAGAAG *Bam*HI *Nco*I
OZM2.r	GCCAAGCTTTTAGCCTCCTCACATAGCGTA *Hin*dIII
MEI20-1.f	GGGTGTAGCCGGGTCAATACG
MEI20-2.f	GGTTGCCTTCACCACTCGAAT
MEI20-4.r	GGTCAGAGCCGGCGACCACTG
MEI20-5.r	TGATTGGTTTGGCTGCGTTCTT
TS1.f	GAGCATTCAGCACTCGGCAAGGT
TS2.r	CAACGTAATATGGGATAGACAAGCAG
MIC12.f	TATTACCAAACGCCGCATTG
MIC14.r	ATATAAACGGGGTAAACGGG
M7.7.f	TATGTGCCACGAGCCTACGAC
M7.7.r	GACAGAAACGTCGTCAGGCC
SL1.f	GGTTTAATTACCCAAGTTTGAG
SL2.f	GTTTTAACCCAGTTACTCAA
YHX1-B1.r	GCACCAAGAATGAGAACCTG

ZINC1 .r	GATGAGAAAGACGTGGAATAC
YHX1.f	GAGGATCCATGGCCACGTTTGTTCCCTTTG *Bam*HI
YHX1.r	GAGTCGACTCAGAAATCTTCATCATCCTC *Sal*I

[1]Primers suffixed with an f are sense stranded, while those suffixed with an r are the complementary strand.
[2]Restriction sites are underlined or italicized.

APPENDIX E. LINKER SEQUENCE UTILIZED IN STUDY

```
5'-       GCGGGCCCAAG       -3'
3'- ACGTCGCCCGGGTTCCTAG -5'
     Pst I    Apa I    BamH I
```