A STRUCUTURE-FUNCTION ANALYSIS OF THE COMPLEX GENE, BLI-4, IN CAENORHABDITIS ELEGANS

by

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B.Sc., University of Alberta 1990

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF

THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

in

THE FACULTY OF GRADUATE STUDIES

(Genetics Programme)

we accept this thesis as conforming
to the required standard

THE UNIVERSITY OF BRITISH COLUMBIA

April, 1995

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Department of **MEDICAL GENETICS**

The University of British Columbia
Vancouver, Canada

Date **MAY 5, 1995**

DE-6 (2/88)
Abstract

The purpose of this study was to elucidate the relationship between the molecular structure of bli-4 in Caenorhabditis elegans and the functional role of the predicted proteins produced by this complex gene. En route to this goal, the molecular structure of bli-4 has been redefined at the 5' and 3' ends of the gene; bli-4 is trans-spliced to the leader sequence SL1 and encodes 21 exons.

Two approaches were adopted to study the relationship between the predicted BLI-4 products and bli-4 mutations. The first employs a systematic search through a portion of the gene in five bli-4 mutant strains using PCR-based heteroduplex analysis. The second approach utilized the technique of germline transformation rescue with injected plasmid DNA. Minigenes, or clones of DNA that contain information necessary to encode a subset of the predicted isoforms of the gene, were constructed for this latter approach. The minigene rescue experiments provide a direct test for the capacity of a given isoform to rescue the different phenotypes of bli-4 (i.e., blistering and/or lethality).

One allele, h199, was detected in the 5' end of the gene as a polymorphism using the PCR-based heteroduplex technique. DNA sequence from homozygous arrested larvae indicated that h199 is the result of a missense mutation, changing a histidine residue to leucine. This amino acid substitution is in the amino terminus, proximal to the protease domain, and in a region that is not particularly well conserved among kex2/subtilisin-like family members.

Genetic analysis suggests that the BLI-4 gene products provide at least two distinct functions: one, which when removed, gives rise to blisters, and the other, which when removed, results in death. Data from transgenic
minigene experiments, however, suggest that the structures of the isoforms are sufficiently similar to be functionally redundant. In light of this new data, it is likely that functional distinction between the bli-4 isoforms is due to pre or post-translational localization and that either or both of these mechanisms are overridden by exogenous copies of minigenes that encode a subset of the total products of bli-4.
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Acknowledgements

I would like to thank my research supervisor, Ann Rose, for guidance and support throughout the course of this work; Colin Thacker for advice, encouragement, and many stimulating discussions; my supervisory committee members, Don Moerman and George Spiegelman for their constructive criticism and advice; past and present members of the Rose lab that have contributed to this work or my understanding of it; Carolyn Brown for comments on the thesis; David Pilgrim for giving me "worm fever"; and my father, Adam, for constant support and inspiration. Most of all, I would like to thank Karalynn Ell for sharing this experience with me.
Introduction

The genetics of bli-4

The bli-4 gene of Caenorhabditis elegans was originally identified as a recessive mutation, e937, that results in fluid-filled separations, or blisters, of the adult nematode cuticle (Brenner, 1974; Peters et al., 1991). Characterization of this mutation revealed incomplete penetrance of the phenotype; approximately 85% of e937 homozygotes exhibit the blistered phenotype. This reduced penetrance is a heritable feature of the mutation since a wild-type worm from an isogenic bli-4 strain produces the same number of blistered progeny as a blistered worm from the same population. The physiological basis for the blistered phenotype is not understood.

Since the isolation of bli-4(e937), thirteen additional recessive alleles of this gene have been identified (Rose and Baillie, 1980; Howell et al., 1987; Peters et al., 1991; Thacker, Srayko and Rose, unpublished data). However, these mutations all result in late embryonic or early larval lethality (Figure 1). Moreover, the host of bli-4 alleles exhibit a complex pattern of intragenic complementation. The thirteen lethal alleles of bli-4 have been grouped into classes II or III, based on the phenotype observed when these lethals are in heteroallelic combination with the only known visible allele of bli-4, e937 (which belongs to class I; Peters et al., 1991). Twelve lethal alleles, when in
Figure 1. Mutant phenotypes of bli-4.

Nomarski photomicrographs of (A) class I blistered phenotype of e937 homozygote, (B) class II q508 homozygote arresting development in late embryogenesis. (C) class III s90 homozygote arresting development as an L1 larva. 70% of s90 homozygotes appear as class II homozygotes (as in B).
heteroallelic combination with \textit{e937}, produce blistered worms that survive to adulthood and are fertile (see Table 1). These non-complementing alleles have been termed class II lethal alleles. A single lethal allele, \textit{s90}, complements \textit{e937} (heteroallelic animals appear completely wild-type) but does not complement any of the class II lethal alleles; \textit{s90} is termed a class III allele.

Certain class II alleles, when placed in heteroallelic combination with \textit{e937}, result in 100\% penetrance of the blistered phenotype (Thacker, \textit{et al.}, 1995; also see Discussion). Because \textit{C. elegans} populations are isogenic, this result suggests that \textit{e937} is hypomorphic and that the class II alleles implicated in this phenomenon are good candidates for null alleles. Furthermore, the phenotype of animals homozygous for class II mutations represents the most severe phenotype of all alleles. Muller's criterion (1937) for designation of the null state requires placement of the allele in question over a deletion that removes the gene. Unfortunately, no such deletion is currently available for unequivocal categorization of \textit{bli-4} alleles at this time.

\textbf{\textit{bli-4} gene structure}

The cloning and molecular characterization of \textit{bli-4} was initiated by Ken Peters (1992). At that time, the complex nature of the gene's structure became apparent (Figure 2). Three different species of cDNAs were known and matched to genomic regions, providing evidence that the \textit{bli-4} gene produced at least three isoforms, arising via differential splicing. The structure of \textit{bli-4} is consistent with the nature of a complex genetic locus. Peters also mapped two mutations of \textit{bli-4} (Figure 2). The blistering phenotype in \textit{e937} homozygotes
Table 1.
Intragenic complementation of bli-4 alleles

<table>
<thead>
<tr>
<th></th>
<th>Class I&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Class II&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Class III&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Class I&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Blistered (85%)&lt;sup&gt;d&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Class II&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Blistered (100%)&lt;sup&gt;d&lt;/sup&gt;</td>
<td>Arrest 3-fold</td>
<td></td>
</tr>
<tr>
<td>Class III&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Wild-type</td>
<td>Arrest 3-fold</td>
<td>Arrest 3-fold/L1 larvae</td>
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</table>

The complementation pattern for 12 lethal alleles and the visible allele, *e937*, is shown. All lethal mutants arrest development in late embryogenesis. 30% of *s90/s90* homozygotes survive until the L1/L2 larval stage. (Peter's, 1992; Thacker, *et al.*, 1995).

<sup>a</sup>Class I mutant is represented by *e937*.

<sup>b</sup>Class II mutants are represented by *h1010* and *q508*.

<sup>c</sup>The class III mutant is represented by *s90*.

<sup>d</sup>Percentage of blistered animals. Approximately 85% of *e937* homozygotes exhibit the blistered phenotype. The blistered cuticle phenotype is fully penetrant in the *e937/class II* animals.
is due to a deletion of 3.5 kb that removes an exon specific to one of the isoforms.  *h1010* is a class II allele, recovered from a strain with transposon-based mutator activity that was mapped to the first 12 exons of *bli-4*.

One of the goals of this thesis was to determine the relationship between the mutations associated with *bli-4* and the molecular structure of this gene. Previous mutational analysis has provided important clues about the function of the predicted products of the gene. Based on Peter's data, the blistering phenotype could be attributed to the loss of an exon that is specific to one of the isoforms produced by *bli-4*. Indeed, it was later shown that the expression of this isoform's transcript is absent in *e937* homozygotes (Thacker, *et al.*, 1995). Therefore, the transcript to which this exon belongs is likely not essential for development. Also, the non-complementing class II allele, *h1010*, is a transposon insertion into exon 9, an exon that all predicted transcripts share (Thacker, *et al.*, 1995). This mutation suggests that one or more of the remaining products of *bli-4* has an essential function.

The region common to all transcripts (the first 12 exons shown in Figure 2) shows sequence similarity to serine endoproteases and overall structural similarity to the kex2/subtilisin-like family of proprotein convertases (Thacker, *et al.*, 1995). This family of enzymes is implicated in the proteolytic activation of many biologically important precursor proteins. It is expected that *bli-4* is involved in enzymatic processing of at least one protein that is essential for the development of the organism. The variable phenotypes associated with this single gene may be due to mutations that alter functionally distinct proteases that arise via alternative splicing.
Figure 2. The complex molecular nature of *bli-4*.

The structure of *bli-4* and the alignment of known cDNA clones by Peters (1992) is shown. At this time, three isoforms were predicted to be encoded by *bli-4*, and the relative positions of unique 3' regions were positioned by sequence and/or hybridization analysis. The transposon insertion mutant *h1010* was mapped to the common region but the exact insertion site was not known. *e937* was found to be a deletion that removes 3.5 kb of DNA, including exon 13.
The kex2/subtilisin-like proprotein convertases

Regulating the activity of gene products is an important issue for any biological system. In some instances, proteins must be produced in a cell (where all transcriptional and translational machinery exists) but not allowed to function until they are safely sequestered in some compartment of the cell, or are exported out of the cell. One of the ways to prevent inappropriate protein activity is to first make a nonfunctional form of the protein that can be activated at some later time. Evidence for this mechanism of post-translational control was inferred from the discovery that pituitary hormones (Chretien and Li, 1967) and insulin (Steiner et al., 1967; Chance, Ellis and Brommer, 1968) are synthesized as inactive precursors. These and other inactive precursor proteins are subjected to limited endoproteolytic cleavage upon their secretion. This cleavage, which occurs most frequently after pairs of basic amino acids such as Lys-Arg or Arg-Arg (reviewed by Docherty and Steiner 1982; Thomas et al., 1988) results in activation of the molecule. One mechanism of endoproteolytic cleavage found in all eukaryotes examined occurs via the processing activity of the kex2/subtilisin-like family of proprotein convertases (reviewed by Barr, 1991; Seidah and Chretien, 1992). Kex2p, the prototype member of the convertases, is a membrane-bound Ca\(^{2+}\)-dependent serine endoprotease that cleaves the yeast pro-\(\alpha\)-mating factor at dibasic residues (Mizuno et al., 1988; Fuller et al., 1989a; reviewed in Fuller et al., 1988). Mammalian members of the convertase family include PC1/PC3 (Seidah et al., 1991; Smeekens et al., 1991), PC2 (Seidah et al., 1990; Smeekens and Steiner, 1990), PC4 (Nakayama et al., 1992; Seidah et al., 1992), PC5/PC6 (Lusson et al., 1993; Nakagawa et al., 1993), PACE4 (Keifer et al., 1991), and furin (Roebroek et al., 1986; Fuller et al., 1989b;
van den Ouweland et al., 1990; Wise et al., 1990). Two genes isolated from Drosophila, called Dfur-1 (Roebroek et al., 1992, 1994; also called dKLIP-1; Hayflick et al., 1992), and Dfur-2 (Roebroek et al., 1992) encode convertases with sequence similarity to furin.

For scientists studying the kex2/subtilisin-like convertases, the relationship between the convertase enzymes and the precursor proteins on which they act is elusive. Establishing the enzyme-substrate connection has been attempted by coexpression studies; one can determine if proprotein processing occurs by exogenous expression of specific convertases with candidate substrates. However, in this artificial context, many different endoproteases are able to process the same substrates. Whether such functional redundancy exists amongst the family members in vivo remains a mystery. One approach to determine possible convertase-substrate interactions involves restricting the endogenous expression pattern and localization of the individual convertases and/or potential substrates. Such analyses have shown that substrate specificity can be influenced by both restricting expression to particular tissues, and compartmentalization of the individual enzymes to specific intracellular locations (Seidah and Chrétien, 1992).

Some members of the family exhibit restricted expression patterns in vivo and participate in the regulated secretory pathway. For instance, PC1/PC3 and PC2 is restricted to endocrine and neuroendocrine tissues (Seidah et al., 1990, 1991; Smeekens and Steiner, 1990; Smeekens et al., 1991), and PC4 is restricted to the testis (Nakayama et al., 1992; Seidah et al., 1992). Therefore, some functional distinction can be inferred from those convertases whose expression pattern does not overlap. A potential substrate that is never localized in the testis would not be predicted to be
processed by PC4, for instance. In contrast, both furin and PACE4 are expressed in a broad range of tissues and participate in the constitutive secretory pathway (Roebroek et al., 1986; van den Ouweland et al., 1990; Van de Ven et al., 1990; Bresnahan et al., 1990; Kiefer et al., 1991). This constitutive expression complicates the task of determining convertase-substrate interactions. PC5/PC6, like furin and PACE4, exhibits a widespread tissue distribution. However, levels of expression are highest in intestinal tissue (Lusson et al., 1993; Nakagawa et al., 1993), indicating that PC5/PC6 may participate in both the constitutive and regulated secretory pathways.

Intracellular compartmentalization may also influence proprotein convertase activity and/or substrate specificity. Localization of the individual processing enzymes is likely intrinsic to the particular convertase examined. For instance, furin is concentrated in the trans-Golgi network (TGN) (Molloy et al., 1994). All members of the kex2/subtilisin-like family are first synthesized as an inactive zymogen (Leduc et al., 1992; reviewed in Seidah and Chrétien, 1992). Furin becomes active at the same time the enzyme is released from the endoplasmic reticulum and transported to the TGN. The enzyme is also observed to cycle back and forth from the cell surface via clathrin coated vesicles, much like EGF receptor molecules. Trafficking signals implicated in this cycling are encoded within the cytoplasmic tail of furin (Molloy et al., 1994).

Defining the function of the proprotein convertases likely requires a system whereby the in vivo context of activity is not perturbed. Therefore, mutational analysis is a powerful method of identifying components of this complicated process of post-translational control. The identification of mutations within a gene that encodes a proprotein convertase is a luxury few researchers in this field possess; only yeast kex2 and C. elegans bli-4 mutants
have been reported. In addition, the bli-4 gene offers an opportunity to examine the function of structurally distinct isoforms that resemble convertase members of both the regulated secretory pathway and constitutive secretory pathway (Thacker, et al., 1995). This gene represents a scientifically challenging target of study due to its genetic and molecular complexity. By taking advantage of the amenable genetics and molecular biology of the C. elegans model system, bli-4 may reveal important insights into enzymatic processing in a wide range of organisms, including humans.
Materials and Methods

Nematode culture conditions and strains

All *C. elegans* strains used in this study were maintained on petri plates containing nematode growth media (NGM) streaked with *Escherichia coli* OP50 at 20°C, unless otherwise indicated (Brenner, 1974).

The canonical wild-type nematode strain (+/+) is *C. elegans*, var. Bristol, strain N2. Mutations in *C. elegans* are assigned two descriptors, a genetic locus, and a corresponding allele that represents the mutational event giving rise to the associated phenotype. For example, the mutation e937 results in a blistered phenotype (worms with blistered cuticles), and is an allele of the *bli-4* gene; this mutation is written as *bli-4(e937)*. In general, genotypes are italicized and phenotypes are not. Upper case letters are used when referring to gene products; for example, *bli-4* encodes four BLI-4 protein isoforms. Nomenclature guidelines for *C. elegans* have been published by Horvitz, *et al.* (1979). Nomenclature of strains and materials used in this thesis is presented in Table 2.
Table 2.
**Abbreviations used in this thesis.**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>bli</td>
<td>Mutations in these genes give rise to a blistered cuticle</td>
</tr>
<tr>
<td>Bli</td>
<td>the Blistered phenotype; fluid filled separations of cuticular tissue</td>
</tr>
<tr>
<td>dpy</td>
<td>Mutations in these genes result in a dumpy phenotype</td>
</tr>
<tr>
<td>Dpy</td>
<td>the Dumpy phenotype: short, fat body morphology</td>
</tr>
<tr>
<td>unc</td>
<td>Mutations in these genes result in uncoordinated movement.</td>
</tr>
<tr>
<td>Unc</td>
<td>the Uncoordinated phenotype: an impairment or abolishment of locomotion or sensation</td>
</tr>
<tr>
<td>rol</td>
<td>Mutations to these loci result in a rolling phenotype</td>
</tr>
<tr>
<td>Rol</td>
<td>the Roller phenotype: a helical twisting of the body around the longitudinal axis, resulting in rolling motion as the worm moves forward</td>
</tr>
<tr>
<td>h</td>
<td>The Rose laboratory allele designation. All alleles, extra-chromosomal arrays, chromosomal rearrangements and DNA constructs (i.e., genotypic alterations) designed in this laboratory are issued an h number.</td>
</tr>
<tr>
<td>KR</td>
<td>The Rose laboratory strain designation. All C. elegans strains isolated in the Rose laboratory are issued a KR number.</td>
</tr>
<tr>
<td>KRp</td>
<td>All oligonucleotides, or primers, designed in the Rose laboratory for the purpose of Polymerase Chain Reaction are issued a KRp number.</td>
</tr>
<tr>
<td>pCeh</td>
<td>DNA constructs are identified as plasmids subcloned from <em>Caenorhabditis elegans</em> in the Rose (h) laboratory.</td>
</tr>
<tr>
<td>hEx</td>
<td>The exogenous DNA construct present within a nematode strain transformed via microinjection in the Rose (h) laboratory.</td>
</tr>
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Restriction enzyme digestion

DNA was digested with restriction enzyme(s) supplied by either Pharmacia or New England Biolabs. Digests were performed with a three-fold excess of enzyme (i.e., 3 units/μg of DNA) in the appropriate buffer (supplied by the manufacturer) for at least one hour at the recommended temperature. Bovine serum albumin (BSA) was also included in restriction digests at a concentration of 100μg/ml when recommended by the restriction enzyme manufacturer.

Agarose gel electrophoresis

DNA was size-separated in 0.7-1.5% w:v agarose gels in 0.5X TBE buffer [1X TBE is 0.89 M Tris, 0.89 M boric acid, 1 mM EDTA (pH 8.0)] with approximately 0.1μg/ml ethidium bromide. Gels were electrophoresed in 0.5X TBE running buffer at approximately 5V/cm. The size standard included in all gels was 1 kb ladder (Bethesda Research Laboratories). DNA bands were detected and photographed under illumination with 300nm UV.

Polymerase Chain Reaction (PCR)

Template DNA from individual worms or arrested embryos homozygous for bli-4 mutant alleles was extracted as described (Barstead, et al., 1991). Most polymerase chain reactions were carried out in a Perkin-Elmer/Cetus PCR machine for 30 cycles of denaturation (94°C, 45 seconds),
annealing (54°C-60°C, depending on primers, 30 seconds), and extension (72°C, 1 minute) followed by extension at 72°C for 7 minutes. Reactions were performed with Taq Polymerase and accompanying buffer system from Promega or with Pfu polymerase and buffer system from Stratagene. Some reactions were carried out in 25μl capillary tubes with a hot-air thermocycler (Idaho) using the Idaho buffer system.

**PCR-based Heteroduplex Technique**

The principle behind the heteroduplex technique for identifying mutations is based on a well-studied property of DNA, the tendency of single-stranded molecules to associate, or hybridize, to their complementary forms. As the title implies, heteroduplex technique involves detecting associated DNA molecules (duplexes) which are not completely complementary. In general, one selects a fragment of DNA to study and mixes this in a 1:1 ratio with the same fragment isolated from a mutant strain. The DNA is denatured into single-stranded form and allowed to slowly reanneal. If a mutation is present in one half of the starting material, three forms of duplex molecules will form: wt:wt homoduplex, mut:mut homoduplex, and wt:mut heteroduplex. Due to structural alteration of the duplex in the wt:mut form, this dsDNA will migrate aberrantly through an acrylamide matrix. Generally, mutations are detected as two bands on a gel, representing both species of homoduplexes migrating at normal position compared with a wt:wt control and a slightly fainter band migrating slightly slower, representing the heteroduplex DNA (see Figure 3) (Keen, et al., 1991).
Figure 3. Heteroduplex method of mismatch detection.

The procedure employed for the detection of class II alleles *h791, h384, h254, h199,* and *h670* is shown. Individual worms that are heteroallelic for each of the five mutations are used as template for PCR (30 cycles), using a set of primers that amplifies a segment of genomic DNA. The resulting PCR products are denatured and slowly reannealed to form stable duplexes. If a mutation is present, three types of duplexes will form: wt:wt, wt:mut, and mut:mut. This DNA is run in a non-denaturing acrylamide matrix. Typically, heteroduplex migration is retarded when compared to homoduplexes and is slightly fainter due to 1/3 representation of the total pool of PCR products (Keen, et al., 1991).
Polymerase Chain Reaction (PCR) was performed on heterozygous individual blistered adult worms of the genotype \textit{dpy-5 bli-4(class II allele)} \textit{unc-13/bli-4(e937)}. The products were checked for size, purity, and approximate concentration by loading 2-3 μl (one tenth) of the total yield in a 1-1.5 % agarose gel along with a size standard (Gibco BRL 1 kb ladder) and a DNA source of known concentration (typically, pRF4 (\textit{rol-6}) plasmid at 50, 100, and 200ng).

Samples were incubated at 95°C for three minutes and slowly (at least 30 minutes) cooled to 37°C. The samples were then removed from 37°C and kept at room temperature prior to loading. MDE™ (mutation detection enhancement) matrix was prepared as suggested (AT Biochemicals). Approximately 200ng of DNA (in less than 14 μl volume) was loaded per well (with 1/10 loading buffer, provided by manufacturer) for each sample. A negative control (N2) and a positive control (\textit{unc-52/+}) were included in each gel. The positive control, CB1012/+ worms, were subjected to PCR using the antisense primer p10 (5'-CTG GTG GGC TAT TCT CTG G-3') and the sense primer peg8 (5'-GAC ATC CAA GTG TTC AGC -3'), which amplify a ~590 bp product of exon 16 and 17 from the \textit{unc-52} gene (primers were a gift from Don Moerman). When using CB1012/+ worms as template for PCR, the mutation \textit{e1012} (a G:C to A:T transition mutation in exon 17 of \textit{unc-52}) is amplified from one half of the starting material. The products were run at 20V/cm for a distance of ~30 cm. The acrylamide matrix was stained with ethidium bromide (1μg/ml) and photographed by illumination with 300nm UV.
Generating heterozygous worms for PCR-based heteroduplex analysis

To generate heterozygosity at the bli-4 locus for heteroduplex analysis, the following cross was performed. Balanced strains carrying lethal class II alleles of the genotype \textit{dpy-5(e61) bli-4(class II lethal) unc-13(e450); sDp2} were crossed to homozygous males of the genotype \textit{bli-4(e937)}. Due to intragenic complementation (Table 1) the resulting F1 heteroallelic progeny are blistered. These blistered worms were used individually for PCR amplification. One exceptional allele, \textit{h199}, did not result in blistering very often when in heteroallelic combination with \textit{e937} (see Results and Discussion); however, only blistered F1 animals (\textit{h199/e937}) were used for heteroduplex analysis to ensure the correct heteroallelic genotype.

\textit{h670} was maintained in strain KR2486 as a heterozygote of the genotype: \textit{+ bli-4(h670) unc-13(e450)/dpy-5(e61) + unc-13(e450)}. Unc animals that gave approximately 1/4 dead progeny were used as template for PCR-based heteroduplex analysis.

Preparation of DNA for germline transformation

Plasmid DNA was purified from bacterial cultures by one of three methods. One method involves standard mini-prep procedure with phenol/chloroform extraction and ethanol precipitation followed by resuspension of the DNA pellet with T.E. + RNAse (10\textmu g/ml). The second method utilizes a commercially available kit (Wizard Mini-Prep, Promega) to isolate plasmid DNA from an overnight culture of bacteria. This procedure, however, did not give any transformants unless the DNA was further
precipitated (70% ethanol, 0.3M NaOAc) and washed with 70% ethanol. The third method is CsCl preparation of plasmid DNA, as discussed in Sambrook, et al., (1989). The most consistent results were achieved with the CsCl preparation.

The concentration of plasmid DNA after preparation was estimated by loading a fraction of the total volume in an agarose gel along with a control plasmid of known concentration, or, in the case of CsCl DNA, estimated by measuring the optical density at 260 nm wavelength with a spectrophotometer. DNA constructs were coinjected with the plasmid pRF4 (a gift from C. Mello), carrying the dominant marker rol-6(su1006) (Kramer, et al., 1990) at a total concentration of approximately 100ng/μl. pRF4 confers a dominant roller phenotype (Rol) to C. elegans strains that carry this plasmid. (Kramer et al., 1990).

**Germline transformation**

DNA was injected into the distal arm of one or both gonad arms in adult hermaphrodites (shown in Figure 4) after the method of Mello et al., (1991). Recombination between separate coinjected DNA molecules has been observed (Mello et al., 1991). This recombination occurs in regions of sequence identity to create large concatemeric hybrid molecules which exist as extrachromosomal forms (see Figure 5).

Approximately ten percent of F1s that carry an extrachromosomal array will transmit that array in a stable, non-Mendelian manner to the F2 generation. Provided the array is large enough, it will be faithfully transmitted to progeny cells in the developing egg after fertilization with
sperm. The inheritance of an array can be detected by the dominant Rol phenotype; rolling F1 animals from an injected hermaphrodite are likely to carry both the pRF4 plasmid and any other coinjected plasmid that bears sequence identity to pRF4. For instance, pBluescript-based clones and pRF4 share sequence identity in at least one location of their plasmid backbone, since both vectors carry the ampicillin resistance gene, $amp^r$. Stable transgenic lines were used in rescue analysis of bli-4 lethal alleles.

**Subcloning of plasmid DNA**

Target DNA and vector were digested with appropriate restriction enzyme(s) supplied by either Pharmacia or New England Biolabs as described above. Enzymes were inactivated as per manufacturer's suggestion when the products were used directly, otherwise, the resulting products were separated from enzymes and extraneous DNA via agarose gel electrophoresis. Extraction of DNA from agarose gels was achieved with the Qiagen purification system. Vector (pBluescript I (SK-) or (KS+)) (Stratagene) and target DNA were ligated at 16°C overnight using T4 DNA ligase (New England Biolabs, as described by Sambrook, *et al.*, 1989). Ligated DNA was used to transform competent *E. coli* strain DH5α (Bethesda Research Laboratories). Transformed cells were identified as being resistant to ampicillin, due to the production of β-lactamase provided by the $amp^r$ gene present in pBluescript. The α-complementation system (Ullmann *et al.*, 1967) was used to distinguish between parent and recombinant plasmids; cells were grown in the presence of a chromogenic substrate, X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) and IPTG (isopropyl thiogalactoside).
Figure 4. Microinjection of plasmid DNA for germline transformation.

DNA prepared for germline transformation was microinjected into the distal gonad arm (arrow) of young adult hermaphrodites, as described in Mello, et al., (1991). Photograph courtesy of J. McDowall.
Formation of hybrid extrachromosomal arrays is driven by homologous recombination.

Plasmid constructs coinjected into the distal arm of hermaphroditic gonads undergo recombination at sites of homology (for instance, the ampicillin resistance gene in pBluescript and pRF4(rol-6) plasmids). Arrays consisting of many copies of each plasmid type are maintained by non-Mendelian inheritance. Strains exhibiting good transmission (20% or greater) are used for subsequent crosses.
Parental plasmid-bearing clones appear blue and recombinants appear white when grown in the presence of IPTG/X-gal (Horwitz et al., 1964). Plasmid DNA was recovered from appropriate clones by conventional alkaline lysis miniprep procedure (Sambrook, et al., 1989).

Reverse transcriptase polymerase chain reaction (RT-PCR)

First strand cDNA was synthesized from 5µg of total RNA using an oligo-dT primer and the Superscript Preamplification system (Gibco BRL). Conditions for synthesis were as described by the manufacture. RT-PCR was performed on poly A+ RNA isolated from wild-type (N2) worms. Reaction conditions for PCR were as described above. RNA was kindly provided by Colin Thacker.

Cloning PCR products

Cloning of PCR products involved size-separation of the appropriate band(s) via agarose gel electrophoresis, followed by purification with either Wizard PCR Prep (Promega) or Qiagen systems. Blunt-end ligation was performed to insert the PCR products into the EcoRV site of the pBluescript(SK) polylinker. When Taq polymerase was used in the PCR reaction, a further step was required to repair the ends prior to blunt-end ligation. This was accomplished by adding 1 unit of T4 DNA polymerase (NEB) in a final volume of 50µl of 1X buffer (supplied) and a final concentration of 200mM dNTPs. PCR reactions using Pfu polymerase
(Stratagene) were cloned directly into the EcoRV site without prior modification.

**Testing for trans-splicing of bli-4 RNA to leader sequences SL1 and SL2**

RT-PCR was performed on N2 RNA with an antisense primer KRp10 (5'-ACT CTC TTC TTC GGT CGC-3') situated in exon III of bli-4, in combination with one of two sense primers, SL1 (plus NotI adapter) (5'-ATA AGA ATG CGG CCG CGG TTT AA TTA CCC AGT TG-3') or SL2 (plus NotI adapter) (5'-ATA AGA ATG CGG CCG CGG TTT TAA CCC AGT TAC TCA-3'). Two bands were observed after amplification with the SL1 primer, no product of expected size was visible with the SL2 primer. The major product was a band of smaller than expected size (~300bp) and was cloned directly after gel-purification; sequencing revealed this DNA was not bli-4 derived. The faint band of expected size (~500bp) amplified with the SL1 and KRp10 primer was gel-purified and this DNA was used as template for a further round of PCR, using SL1 primer and a nested primer, KRp11. A resultant single band of expected size (~450bp) was gel-purified. This fragment was cloned and sequenced (shown in Figure 15, Results).

**DNA sequencing**

Sequences of pBluescript plasmid inserts were obtained with Sequenase Version 2.0 (United States Biochemical (USB)), with $^{35}$S-dCTP encorporation for detection of DNA by autoradiography. Preparation of
dsDNA template was accomplished by purifying DNA from 3-4.5 ml overnight bacterial cultures with Wizard Purification preparations (Promega). Subsequent steps performed to prepare template were as suggested by USB. Primers used for sequencing were T7, T3, universal, reverse, SK, and KS, obtained from Stratagene.

**DNA sequence analysis**

DNA sequences were translated and restriction mapped with the DNA Strider program for Apple Macintosh. Amino acid sequences generated from Strider were used to search SWISSPRO and GENBANK databases using the BLAST network service (email address: blast@ncbi.nlm.nih.gov). Computations for searches were performed at the National Centre for Biotechnology Information (NCBI).

**Subcloning and the construction of bli-4 minigenes**

Most minigenes were constructed from four existing plasmid clones of bli-4, pCeh180, pCeh181, pCeh220, and pCeh221 (courtesy of Ken Peters and Colin Thacker). Most 3' exons were subcloned from the parent clone pCeh180. The putative 5' promoter region and 5' exons included in all minigenes were derived from pCeh220, pCeh221, pCeh181 and subclones thereof. Appendix 1 shows all relevant subclones used in this thesis. The cloning steps for constructing minigenes are outlined in Figures 6 to 11.
Rescue of bli-4 lethal alleles with transgenic arrays

In order to determine whether a transgenic array rescued bli-4 lethality, the following crosses were performed. Each of the lethals, maintained with the balancer sDp2, were crossed to N2 males. q508 was maintained in the strain KR2572 which has the genotype: \textit{dpy-5(e61) bli-4(q508); sDp2}. h199 and s90 were maintained in strains KR513 and KR2728 respectively and have the corresponding genotypes: \textit{dpy-5(e61) bli-4(h199) unc-13(e450); sDp2}, and \textit{dpy-5(e61) bli-4 (s90) unc-13(e450); sDp2}. After allowing mating to occur (typically 16 hours) hermaphrodites were placed on individual plates. From these plates, F1 males (heterozygous for the lethal mutation and possibly carrying sDp2) were crossed to Rollers from the strain carrying the extrachromosomal array. Males which carry sDp2 are known to mate very poorly so their presence in the F1s was not problematic (Rose, \textit{et al.}, 1984). Rollers were removed from the mating plate after 16 hours and allowed to lay eggs on individual plates. Only those Rol hermaphrodites which gave male progeny were used for further analysis. From plates with male progeny, hermaphrodite Rol F2s were picked to individual plates. Only plates of F2s with some arrested embryos (evidence for the lethal bli-4 allele) were scored for rescue; even if rescue is possible, only a fraction of the lethal homozygotes will receive an unstable extrachromosomal array, thus arrested embryos should be present regardless of the rescuing capability of the array. All F3 progeny were scored from these F2 parents. For alleles h199 and s90, rescue was evidenced by the presence of Dpy Unc animals in the F3 generation. For q508, Dpy animals were present if rescued by the transgenic array. Both Dpy-5 and Unc-13 are epistatic to Rol-6, therefore, the Rol
Figure 6. Construction of pCeh226.

A) The substrate clones used in the construction of pCeh226 are shown.

B) A schematic representation of the cloning steps involved in the construction of pCeh226.
Figure 7. Construction of pCeh229.

A) The substrate clone pCeh226 used in the construction of pCeh229 is shown.

B) A schematic representation of the cloning steps involved in the construction of pCeh229.
Figure 8. Construction of pCeh230.

A) The substrate clones used in the construction of pCeh230 are shown.

B) A schematic representation of the cloning steps involved in the construction of pCeh230.
Figure 9. Construction of pCeh236.

A) The substrate clones used in the construction of pCeh236 are shown.

B) A schematic representation of the cloning steps involved in the construction of pCeh236. Arrows indicate direction of bli-4 reading frame.
Figure 10. Construction of pCeh238.

A) The substrate clones used in the construction of pCeh238 are shown.

B) A schematic representation of the cloning steps involved in the construction of pCeh238.
Figure 11. Construction of pCeh252.

A) The substrate clones used in the construction of pCeh252 are shown.

B) A schematic representation of the cloning steps involved in the construction of pCeh250. pCeh244 is a PCR-derived product from single h199 homozygous arrested embryos, amplified with Pfu polymerase using primers KRp29 and KRp45 (Figure 15). The amplified product was gel-purified and digested with ClaI and SacI, and cloned into BSSK (ClaI/SacI). A parallel series of experiments using a PCR product from wild-type animals were also performed. pCeh250 was subsequently used to construct pCeh252, a subclone analogous in structure to pCeh229 (Figure 7). The procedure was as follows: pCeh229 was digested with PstI and KpnI (KpnI is present in the polylinker, 3' of the SalI site), and the ~2 kb fragment containing exon 13 was ligated into pCeh250 (digested with PstI and KpnI) to produce pCeh252. Wild-type subclones analogous to h199 subclones are: pCeh244(h199) and pCeh245(N2); pCeh246(h199) and pCeh247(N2); pCeh250(h199) and pCeh251(N2); pCeh252(h199) and pCeh253(N2).
A

\[ Xb \quad E \quad Sac \quad Xh \quad C \quad C \quad Sac \quad E \quad E \quad P \quad E \quad Sal \quad Sal \quad E \quad Sal \quad Xh \quad P \quad Xb \quad E \quad K \quad Sac \quad K \quad E \quad E \quad P \quad Sal \quad 1kb \]

pCeh244

pCeh220 & pCeh221

pCeh181

pCeh249

B

pCeh221

\[ Xh \]

C (partial)

1.1 kb

pCeh224

\[ C / Sac \]

\[ Xh \quad C \quad C \quad C \quad * \quad Sac \]

two factor ligation into Xh/Sac BSSK

pCeh246

\[ Xh \quad C \quad C \quad * \quad Sac \]

pCeh249

\[ X / Sac \]

1.6 kb

pCeh249 (Xh/K)

\[ Xb \quad E \quad Sac \quad Xh \quad K \]

\[ * = h199 alteration \]

A \rightarrow T

pCeh250

substrate for pCeh252
phenotype is masked in the event of rescue by the transgene. Stable Dpy or DpyUnc lines were established for each rescue and given a strain designation. Furthermore, a few animals from each rescued line were crossed to N2 males to ensure that Rol progeny resulted, proof that the extrachromosomal array was present and faithfully propagated in rescued animals. A general scheme is presented in Figure 12.

Scoring the blistered phenotype

Blistering was scored as either positive or negative, with no attention paid to severity of individual blisters (i.e. size or volume) or to the number of blisters per animal. In cases where a transgenic array was tested for ability to rescue, the dominant marker rol-6(su1006) was used to delineate worms carrying the transgene from those that do not (see germline transformation, above). Adequate mobility by the worm is required to observe the Rol phenotype conferred by the rol-6 marker. However, the blistered phenotype, when severe, can greatly impede movement, making it difficult to score the Rol phenotype. Therefore, rollers were picked off onto a new plate as L4 or earlier larvae (prior to the adult stage when blistering occurs) and then scored for presence or absence of blistering.
Lethal alleles balanced by sDp2 (e.g., h199, q508, and s90) were crossed into Rol strains carrying the extrachromosomal array. Rol hermaphrodites were picked from the F2 and set up individually for scoring. Rol F2s that did not produce any dead progeny were presumed to not carry the lethal allele and were not scored.
WT

Unc

\[ \text{dpy-5} \text{ bli-4(class II)} \text{ unc-13} \]

\[ \text{dpy-5} \text{ bli-4(class II)} \text{ unc-13} \]

\[ \text{sDp2} \]

\[ \text{dpy-5} \text{ bli-4(class II)} \text{ unc-13} \]

\[ + \quad + \quad + \]

pick wild-type \( \text{♂'s} \)

(may also carry sDp2)

WT

Rol

\[ \text{dpy-5} \text{ bli-4(class II)} \text{ unc-13} \]

\[ \text{+ bli-4(e937) +} \]

\[ \text{+ bli-4(e937) +} \]

\[ \downarrow \downarrow \downarrow \text{hEx (includes rol-6)} \]

Bli

\[ \text{dpy-5} \text{ bli-4(class II)} \text{ unc-13} \]

\[ + \text{ bli-4(e937) +} \]

\[ \text{+ bli-4(e937) +} \]

\[ \downarrow \downarrow \downarrow \text{hEx (includes rol-6)} \]

Rol

\[ \text{dpy-5} \text{ bli-4(class II)} \text{ unc-13} \]

\[ + \text{ bli-4(e937) +} \]

\[ \text{+ bli-4(e937) +} \]

\[ \downarrow \downarrow \downarrow \text{hEx (includes rol-6)} \]

pick Rol \( \text{♀} \) for scoring
Results

SECTION I. The 5' region of the bli-4 gene.

A. Determination of the 5'-most sequence of bli-4

The 5' end of bli-4 was determined by performing RT-PCR on poly A+ RNA isolated from N2 worms (see Materials and Methods). The relative positions of primers used for this task is presented in Figure 13. A single band of expected size (~450bp), amplified by the sense primer derived from SL1 sequence and the bli-4 antisense primer KRp11 was gel-purified, cloned and sequenced. The sequence of both ends of this product are presented in Figure 14. This experiment indicated that bli-4 poly A+ RNA is trans-spliced to the leader sequence SL1 and that the 5' portion of the gene begins with the adenine residue immediately following the SL1 sequence. This adenine is not present at the 5'-most position of the cDNA containing the longest 5' end, pCeh197. A parallel experiment performed with an SL2 trans-splice leader primer failed to amplify bli-4 sequences, as determined by sequencing cloned DNA of slightly smaller than expected size.
Figure 13. A test for trans-spliced leaders SL1 or SL2 in the bli-4 transcript.

The primers shown were utilized in reverse transcriptase-PCR to determine if bli-4 RNA was trans-spliced to leader sequences SL1 or SL2. Arrows above (directed right, or 3') indicate sense primers and those below (directed left, or 5') indicate antisense primers. The predicted 5'UTR of bli-4 is shown in white; the protease domain is in grey; only those exons (1-12) common to all bli-4 transcripts are shown.
SL1 \textit{ggtttaattacccaagtttgagacgttcgtctcgggtcgatgctag}
CGTCGCTTCGGGGTGAGTCTAGAG
GCAAGCGAAGGCCAGCTACAGTC

\textit{aataaacgaaaaagaataacccttgccacccagcgaatgctgaaacat}
AATAAACGAAAAAGAATAACCCGTTGCAACCAGCGAATCGTGAACATTT
TTATTGTCTTTTCTTTATTTGGCAACGGTGGTCGTTTACGTCGCTTAA

\textit{tcaatctcaccatcactcgtcaccaaacggatattatattattatatt}
TCAATACCTACCATATCAGTCACCCCCAAAAACGGATTTATTATTATATA
AGTTATGAGTGCGTAGTGCGTGGTTTGGCCTAAATAATAATAATA

\textit{gacccatatctcaccatcttctcagctcaacggctagtctatctgcag}
GACCATATCATCCCATTTTTATTCCCACAACGCTGATGCTAGATAG
CTCGTATAGTATGGTTGAAATAAGAGTTTGCACATCACATATAGCTAT

\textit{gccggatacatggcaaa}
GCGCGTATAGCATTGCCAAATTTCTGGCAGTTTTAACTCGCAGTTGCACTACT
CGGCTATACGTTACGTTAAGAGCGTCAAAATTAAGCGTCACGTAAAGTGA

\textit{ATGGAACATGATTTCCATTTTGCGATGAAATAGTATAGGTCTGCTGTGGGAAACC}
TAACTTGTACTAAGTAAACGCCATCTTTATCATATCCAGACACCACTCTGG

\textit{AATACATACCCATATAATCGTATTGCGAAGATATGGATAGCGCTGTGCCG}
gccattatgcaaatc????tctctactactcgaagct????

\textit{GAATAGCCTGACGACATGATCATGATATGAAAGGTGATCCGTTTTTGGAT}
CTTATCGACGACTAGTACTGTACGTACATTCTGGCAGTTTTCCATAGCCCAGAAACC
CATTACGAGCATGATGCTATGACTGATATGAAAGGTGATCCGTTTTTGGAT

\textit{ACCTACACTCTCCCTTTATGACTCGGAAACAACAAAGGACAC}
TGAGTGATGAAGGAAATAGTGAGCCCTTTGTGTGTCTGCTG KRp11
tgagtgatgaagggaaatagtgagccctttgtgttt

Figure 14. \textit{bli-4} is \textit{trans}-spliced to SL1 (spliced leader 1).

The sequence of the ~450 bp RT-PCR product amplified from N2 poly A+ RNA with primers SL1 and KRp11 is shown. Italics indicates sequence obtained from the cloned PCR product and the relative position of the sequence is matched with the cDNA that is most complete at the 5' end, pCeh197. SL1 and KRp11 sequences are underlined. The sequencing gap between each end of the cloned PCR product is shown; question marks indicate unreadable sequence.
B. Mapping mutations in the 5' region of bli-4

The technique of PCR-based heteroduplex analysis was employed to search for polymorphisms in a subset of class II mutant strains. Since the genetics of bli-4 suggests that class II alleles affect the production and/or function of all transcripts encoded by the gene, it was believed that the region common to all transcripts would be a good place to start searching for these mutations. The following class II lethal alleles were chosen for the analysis: h670, h199, h791, h384 and h254. Primers for PCR were constructed to amplify overlapping fragments of the common region of bli-4 (Figure 15). The PCR products made from heteroallelic worms for the above stated lethal mutations were tested for polymorphisms (see Materials and Methods, Figure 3). The 3' end of the common region was not tested due to the lack of primers to amplify a fragment small enough to be useful for mutation detection by this technique. A summary of the results for PCR-based heteroduplex detection of mutations is shown in Table 3.

B.1. h199 is a missense mutation

A single polymorphism was detected in h199/e937 heterozygotes from the PCR product of primers KRp44 and KRp45 (Figure 16). To confirm the existence of a base pair alteration, h199/h199 dead embryos were used as template for PCR. Products from three independent PCR reactions were cloned and sequenced in both directions. As shown in Figure 17, all three clones show an A:T to T:A transversion in h199 but not in e937 or N2 controls.
Figure 15. Location and sequence of primers designed to amplify the 5' end of bli-4.

Overlapping fragments of the bli-4 common region were amplified by using combinations of sense primers (drawn above the gene structure) with antisense primers (drawn below the gene structure). The predicted 5'UTR of bli-4 is shown in white; the protease domain is in grey.

A) The host of primers available for heteroduplex analysis, determination of SL1/SL2 splicing and 3' RACE experiments.

B) Four additional primers were constructed to further divide the protease domain (shown as grey boxes).
Table 3.
Summary of PCR-based mismatch detection

<table>
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<th>primers</th>
<th>exons</th>
<th>h670</th>
<th>h199</th>
<th>h791</th>
<th>h384</th>
<th>h254</th>
</tr>
</thead>
<tbody>
<tr>
<td>KRp29 &amp; KRp10</td>
<td>1-3</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>KRp44 &amp; KRp45</td>
<td>3-5</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>KRp34 &amp; KRp35</td>
<td>5-7</td>
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<td>-</td>
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<td>-</td>
</tr>
<tr>
<td>KRp36 &amp; KRp37</td>
<td>8-10</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<td>-</td>
</tr>
</tbody>
</table>

Individual worms heteroallelic for each of the alleles presented were tested for polymorphisms via the heteroduplex method of mismatch detection (see Materials and Methods).

+ : polymorphism detected by PCR-based heteroduplex analysis
- : PCR fragment appeared as homoduplex control
A polymorphism associated with h199 was observed upon heteroduplex analysis of the PCR product of KRp44 and KRp45. Four other alleles of bli-4 did not exhibit a polymorphism for this same region. The positive control primer set (peg8 and p10) on CB1012/+ (unc-52 heterozygote) was included; N2 control for KRp44 and KRp45 was included in this experiment and did not reveal a polymorphism (not shown).

Figure 16. A polymorphism detected by heteroduplex analysis in h199/e937 animals.
Figure 17. Sequence of KRp44/KRp45 PCR products from arrested h199 homozygotes.

A) A summary of sequenced clones from three independent PCR products of arrested h199 homozygotes. The three clones are listed in (B). KRp44 and KRp45 sequences are underlined, as is the location of the transversion mutation.

B) i) Two h199 clones from the fragment amplified with KRp44 and KRp45 primers using Taq polymerase were cloned and sequenced. Sequence differences found in these clones, when compared to the e937 and N2 clones, are bold-faced.

ii) One h199 and one N2 clone from the fragment amplified with KRp29 and KRp45 primers using Pfu polymerase were cloned and sequenced (see Materials and Methods). The h199 clone was utilized for construction of a minigene that contains the h199 A-T transversion, pCeh252 (Figure 11).
A

KRp44

GTTGAACGATTTGGAATCCACGTCGACCGTGAATGGGTTGAAGAC
CAACTTGCTAACCTAAGTGTAGGTCGGCAGCTTACCCAACTTCT
ACAGCGACCGAAGAAGAGAGTCAAAAGAGATTATATTCTCCTGG
TGTCGCTGGCTTCTTCTCTCAGTTTTTCTCTATAATATAAGGACC
ATAATgttagttttttatactccccctatatgatttaggttt
ccagaatcattacggttttctttttatcttagcttttagagttt
ggtttagtaatggcaaagaagaaaaataagatcgaatctcaaa
cttttagtctttgttctacaattaccattctttttatcattactttt
agtcCTACAAGTDAAGATGGGAAGGGGCAAGCCAAAAACT
ACCGTGATGGTACTCGTAGACCTGACAGCAGCAGTCCTGGCTTTTGA
aagtcCTACAAGTDAAGATGGGAAGGGGCAAGCCAAAAACT
CCAGGAGAAAATCCATACCTCCCATTTCGATCCACTTTTATAAA
GGTCTCTTTTTAGGTAGTGAAAGGACTAGGTGAATATT
AGACCAGTGTTATTTGgtagttttcaataattcataatcttttta
TCTGTGTCACCATAAACcactcaaatatttaagtttaaagaaatt
agagaaaaaaaccaactgatacatttacagCATGGTGAGCA
GTTGGTTGATATGATATCAACCACCTTATATATAKRp45

B

i) pCeh240 (h199)  ...tttcagGAT GTT CAC CTT TCT AAC CCC TTC CGC...
pCeh241 (h199)  ...tttcagGAT GTT CAT CTT TCT AAC CCC TTC CGC...
pCeh242 (e937)  ...tttcagGAT GTT CAT CTT TCT AAC CCC TTC CGC...
pCeh243 (WT)  ...tttcagGAT GTT CAT CTT TCT AAC CCC TTC CGC...

ii) pCeh244 (h199)  ...tttcagGAT GTT CAT CTT TCT AAC CCC TTC CGC...
pCeh245 (WT)  ...tttcagGAT GTT CAT CTT TCT AAC CCC TTC CGC...
However, one of the $h199$ clones also revealed a second alteration in the same region. It was suspected that $Taq$ polymerase introduced an error at this site during the amplification process. $Taq$ polymerase is known to have a significant rate of misencorporation of nucleotides during amplification. Therefore, one reaction was performed on $h199$ and N2 template using $Pfu$ polymerase for PCR; $Pfu$ has approximately 12 fold higher fidelity than $Taq$ polymerase (Lundberg, et al., 1991). As shown in figure 18B, the A:T to T:A change alone is present in the $Pfu$ amplified $h199$, but not the N2 control.

**B.2. $h199$ is a weak class II allele**

Previous work on intracomplementation between alleles of the $bli-4$ locus revealed that penetrance of blistering increases when $e937$ is placed in heteroallelic combination with class II alleles (Thacker et al., 1995). However, in performing crosses to acquire heterozygous hermaphrodites for PCR-based heteroduplex analysis, it was observed that $h199/e937$ hermaphrodite worms rarely blister. For instance, when an Unc hermaphrodite of the genotype $dpv-5$ $bli-4(h199)$ $unc-13$; $sDp2$ was crossed to $e937$ homozygous males, the following non-Unc outcross progeny resulted: 58 blistered males, 3 blistered hermaphrodites, 4 non-blistered males, 62 non-blistered hermaphrodites. These outcross progeny should have been heteroallelic for $h199$ and $e937$ at the $bli-4$ locus, unless they carried the $sDp2$ balancer. Since the $sDp2$ balancer would have been transmitted to some of the progeny, incomplete penetrance was expected. However, this does not explain why so few of the hermaphrodites blistered, since $sDp2$ should have been transferred to both gametic lines (Rose, et al., 1984). In order to
determine whether this phenomenon was due to the \textit{h199} allele itself, and not to preferential transmission of \textit{sDp2} to hermaphrodite outcross progeny, 3 blistered and 4 non-blistered hermaphrodites from the above cross were set up on individual plates and their progeny scored. All seven worms gave some dead progeny, suggesting that each hermaphrodite set up contained the \textit{h199} lethal allele. Two of the non-blistered animals gave many Unc progeny, which was evidence for the presence of \textit{sDp2}; this balancer covers both \textit{dpy-5} and \textit{bli-4} loci, but not \textit{unc-13} (Rose, et al., 1984). The progeny from these two animals were not included in the summary shown in Table 4. If the \textit{h199/e937} animals in this experiment always blistered, we would have expected approximately 1/2 of 791 (=396) plus 85% of the \textit{e937} homozygotes (1/4 of 791, or 0.85 X 198) to be blistered (i.e., 564 animals). If the \textit{e937/h199} animals never blistered, we would have expected only the \textit{e937} homozygotes, or approximately 85% of (1/4 of 791) = 169 animals to blister. The data in Table 4 suggests that \textit{h199} contributes very little to the blistered phenotype in heteroallelic animals.

Males of the genotype \textit{h199/e937} appeared to blister with complete, or near complete penetrance (58/62 males blistered, although some of these non-Bli males may have carried \textit{sDp2}). Similarly, \textit{e937} homozygous males were always observed to blister in an isogenic mating strain even though only about 85% of the hermaphrodites blistered in the same population.
Table 4.
Progeny from 5 h199/e937 hermaphrodites scored.

<table>
<thead>
<tr>
<th>Blistered</th>
<th>Wild Type</th>
<th>Dead(egg)</th>
<th>Dead(larva)</th>
<th>Dpy</th>
<th>Unc</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>192</td>
<td>428</td>
<td>150</td>
<td>17</td>
<td>4</td>
<td>0</td>
<td>791</td>
</tr>
</tbody>
</table>

*dpy-5 bli-4(h199) unc-13; sDp2* hermaphrodites were crossed to *e937/e937* males, resulting in non-Unc outcross progeny (F1) of the genotype: *dpy-5 bli-4(h199) unc-13/+ e937 +*, or, *dpy-5 bli-4(h199) unc-13/+ e937 +; sDp2*. 7 non-Unc F1s (two of which were also blistered) were plated on individual plates to score the F2 progeny. 2/7 F1s were observed to give many Unc F2s. This indicated the presence of *sDp2* in those parent F1s. These two parents' progeny were not included in the summary.
SECTION II. The 3' region of the bli-4 gene.

A. Sequence determination of pCeh207

pCeh207 is a plasmid clone containing the 3' exons of the bli-4 transcripts, blisterase B, C, and D (Peters, 1992). This clone was restriction mapped and subcloned into smaller fragments for dsDNA sequencing (Figure 18). This sequence information allowed the construction of primers within intronic sequences and also established the genomic arrangement of these 3' exons. A summary of the regions sequenced is shown in Figure 18B. Much of the sequencing was performed on one strand since the coding sequence for this region had been previously determined by cDNA analysis (Peters, 1992). Furthermore, the construction of primer pairs (shown in Figure 20) for PCR was rapidly accomplished and employed for other purposes (see below) including the confirmation of sequence information for this region.

B. Searching for the s90 lesion in the 3'-specific exons of bli-4

Primer pairs constructed from sequence obtained with pCeh207 subclones were applied to amplify genomic DNA from arrested s90 larvae. A summary of the sequenced regions as well as the overlapping investigative sequencing by Colin Thacker is shown in Figure 20. The procedure for cloning and sequencing PCR products for this purpose is described in Materials and Methods. To date, the molecular lesion
A) The initial status of 3'exons (Peters, 1992). cDNA sequence was known (boxes) but genomic gaps prevented placement of intron/exon boundaries. Genomic sequence is represented by horizontal lines through the exons, which are numbered below.

B) Restriction map and sequencing strategy of pCeh207. The restriction map for pCeh207 was determined. This allowed the placement of genomic sequence gaps (approximate size of each given) and provided information to produce subclones of pCeh207 for dsDNA sequencing. Each arrow represents direction and length of sequence obtained from subclones (lighter arrow represents sequence obtained from Colin Thacker). The polylinker of the plasmid is shown as an angled line at each end.

C) The complete genomic arrangement of 3' exons in pCeh207. Exon 18 was placed as a result of the identification of a fourth blisterase transcript (Thacker et al., 1995).

Figure 18. Establishing the genomic arrangement of 3' exons in pCeh207.
Figure 19. Location of primers designed to amplify the blisterase B, C, and D 3' exons.

PCR fragments of the blisterase B, C, and D specific 3' exons were amplified using combinations of sense primers (drawn above the gene structure) with antisense primers (drawn below the gene structure). KRp39 anneals within exon 18 but was designed previous to the discovery of the transcript to which this exon belongs (Thacker, et al., 1995). The predicted 3'UTRs for blisterases B, C, and D are shown in white.
Figure 20. Sequencing of 3' exons in s90 homozygotes.

The entire sequence of pCeh207 plus sequence extending 5' of pCeh207, to include exon 14 is shown. PCR amplified fragments from s90 arrested larvae that were cloned and sequenced are shown with underlines. Single underlines represent *Pfu* polymerase-based PCR reactions, performed by Colin Thacker; double underlines represent *Taq* polymerase-based PCR reactions and repeated *Pfu* polymerase-based PCR reactions. No sequence differences between s90 and N2 have been detected in the regions tested. Lower case indicates introns, upper case indicates exons. The exon positions are as follows: exon 14 (139-425), exon 15 (721-793), exon 16 (1646-1824), exon 17 (1875-1983), exon 18 (2076-2209), exon 19 (2608-2835), exon 20 (2889-3092), and exon 21 (3143-3476).
tttttagctaatatttttttatgtttctataagttttactgaaaagatg
aatttcgcacgtcaatatcaagatccccctgcaaatattttattttgca
attaaatattttcatgttcttaattgtactGTTGATGAGGTGTTGAAAGA
ATTGAAATCATTTGGAAGTGACATTAGAAGAGAGTGTCACATTTGGAATTG
GGAGCATGCTGTAACATATAACATATTACAAAGAGAATGGAATCTTCTCTC
GTACCCTAGTTTGTATTACTCTTTCAACCAATACCGATTTTCTTTG
ATTATTCTTTCTCTTATTGATGCCATCGCCAAATTTCCGCGTTTGT
AGACTATATGAAATCATTGGGTGCTgtgaataatcgttctttcaattgtq
tcccatcaaaattttcagctttcctcatcagatctccgtaataccccaaattt
gtgcctcatattatatgtgtacacgctgtgtgtgtgtgtgtgtgt
ctattgtactatatgtcaattttctcaaatatcaaaaaattttttttt
tttccgtcatcagaaacaattaacccgattttcttatatttacaataattttcaaaaaagtagtqagaqgatagtgaggtacgcagacacacaacacacaccac
ctattgtcctattttttgtcaatattgtaacacgcagattatattac
qtqagatcgtqtttttacagGTGGAAGAGTCTGCTCCTGACATTTTCCA
GATTGAGCTGGCTGGAATTGCTATGATGATGGACTGAGGgtgagga
atqqatccccctgtactataaatgtttaaaagatattttctgttttttt
tcagaaaaattttcaaaaggcaaaaaattaacattgcagaaaatttc
ctgagaataattgtcagatgcgaattttgcatattgtcgaaaaaata
cggtagccccgtctcgacacggccgatttttttcatgcaaaagagagtgccg
ccttaagggaatactgtgttctccacatttttcgccgttgctgtttttct
tctatattttcaatttttcactgtattccccaaatcattgaagaatgtgt
tctatattgcatcatttttcactacaaatcattcaggcagagtaacat
ggaaaaatctataaaaaatggactacagtactgttttgctcgtagtcc
agtggattttttatatagatatatcagaactacagtttggactctacaag
gatttattatagacacacttagtaacactaagcagaagcagaacacta
taatagcagactgtcggagaattcctcccaaggttgccaatatgtctct
tgtttttgttagcactgactgtgtgtttcccttgagcagttgtgtagatg
aatctgcgtcgtgttgtagcgcctcatgtagcatctatatttgtcaaat
atatagataaatataagttcgtgaataatcgggacacctgctctcattgttat
gcgcagactactctcaatgttaaaacttttgcgtttaaatattgggttg
agtctgatatcctgtactgttatttttgattaaatctgaaatcagaatagc
acatagacatatcattttctgtaatatttttcatattttacagGTGCC
ACAGAATCGACGTCAACACATCATGTTTCCGGATAACACATTAACTCA
AATTCCTTCAAAATAAAGGAGGAAGTGGGATTCAAGTGGTGGTCCCCAAATGCG
ATGATACTATTACTGGTAGTGACAGTGTAAAAATGTGCTCATCCCCAT
responsible for the s90 mutant phenotype has not been discovered. However, the search has not yet included the exons common to all isoforms.

SECTION III. The structure of the bli-4 gene.

The results obtained for the 5' and 3' regions of bli-4 allowed the construction of the molecular structure map of the gene shown in Figure 21. This schematic representation also serves as an indicator of the progress made towards characterizing the bli-4 gene during the course of this study by comparison to the former structure status depicted in Figure 2. The high resolution map for the bli-4 gene enabled the construction of subclones encoding subsets of blisterase isoforms, as discussed below.

SECTION IV. Transformation rescue experiments with subsets of bli-4 coding information.

In order to test for rescue by subclones of bli-4, it was necessary to first establish the 5' promoter region of the gene. Although defining the realm of the promoter is not required at this level of analysis, it is important to include a sufficient amount of 5' sequences to ensure expression of the exogenous gene. This was accomplished by fusing the putative promoter region of bli-4 and a small amount of bli-4 coding sequences with the lacZ coding sequences included in the expression vector pPD21.28 (Fire, et al.,
pH-4 is trans-spliced to SL1 as shown. 5' and 3' untranslated sequences (UTRs) are shown as white boxes.

The structure of the pH-4 gene is shown. The gene encodes at least four transcripts that arise via differential splicing of the first twelve exons onto different downstream 3' exons or sets of exons. The predicted products of pH-4 have been termed bistrasense A, B, C, and D. The molecular lesions responsible for pH199 explain the absence of bistrasense A. The bistrasense C, B, and D have been termed bistrasense C, B, and A. The molecular lesions responsible for pH199 explain the absence of bistrasense A. The bistrasense C, B, and D have been termed bistrasense C, B, and A.
Sequence extending from the XbaI site approximately 5 kb upstream of bli-4 exon 1, to the ClaI site in exon 2 was found to express β-galactosidase (β-gal) in hypodermal cells, the ventral nerve cords, and vulval cells (Thacker, et al., 1995). Expression was first observed at the two-fold stage of embryonic development, the stage at which most class II lethal homozygotes arrest. A second lacZ fusion was made with 5' putative promoter sequences starting at the XhoI site just 700 bp upstream of exon 1 to the ClaI site in exon 2. This construct did not express β-gal in any tissue. Therefore, the XbaI (5 kb) 5' upstream sequence was included in all minigenes used in this study. Because this is a fairly large amount of sequence, (certainly many C. elegans genes are 5 kb or smaller) it was necessary to ensure that another gene in this region was not responsible for rescue of bli-4 phenotypes. pCeh238 did not rescue the lethal mutants q508, h199, or s90, and pCeh239 did not rescue blistering (see below). Both of these constructs carry the identical upstream promoter sequences. This argues against another gene existing in this upstream region that is responsible for the rescues (see below).

Minigenes derived from coding sequences of bli-4 were tested for their capacity to rescue each class of mutant bli-4 phenotype. The alleles e937 (class I), q508 and h199 (class II) and s90 (class III) were tested against each minigene constructed, as summarized in Table 5. For simplicity, e937 homozygous hermaphrodites were injected with the subclones (see Materials and Methods) and rescue of blistering was assessed from stable lines. The arrays were then crossed into lethal bearing strains mentioned above (Figure 12). All minigenes except for pCeh238, and pCeh239 rescued all three allelic classes of bli-4 mutations; this data is summarized in Figure 22. The dominant marker, rol-6(su1006), did not rescue lethality (since this marker was present in arrays that do not rescue) and did not significantly contribute
to reduction of blistering when present alone (Table 7). Rescued homozygotes for each lethal allele were maintained and given strain designations (Table 6). All stable rescued lines gave some dead progeny due to less than 100% transmission of the extrachromosomal array. After many generations, rescued lines flanked by markers epistatic to rol-6 (e.g. dpy-5) still gave Rol progeny when outcrossed to N2 males, suggesting selective maintenance of the extrachromosomal array in these rescued lines, as expected. pCeh221 and pCeh230 were observed to poorly rescue both hl99 and s90, as evidenced by very few progeny recovered in each generation. Death of most animals was approximately late L2 - early L3 larval stage for each of the strains. Since this is later than the typical arrest stage for these two alleles alone, it is likely that bli-4 activity is reduced, but not absent, in these animals. Due to their severely reduced viability, these strains were lost before a population could be archived at -70°C.

A. Subclones encoding blisterase A rescued blistering and lethality

Subclones pCeh226, pCeh229, and pCeh230 were injected into CB937 (e937/e937) hermaphrodites. Stable transgenics were obtained for each of the constructs injected (Table 6). Rol animals were never observed to blister in any of the transgenic strains. Upon outcrossing to the lethal (q508, h199, s90) bearing strains, each construct was found to rescue both classes of bli-4 lethal alleles, however, as mentioned previously, pCeh230 transgenic lines had reduced viability and were lost for h199 and s90 alleles. A summary of rescue experiments is provided in Table 5 and Figure 22.
Table 5.
Summary of rescue results for subclones of bli-4.

<table>
<thead>
<tr>
<th></th>
<th>ClassI</th>
<th>ClassII</th>
<th>ClassIII</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>e937</td>
<td>q508</td>
<td>h199</td>
</tr>
<tr>
<td>pCeh226</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>pCeh229</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>pCeh230</td>
<td>+</td>
<td>+</td>
<td>+a</td>
</tr>
<tr>
<td>pCeh238</td>
<td>+/-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>pCeh236</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>pCeh221</td>
<td>+</td>
<td>+</td>
<td>+a</td>
</tr>
<tr>
<td>pCeh239</td>
<td>-</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

*a* animals survived but exhibited very poor viability and fecundity; strains were lost as a result.

 +/- : frequency of blistering is reduced, but not eliminated.

n.d. : not done
Table 6.

Strains constructed for transgenic rescue.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Method</th>
<th>Genotype</th>
<th>Plasmid</th>
</tr>
</thead>
<tbody>
<tr>
<td>KR2859</td>
<td>injection</td>
<td>bli-4(e937);hEx54</td>
<td>pCeh226</td>
</tr>
<tr>
<td>KR2860</td>
<td>cross</td>
<td>dpy-5(e61) bli-4(q508);hEx54</td>
<td>pCeh226</td>
</tr>
<tr>
<td>KR2861</td>
<td>cross</td>
<td>dpy-5(e61) bli-4(s90) unc-13(e450);hEx54</td>
<td>pCeh226</td>
</tr>
<tr>
<td>BA5h199</td>
<td>cross</td>
<td>dpy-5(e61) bli-4(h199) unc-13(e450);hEx54</td>
<td>pCeh226</td>
</tr>
<tr>
<td>KR2862</td>
<td>injection</td>
<td>bli-4(e937);hEx55</td>
<td>pCeh229</td>
</tr>
<tr>
<td>KR2863</td>
<td>cross</td>
<td>dpy-5(e61) bli-4(q508);hEx55</td>
<td>pCeh229</td>
</tr>
<tr>
<td>KR2864</td>
<td>cross</td>
<td>dpy-5(e61) bli-4(s90) unc-13(e450);hEx55</td>
<td>pCeh229</td>
</tr>
<tr>
<td>SalRLh199</td>
<td>cross</td>
<td>dpy-5(e61) bli-4(h199) unc-13(e450);hEx55</td>
<td>pCeh229</td>
</tr>
<tr>
<td>KR2865</td>
<td>injection</td>
<td>bli-4(e937);hEx56</td>
<td>pCeh230</td>
</tr>
<tr>
<td>KR3028</td>
<td>cross</td>
<td>dpy-5(e61) bli-4(q508);hEx56</td>
<td>pCeh230</td>
</tr>
<tr>
<td>KR2868</td>
<td>injection</td>
<td>bli-4(e937);hEx46</td>
<td>pCeh238</td>
</tr>
<tr>
<td>KR2869</td>
<td>injection</td>
<td>bli-4(e937);hEx47</td>
<td>pCeh238</td>
</tr>
<tr>
<td>KR2870</td>
<td>injection</td>
<td>bli-4(e937);hEx48</td>
<td>pCeh238</td>
</tr>
<tr>
<td>KR2871</td>
<td>injection</td>
<td>bli-4(e937);hEx49</td>
<td>pCeh238</td>
</tr>
<tr>
<td>KR2998</td>
<td>injection</td>
<td>bli-4(e937);hEx58</td>
<td>pCeh236</td>
</tr>
<tr>
<td>KR2999</td>
<td>cross</td>
<td>dpy-5(e61) bli-4(q508);hEx58</td>
<td>pCeh236</td>
</tr>
<tr>
<td>KR3001</td>
<td>cross</td>
<td>dpy-5(e61) bli-4(s90) unc-13(e450);hEx58</td>
<td>pCeh236</td>
</tr>
<tr>
<td>KR3000</td>
<td>cross</td>
<td>dpy-5(e61) bli-4(h199) unc-13(e450);hEx58</td>
<td>pCeh236</td>
</tr>
<tr>
<td>KR3006</td>
<td>injection</td>
<td>bli-4(e937);hEx59</td>
<td>pCeh221</td>
</tr>
<tr>
<td>KR3014</td>
<td>cross</td>
<td>dpy-5(e61) bli-4(q508);hEx59</td>
<td>pCeh221</td>
</tr>
<tr>
<td>KR3003</td>
<td>injection</td>
<td>bli-4(e937);hEx62</td>
<td>pCeh181</td>
</tr>
<tr>
<td>KR3002</td>
<td>injection</td>
<td>N2;hEx61</td>
<td>pCeh239</td>
</tr>
</tbody>
</table>
B. A subclone encoding blisterase B partially rescued blistering, but not lethality

The minigene pCeh238 was injected into CB937 (e937/e937) animals. Four stable Rol lines were established and each was assayed for penetrance of blistering. pCeh238 was found to only partially rescue blistering as an extrachromosomal array; in each of the four lines, some Rol progeny did blister. After outcrossing pCeh238 to lethals q508, h199, and s90, no rescue was evident, indicating that blisterase B was unable to supply sufficient function to rescue lethality but it did reduce the penetrance of blistering. Table 7 presents a summary of the results obtained for the partial rescue of blistering by pCeh238.

C. A subclone encoding blisterases B, C, and D rescued blistering and lethality

pCeh236 was injected into CB937 (e937/e937) animals and a stable line was recovered. Rol animals from this strain were never observed to blister, indicating complete rescue of blistering by pCeh236. This construct was also found to rescue q508, h199, and s90. A summary of rescue results for pCeh236 is shown in Table 5 and Figure 22.

D. Transmission frequency of rescuing subclones verses proportion of rescued animals.

In order to better assess the rescuing capacity of a given isoform, data for every rescue experiment was compiled. It was suspected, for instance, that the reason for loss of some of the transgenic lines that carried pCeh230
Table 7.
Partial rescue of blistering by pCeh238

<table>
<thead>
<tr>
<th>DNA injected in CB937</th>
<th>Stable line</th>
<th>% Blistering of Rollers</th>
<th>Bli,Rol / total Rol</th>
</tr>
</thead>
<tbody>
<tr>
<td>rol-6</td>
<td>KR2872</td>
<td>79</td>
<td>110/139</td>
</tr>
<tr>
<td>pCeh238 + rol-6</td>
<td>KR2868</td>
<td>43</td>
<td>79/185</td>
</tr>
<tr>
<td>pCeh238 + rol-6</td>
<td>KR2870</td>
<td>17</td>
<td>17/101</td>
</tr>
<tr>
<td>pCeh238 + rol-6</td>
<td>KR2869</td>
<td>8</td>
<td>41/523</td>
</tr>
<tr>
<td>pCeh238 + rol-6</td>
<td>KR2871</td>
<td>3</td>
<td>7/206</td>
</tr>
<tr>
<td>pCeh226 + rol-6</td>
<td>KR2859</td>
<td>0</td>
<td>0/ &gt;1000</td>
</tr>
</tbody>
</table>

Four independent lines were recovered from a single round of injection experiments. A few animals from each line were picked and their progeny scored for penetrance of blistering among Rol progeny (see Materials and Methods). rol-6 marker was introduced with pRF4 (Mello, et al., 1991).
and pCeh221 was due to very weak rescuing capacity by these constructs. The frequency of Rol animals was calculated for each line, as was the percentage of rescued animals (evidenced by the flanking marker(s) phenotype) from the total number of homozygotes for the lethal allele. A comparison between the frequency of transmission of the array [Rol/(WT + Rol)] and frequency of rescue [Rescued/(Rescued + dead)] provides an indication of the rescuing ability of a given array. Data from transgenic rescue experiments is summarized in Table 8 and a graphical representation of relative rescuing ability of each construct is presented in Figure 23.
Table 8.
Progeny scored from transgenic lethal rescue experiments

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>Rol</th>
<th>Dpy</th>
<th>Unc</th>
<th>Dpy, Unc</th>
<th>dead egg</th>
<th>dead L1</th>
<th>total</th>
<th>% Rol</th>
<th>% Rescue</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>q508</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pCeh226</td>
<td>748</td>
<td>165</td>
<td>40</td>
<td></td>
<td>131</td>
<td>33</td>
<td>1117</td>
<td>18</td>
<td>20</td>
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Progeny from parents of the genotype *dpy-5 bli-4(q508)/bli-4(e937);hEx"n" and *dpy-5 bli-4(h199) unc-13/bli-4(e937);hEx"n", and *dpy-5 bli-4(s90) unc-13/bli-4(e937);hEx"n", were scored for transgenic rescue; n = one of the bli-4 subclones presented in Figure 23. For simplicity, blistering was not scored. WT is wild-type; %Rol was calculated as Rol/(WT + Rol); %Rescue was calculated as Dpy/(Dpy + dead egg + dead L1) for q508, and as Dpy,Unc/(Dpy,Unc + dead egg + dead L1) for h199 and s90.
Figure 22. Summary of results for transgenic rescue using subclones of bli-4.

A) The structure of bli-4 (see Figure 21 for description)

B) The bli-4 minigenes, pCeh226, pCeh229, pCeh230, pCeh238, pCeh236, pCeh221, and pCeh239 were tested for rescue of blistering (Bli) by injecting into e937 homozygous hermaphrodites. Each of the constructs was then crossed into h199, q508, and s90 strains (see Materials and Methods) to test for rescue of lethality (Let). Thin lines indicate genomic sequence gaps in plasmid constructs, compared to endogenous sequence. Construction details are provided in Figures 6-11.

a strains for h199 and s90 were not able to be maintained with these constructs

+/− frequency of blistering is reduced but not eliminated

nd not done
Figure 23.
The relative rescuing ability of different subclones of bli-4.

The subclones that rescued the lethal alleles q508, h199, and s90 were graphed according to % animals that Rol (Rol/Rol + Wild-type) and % animals rescued (e.g. DpyUnc/DpyUnc + dead animals) shown along left margin. Data derived from Table 8. The Blistered phenotype was not scored, any Bli progeny were scored as wild-type; no BliRol were observed. Error bars represent 95% confidence interval calculated by the formula: $100 \times p \pm 1.96(p(1-p)/n)^{1/2}$; where $p = \%$Rol/100 or $\%$Rescue/100 for each strain and $n =$ total number of animals used to calculate $\%$Rol or $\%$Rescue respectively.
Discussion

*bli-4* is trans-spliced to SL1

Previous examination of *bli-4* cDNAs predicted that the 5' end of the transcript (exons 1 to 12) is common to all four isoforms (Peters, 1992; Thacker, *et al.*, 1995). The cDNA with the most complete 5' sequence identified (pCeh197), contains 176 nucleotides of untranslated sequence (5' UTR), suggesting that this cDNA represents a complete, or nearly complete message at the 5' end (Peters, 1992). However, the 5'-most sequence of the processed transcript cannot be confirmed on this information alone. In order to determine the 5' end of the *bli-4* RNA, an experiment was conducted to determine if the RNA is *trans*-spliced to the leader sequences SL1 or SL2.

Approximately seventy-percent of *C. elegans* transcripts are *trans*-spliced to leader sequences SL1 or SL2 (Spieth, *et al.*, 1993). Although the molecular function of such leader sequences is not known, a deletion that removes many copies of the SL1 gene results in embryonic lethality, suggesting an essential component of at least some genes' expression (Kimberly Ferguson, Paul Heid, and Joel Rothman, pers. comm.). A benefit of the *trans*-splicing phenomenon to *C. elegans* researchers is that it enables rapid determination of the 5' most sequence of processed transcripts for genes *trans*-spliced to either of these leader sequences. The general procedure to
detect trans-splicing to either SL1 or SL2 leaders utilizes RT-PCR on poly A+ RNA with primers that are situated in the leader sequence and within the 5' portion of the gene of interest. This approach led to the discovery that bli-4 is trans-spliced to SL1 and established the 5'-most sequence of the processed bli-4 transcripts, which was one nucleotide longer than pCeh197 (Figure 14). Although it is possible that not all transcripts of bli-4 receive the SL1 leader, transcript-specific differential trans-splicing has never been reported for other genes whose products undergo alternative splicing. RT-PCR experiments to test this possibility would necessitate the use of antisense primers located in the distant 3' specific exons for each of the isoforms. This would involve amplification of sequences approximately 3 kb in length, or longer. Considering the relatively high level of background amplification often observed with the SL1 and SL2 primers, this task may not be particularly easy.

**Mapping mutations in the 5' region of bli-4**

In this thesis, the technique of PCR-based heteroduplex detection was applied to five class II alleles of bli-4 to search for the molecular lesions responsible for each mutation. Based on intracomplementation analysis of bli-4, class II lethal alleles are expected to interfere with the expression and/or function of all isoforms. Therefore, the initial search for class II mutations began in exons 1 to 12, the coding information that all isoforms share. Heteroduplex technique was employed for this task. By using MDE™, an acrylamide-based matrix through which DNA fragments are electrophoresed, one can rapidly detect single base pair alterations with relatively high resolution (Keen, et al., 1991). This is an important consideration, since
most of the alleles of bli-4 were induced with EMS, which is known to preferentially cause single base pair alterations, most of which are G:C to A:T transitions. Also, bli-4 spans a relatively large genomic region (approximately 15 kb); considerable time and expense would be required to sequence the entire region for each mutation. According to the manufacturers, the resolving power of the heteroduplex technique with the MDE™ product is dependent on three main variables, the size of the DNA fragment (mismatch detection has been observed for a 900 bp fragment, but 400 bp is optimal), the location of the mismatch in the DNA fragment (a mismatch in the centre of the fragment is optimal), and the type of mismatch (a G:G mismatch is optimal). Based on the size criterion, exons 11 and 12 were excluded from the analysis, since the only primers available for this region amplify a product of 1011 bp.

PCR primer pairs (KRp34/KRp35 and KRp36/KRp37) amplify the protease domain of bli-4 in two overlapping products of sizes 816 bp and 755 bp (Figure 16). Both products were found to smear slightly in the gel matrix. No polymorphisms were apparent in any of the class II mutants analyzed in the protease domain. However, smearing of the DNA bands, combined with the relatively large product size, reduces the confidence of interpreting these results as evidence for the absence of mutations. Therefore, four additional primers that further divide this region into smaller products were designed for future analysis (Figure 15B). These primers have been tested and found to work well with the previous four, but have not been applied to search for mutations at this time.
The \( h199 \) mutation was induced with EMS and recovered in a screen to identify lethal alleles balanced by the free duplication \( sDp2 \) (Howell, 1989). Complementation analysis revealed that \( h199 \) fails to complement the blistered phenotype of \( bli-4(e937) \), indicating that it is an allele of the \( bli-4 \) gene. Subsequent mapping data obtained by Howell (1989) and Peters et al., (1991) suggested that the original \( h199 \) mutation (in strain KR513) was linked to a second lethal mutation towards the left end of LGI, near \( unc-40 \). However, recent mapping of \( h199 \) in KR513 indicated that the second mutation no longer exists (mapping data obtained from R. Johnsen presented in Appendix 2). Furthermore, subclones of \( bli-4 \) rescued the \( h199 \) mutation (Table 8, and Figure 22), which would not be expected if a second, linked lethal mutation was present in the strain. A new strain designation (KR2997) has been assigned to distinguish this strain from KR513.

I have mapped the \( h199 \) lesion by heteroduplex mismatch detection and have sequenced the region of DNA containing the molecular lesion from \( h199 \) homozygotes. An A:T to T:A transversion was found in three independent \( h199 \) clones from PCR products of exons 3 to 5 (Figure 17). Although EMS most often induces G:C to A:T transitions (Griffiths, et al., 1993), A:T to T:A transversions resulting from EMS exposure have been reported in \( C. elegans \) (Perry, et al., 1994).

The transversion mutation results in an amino acid substitution of His127 (\( CAT \)) to Leu (\( CTT \)). This alteration is in the amino terminus of the common region of predicted \( bli-4 \) isoforms, proximal to the protease domain. The function of this region of the predicted \( bli-4 \) proteins is unknown and not well conserved among proprotein convertase family members; \( h199 \) may
reveal a previously unrecognized functional component specific to bli-4 convertases. However, one cannot rule out the possibility that a common function exists for this region among all family members, in which case this mutation may provide novel insights into the function of this region in other proprotein convertases.

**h199/e937 hermaphrodites rarely blister**

Throughout the course of this work, it has been observed that e937/q508 and e937/h670 worms blister with 100% penetrance and heightened expressivity, when compared to e937/e937 worms. For instance, non-Unc progeny from the parent unc-63 bli-4(h670) unc-13/let(h661) bli-4(e937); hT2 were always blistered; no wild-type progeny were ever observed. hT2 is a translocation between LGI and LGIII (McKim, et al., 1992) that carries the e937 mutation and has been used to identify class II alleles by precomplementation screens (Peters, et al., 1991; Thacker, Srayko, Rose, unpublished). e937 translocation homozygotes do not survive in this strain due to the existence of a linked lethal mutation (h661). Therefore, any blistered animal in the above strain is a result of e937/h670. The absence of wild-type animals indicates 100% penetrance of the blistered phenotype when e937 is in heteroallelic combination with class II alleles such as h670. q508/e937, h670/e937, and h1010/e937 animals have been observed to blister with complete penetrance when obtained from crossing e937 males to hermaphrodites that carry the lethal class II alleles (C. Thacker, M. Srayko, unpublished observations). Also noted with these strains was that blistering was very severe in heteroallelic animals; for instance, a large blister usually
enveloped the head soon after the fourth larval molt, rapidly extending down the length of the worm, resulting in reduced viability. Although a rigorous quantification of this phenomenon was not performed, \( h42, h791, h384, \) and \( h254, \) also appeared to behave as \( q508, h670, \) and \( h1010 \) when in heteroallelic combination with \( e937. \) As presented in this thesis, \( h199/e937 \) hermaphrodites rarely blistered (Table 4). This data suggests that the basis for lethality in \( h199 \) may be somewhat different than the other class II alleles.

Class II alleles are thought to affect the expression and/or function of all products produced by the \( bli-4 \) gene; consistent with this hypothesis, three class II alleles have been mapped to the region shared by all transcripts of \( bli-4 \) (Figure 2). Based on Northern and RT-PCR analysis, \( e937 \) abrogates expression of blisterase A, but does not eliminate the expression of blisterases B, C, and D (Thacker, et al., 1995). One possible explanation for complete penetrance of blistering in \( e937/class \) II worms is that there is one less copy of a gene capable of producing blisterase B, C, and D in \( e937/class \) II worms than in \( e937/e937 \) worms. If slight functional redundancy exists between some of the isoforms of this gene, one might predict an overall reduction in activity of the \( bli-4 \) gene in \( e937/class \) II animals, when compared to \( e937/e937 \) animals. By similar reasoning, it appears that \( h199 \) does not reduce the activity of \( bli-4 \) as much as these aforementioned class II alleles because \( e937/h199 \) worms blister less often than \( e937/e937 \) worms. However, this weak intracomplementing ability is not paralleled by a weakness in lethality; \( h199 \) homozygotes arrest with the same phenotype as other class II alleles examined and the penetrance of lethality appears complete (data not shown).

It is evident from the intracomplementation data that \( h199 \) does not behave like these other class II alleles. The fact that \( h199 \) appears to be a somewhat weaker class II allele is consistent with the nature of the lesion
responsible for this mutation. A missense mutation could give rise to a protein with partial function. Perhaps the h199 mutation affects the function of the predicted essential bli-4 product(s) more severely than the predicted non-essential bli-4 product(s) implicated in the adult cuticular function. If true, this would be a particularly informative mutation, considering it is located in a region common to all predicted BLI-4 isoforms.

A further complication is that males of the genotype h199/e937 do seem to blister with complete, or near complete penetrance (see Results, p49). However, it is possible that the male morphology is more susceptible to blistering and therefore, responsible for the differing penetrance of blistering between the two sexes of h199/e937 animals. For instance, e937 homozygous males are always observed to blister in an isogenic mating strain even though only about 85% of the hermaphrodites blister in the same population (C. Thacker, M. Srayko; unpublished observations). This argues against an extragenic basis for the difference, such as an X-linked suppressor of blistering. Another possible explanation is that bli-4 expression is different between males and hermaphrodites; a sex-specific Northern blot could be performed to test for this.

pCeh250 is a minigene that contains the h199 mutation and encodes only the blisterase A isoform (similar to pCeh229). This minigene may be used in the future to assay the rescuing capability of a blisterase A isoform that carries the missense mutation. This experiment will provide information about the effect of the mutation on the function of the predicted proteins. For instance, pCeh250 may rescue blistering completely, since h199 seems to not affect this function as much as other class II alleles in e937/class II animals. However, one would not expect pCeh250 to rescue lethality since there is no evidence that h199 is weak with respect to that phenotype.
addition, an epitope tag could be added to the h199 exogeneous construct and a transgenic animal producing this flagged protein could be used in immunolocalization studies to determine if the mutation affects intracellular trafficking, such as exit from the endoplasmic reticulum. It has been observed, for example, that pro-furin containing a defective kex2/subtilisin-like cleavage motif does not exit the endoplasmic reticulum (Molloy et al., 1994). Similarly, the h199 proteins may not be properly processed; this could be detected by standard Western blot analysis on protein isolated from transgenic worms. The His to Leu change is 11 amino acids carboxyl to one of three potential kex2/subtilisin-like cleavage motifs (R-V-K-R) present in the common region of predicted bli-4 isoforms.

**Searching for the s90 lesion in the 3'-specific exons of bli-4**

The sequence determination of pCeh207 not only established the molecular structure of the 3' end of bli-4, but also allowed the design of primers for mutation searching in this region of the gene. These primers were employed to begin testing a hypothesis originally proposed by Peters (1992), suggesting that the class III mutation, s90, affects isoform(s) other than blisterase A (the transcript that is eliminated in e937 homozygotes). This hypothesis was based on intracomplementation analysis, which indicated that s90 complemented the blistered phenotype of e937; e937/s90 worms were wild-type in appearance. This complementation result would normally suggest that these two mutations affect different genes, however, at least three pieces of evidence support the hypothesis that s90 is an allele of bli-4: 1) s90 failed to complement all class II alleles (Peters, 1992; Thacker, et
al., 1995); 2) the allele mapped to the bli-4 region genetically (Peters, et al., 1991); and 3) as shown in this thesis, s90 was rescued by many subclones of the bli-4 gene. Therefore, given that s90 is a bli-4 allele, one might predict that the s90 lesion will be found within a region that affects the expression and/or function of blisterase B, C, D, or some combination thereof. One possible location for such a mutation would be the 3'-specific exons for these isoforms.

Sequencing of exons in the 3' region of bli-4 was initiated by subcloning PCR fragments obtained with Taq polymerase. After sequencing through both mutant and wild-type clones for exons 16, 19 and 20, it was decided that Pfu polymerase should be used to generate the amplified DNA from s90 homozygotes, especially after discovering a second nucleotide alteration in the h199 clone pCeh240, which was likely attributable to Taq polymerase (Figure 17). However, no mutations were detected in any of the regions in the s90 clones amplified with Taq polymerase. Subsequent cloning and sequencing of Pfu-generated PCR fragments was performed by Colin Thacker. A summary of some regions sequenced is given in Figure 21. To date, all 3'-specific exons for each of the four transcripts, except 94 nucleotides at the end of exon 18 have been sequenced from s90 homozygotes (C. Thacker, pers. comm.).

The absence of the s90 mutation in a 3'-specific exon would promote a revision of the current hypothesis explaining the intragenic complementation between s90 and e937. However, a mutation that specifically affects a subset of the products produced by bli-4 does not have to reside in a coding region of bli-4. For instance, a mutation in a cis-acting regulatory element that directs expression of the gene in early development, could result in the lethal phenotype associated with s90. If the same allele
allowed normal expression of the gene at the adult stage, the blistering phenotype of e937 could be complemented. Another way to affect a subset of bli-4 products without mapping in the 3' exons is if s90 resides in one of the unsequenced introns of the 3' region, such that the post-transcriptional processing of specific transcripts is affected. One example of an alteration implicated in this phenomenon in C. elegans is the intragenic revertant of unc-52(e669), su250 (Rogalski, et al., 1995). su250 is a single nucleotide alteration that resides in the centre of intron 16 of the unc-52 gene, and reverts the paralyzed phenotype of the e669 mutation (which is a point mutation in exon 17).

The s90 mutation may also reside in the region common to all isoforms. In fact, the data from h199/e937 animals (Table 4) suggests that at least some distinction between the adult cuticular function and the early developmental function can be separated by a mutation in the common region. Complementation of blistering in s90/e937 may be a severe example of the same mechanism responsible for the reduction in blistering observed in h199/e937 hermaphrodites.

The structure of the bli-4 gene

Our understanding of the function of the predicted kex2/subtilisin-like proprotein convertases encoded by the bli-4 gene relies on a thorough understanding of the molecular structure of the gene. Most of the genomic DNA for the bli-4 gene has now been sequenced and a high resolution restriction map is available. Future work on bli-4 will likely include
completing the sequencing of the gene, in particular, the ~6 kb intron separating exons 13 and 14.

**Transformation rescue experiments with subsets of bli-4 coding information**

Genetic evidence, combined with mapping data for molecular lesions of bli-4 suggest that at least some of the predicted isoforms of this gene perform distinct functions. In an attempt to further elucidate the relationship between the structure and function of the gene, subclones of bli-4 were assessed for transgenic rescuing ability against four alleles, representing each class of bli-4 mutant phenotype. The preliminary results indicated that the isoforms of bli-4 are sufficiently similar to provide overlapping function: exogenous blisterase A rescued lethality as well as blistering, and exogenous blisterases B, C, and D rescued blistering and lethality when expressed from the same construct. This latter result was not expected, since e937 homozygotes do not produce the blisterase A transcript, but do make blisterase B, C, and D transcripts (Thacker, et al., 1995). This apparent contradiction in the predicted functional roles of the blisterases is perhaps due to aberrant expression of the exogenous gene, rather than functional equality. In support of this reasoning, pCeh221 rescued blistering and lethality even though it only encodes the first 12 exons of the gene and therefore, cannot make a protein identical to any of the predicted endogenous products. Also, incomplete penetrance of blistering in e937 homozygotes suggests that endogenous levels of the non-blisterase A isoforms can rescue blistering in a small percentage of animals.
Transgenic rescue is a widely used method of determining the smallest fragment of DNA that is capable of reverting a mutant phenotype. This is a convenient way to determine the physical location of a gene and, in some instances, the regulatory elements necessary for its expression. However, gene expression and control of many exogenous copies present in the extrachromosomal array is a poorly understood process. Rescue data derived from transgenic experiments involving a well characterized gene is sometimes contradictory to expectations. For instance, mutant rescue with exogenous constructs that do not encode the complete gene (like pCeh221) are not uncommon (e.g., Bargmann, et al., 1995; Babity, 1992). For this reason, the conclusions made from transgenic rescue experiments are often limited to "positive" or "negative", especially when dealing with lethal mutations. In general, the presence of a homozygous marker that flanks the lethal mutation in the F1 progeny from a heterozygote carrying the array is sufficient to conclude that the array rescues lethality.

In this thesis, however, I have scored all of the progeny from lethal transgenic heterozygotes, in an attempt to carefully measure the rescuing capacity of the subclones of bli-4. It was suspected that, if slight functional redundancy did exist between isoforms in vivo, exogenous expression of inappropriate isoforms may have resulted in incomplete rescue of lethality. This incomplete rescue would have been observed as lethal homozygotes that survived and produced progeny, but their frequency in the population would have been lower than expected, much the same way that incomplete penetrance of blistering in e937 was evident in a population of worms, not in an individual blistered worm. For instance, a comparison between the transmission frequency of the array (Rol/Rol+WT) and the frequency of rescue (Dpy/Dpy+dead) provides the information necessary to determine the
penetrance of lethality in transgenic worms. In this manner, if frequency of
transmission of the array and frequency of rescue by the array are equivalent,
the conclusion would be complete rescue. In two cases, the frequency of
transmission of the array was consistently higher (for all three alleles) than
frequency of rescue by the array (Figure 24). By this criterion, pCeh221, and
one of the minigenes that encodes blisterase A, pCeh230, did not rescue
lethality as well as the blisterase B, C, D minigene (pCeh236) or other
blisterase A constructs (pCeh226 and pCeh229). If the reason for overlapping
function from exogenous constructs was due to over-expression or aberrant
expression, perhaps the reduced rescuing ability of pCeh221 and pCeh230 was
a result of the removal of a large portion of intron 12. Since the blisterase B,
C, D minigene, pCeh236, also lacks this region, it may be that the predicted
isoforms encoded by this construct are more closely associated with the
essential role predicted for the bli-4 gene, and that even reduced expression
still allowed complete rescue. This interpretation is consistent with the
intracomplementation data for the bli-4 gene.

Blisterase B partially rescued blistering but not lethality

The minigene pCeh238 can code for the blisterase B isoform. Transgenic animals homozygous for the e937 mutation were observed to
blister at reduced penetrance, indicating that blisterase B partially rescued this
phenotype. Upon introduction of pCeh238 into the lethal strains, it was
discovered that blisterase B was unable to rescue any of the lethal alleles,
q508, h199, or s90. In light of the apparent functional redundancy exhibited
by the other isoforms of bli-4, these results were somewhat surprising. The
carboxyl-terminus of blisterase B is quite similar to the carboxyl-terminus of blisterase A; both regions are approximately equal in length and contain several hydrophobic residues. Therefore, it was expected that blistering would be completely rescued, such as with pCeh236 (blisterase B, C, and D, together).

The blisterase B specific exon (exon 14) has a 3' untranslated region (3'UTR) of just 23 nucleotides, which is extremely small when compared to most C. elegans genes. This, combined with Northern blot analysis indicating blisterase B is weakly expressed in mixed stage RNA (Thacker, et al., 1995), suggests that low endogenous blisterase B expression may be a result of reduced stability of this transcript. Therefore, it is possible that blisterase B levels from the array may be lower than other isoforms even if pCeh238 is transcribed as efficiently as the rescuing constructs. An additional feature of pCeh238 that may have contributed to its lack of rescuing ability is that it contains the first exon of blisterase C/D. The presence of exon 15, which has a canonical splice acceptor sequence (TTTACAG) may result in the production of an aberrant transcript, further reducing the amount of blisterase B. Further evidence that low exogenous expression of blisterase B was responsible for its inability to completely rescue blistering is that one strain believed to contain an integrated pCeh238 array does rescue blistering completely (data not shown).

The negative result for lethal rescue by pCeh238 and the negative rescue result for blistering by pCeh239 (C. Thacker, pers. comm.) indicates that the common region of bli-4 is necessary for rescue of the bli-4 mutant phenotypes. However, pCeh221 rescue data suggests that this region is also sufficient to rescue the mutant phenotypes (albeit at a reduced level for lethality) when in the context of this exogenous construct. This apparent
contradiction implies a difference in function or expression between the products of these constructs. Although the function of the 3' ends of the predicted bli-4 isoforms still remains a mystery, these structural features may account for the differing rescuing capacity of pCeh221, pCeh238 and pCeh239. Future analysis of the distribution of the predicted bli-4 isoforms in the worm via immunolocalization may provide some evidence for their function. For instance, individual isoforms may be targeted to different tissues. It would be informative to subject the transgenic worms to immunolocalization studies to determine if inferred functional redundancy is due to aberrant localization of the exogenously produced proteins.

bli-4 potentially produces at least four protein isoforms, each of which are identical at the amino-terminus (Figure 21) but differ in their relatively small carboxyl-termini. Therefore, the question of whether each isoform performs a distinct in vivo function is difficult to assess with transgenic rescue experiments. Aberrant expression from an extrachromosomal array could override endogenous bli-4 function, especially if the distinction between bli-4 isoforms in vivo is due to temporal and/or spatial expression differences.
Conclusions

1. A refined structure of bli-4 was constructed. The gene encodes 21 exons and is trans-spliced to the leader sequence SL1.

2. The class II lethal mutation h199 was mapped by PCR-based heteroduplex technique. h199 is an A:T to T:A transversion that results in a His to Leu substitution in a region proximal to the protease domain, which all isoforms share. h199/e937 animals rarely blister, suggesting that h199 retains some activity, at least with respect to the adult cuticular function associated with this gene.

3. The s90 mutation has not been found, despite a search through most of the coding region from the 3' specific exons of the gene. A revision of the current hypothesis regarding the location of this complementing allele may be required.

4. Transgenic minigene experiments suggest that the isoforms produced by bli-4 are sufficiently similar to be functionally redundant, at least when exogenously expressed. The penetrance of lethality of rescued transgenic lines was measured, providing evidence that not all minigenes are equivalent in their ability to rescue lethality.
References


Figure 24. Subclones of the bli-4 region.

The relative positions of subclones from the bli-4 region are shown. Relevant restriction sites are indicated, brackets indicate blunt-end loss of a site during the cloning procedure.
Appendix 2.
Evidence that KR513 no longer contains a linked lethal allele within sDf4.

The following cross was performed by R. Johnsen:

\[ dpy-5 \text{ let(?) bli-4(h199)} \text{ unc-13; sDp2} \times \text{ unc-11 dpy-14; szT1 (Lon males)} \]

WT progeny recovered:

\[
\begin{array}{cccc}
+ & dpy-5 & \text{ let(?) bli-4(h199)} & + \\
unc-11 & + & + & dpy-14 & +
\end{array}
\]

cross the above animals to sDf4/hT2:

\[
\begin{array}{cccc}
dpy-5 & \text{ let(?) bli-4(h199) unc-13} \text{ Dpy progeny} \\
sDf4 & \text{ if let(?) is outside} \\
\end{array}
\]

Many Dpy progeny were recovered (four were set up and each gave only Dpy progeny). Therefore, if there is a lethal mutation other than bli-4(h199), it is outside of sDf4.

Mapping data:

KR513 X N2 males,

set up heterozygous F1s for scoring (discard any F1s that give many Unc-13 F2 progeny, which is evidence for sDp2 in F1)

<table>
<thead>
<tr>
<th>wild-type</th>
<th>Dpy</th>
<th>Unc</th>
<th>Dpy,Unc</th>
</tr>
</thead>
<tbody>
<tr>
<td>195</td>
<td>3</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>246</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>270</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
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<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>205</td>
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<td>1</td>
<td>0</td>
</tr>
<tr>
<td>103</td>
<td>1</td>
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<td>0</td>
</tr>
<tr>
<td><strong>total</strong></td>
<td><strong>1237</strong></td>
<td><strong>3</strong></td>
<td><strong>0</strong></td>
</tr>
</tbody>
</table>

Map distance = 0.3 m.u. between dpy-5 and h199, as compared to 0-0.1 (Peters, et al., 1991), indicating that the lethal once linked to h199 in KR513 is no longer in this strain. This strain was reassigned as KR2997.

Recombination frequency was calculated by \( p = 1-(1-2R)^{1/2} \) where \( R \) is the fraction of recombinant progeny over total progeny (Brenner, 1974) and total progeny is 4/3 (wild-type + Dpy) (Rose and Baillie, 1979)