FUNCTIONAL DISSECTION OF THE GENE, *bli-4*, IN
CAENORHABDITIS ELEGANS

by

READMAN YUE-BOK CHIU

B.Sc. Simon Fraser University 1995

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF

THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

in

THE FACULTY OF GRADUATE STUDIES

(Department of Medical Genetics)

we accept this thesis as conforming
to the required standard

THE UNIVERSITY OF BRITISH COLUMBIA

April, 1997

© Readman Yue-Bok Chiu, 1997

In presenting this thesis in partial fulfilment of the requirements for an advanced degree at the University of British Columbia, I agree that the Library shall make it freely available for reference and study. I further agree that permission for extensive copying of this thesis for scholarly purposes may be granted by the head of my department or by his or her representatives. It is understood that copying or publication of this thesis for financial gain shall not be allowed without my written permission.

Department of _Medical Genetics._

The University of British Columbia
Vancouver, Canada

Date _April 29, 1997_

DE-6 (2/88)

Abstract

The *bli-4* gene of *Caenorhabditis elegans* encodes at least four gene products by the mechanism of alternative splicing. Termed blisterases A-D, these isoforms contain unique carboxyl termini and exhibit distinct structural homology to different members of the kexin family of proprotein convertases. Two different kinds of phenotypes have been identified in our collection of *bli-4* mutants: blistering at the adult stage of the allele *e937*, and embryonic (or early larval) lethality of thirteen alleles, suggesting *bli-4* plays a functional role not only in the assembly or maintenance of the adult cuticle but also in the early development of the animal. The goal of this thesis is to investigate the functions of the individual blisterases using isoform-specific minigenes in a *bli-4* mutant background. Three minigenes were constructed. Together with pCeh226 containing the carboxyl terminus for blisterase A, these constructs provide minigenes specific for three of the isoforms, pCeh299 for blisterase B, pCeh308 and pCeh309 for blisterase C. The blistered mutant lacks the 3′ exon of blisterase A. As expected, a high copy number of the minigene providing the blisterase A isoform rescued this phenotype. In addition, the blisterase A minigene also rescued the lethal mutants, suggesting either that blisterase A is required early in development or inappropriately provides the function of the other isoforms. At high copy number, the blisterase B minigene also rescued both the blistering and the

lethal phenotypes whereas a modified blisterase C minigene (pCeh309) only rescued the blistering phenotype. At low copy number, three out of five transgenic lines containing the blisterase B minigene and one putative integrated line containing blisterase C (pCeh308) also rescued blistering. None of the arrays carrying a low copy number of minigenes rescued the lethal mutants tested. The results support the hypothesis that high copy number lines can inappropriately provide the function of other isoforms. This is compatible with the suggestion that there is functional redundancy between the isoforms, with blisterases A and B probably playing a more important role than blisterase C as indicated by the present results. Biological specificity may result from the developmental, or tissue-specific distribution of the processing enzyme with its substrate.

Table of Contents

List of Tables

List of Figures

Acknowledgments

I would like to thank my research supervisor, Dr. Ann Rose, for giving me the opportunity to work on the *bli-4* project, and for her support and guidance throughout the course of this work. I would like to especially thank Dr. Colin Thacker for his invaluable help, care, and support in both good and bad times. I thank my supervisory committee, Dr. Fred Dill and Dr. Carolyn Brown, for their constructive advice and support. Thanks to all the past and present members of the Rose lab for making it a nice environment to work in. Finally, thanks to my parents, God, and Ada for her love and support for my pursuit of this degree.

Introduction

The genetics of *bli-4*

The *bli-4* gene of *C. elegans* was originally identified as a recessive mutation, *e937*, that results in fluid-filled separations, or blisters, of the adult cuticle layers of the nematode (Brenner 1974; Figure 1). The phenotype is incompletely penetrant, as 10-20% of a population of isogenic *e937* homozygotes do not develop blisters. This feature of reduced penetrance is heritable, as a "wild-type-looking" and a blistered *e937* sibling both continue to produce progeny of similar blistering population frequency in the next generation. In addition, *e937* homozygotes display variable expressivity of the blistering phenotype. Blisters may be restricted to one location or cover the entire length of the animal.

Subsequent to the identification of *e937*, thirteen additional recessive mutations were mapped to the *bli-4* locus on chromosome I by complementation testing (Howell et al., 1987; Peters et al., 1991., Thacker, Srayko and Rose, unpublished data). These alleles, however, result in either embryonic or early larval lethality, suggesting a functional role for *bli-4* in early development in addition to adult morphology. Based on a complex heteroallelic complementation pattern, the current collection of *bli-4* alleles are grouped into three classes (Peters et al., 1991; Figure 2). Class I is
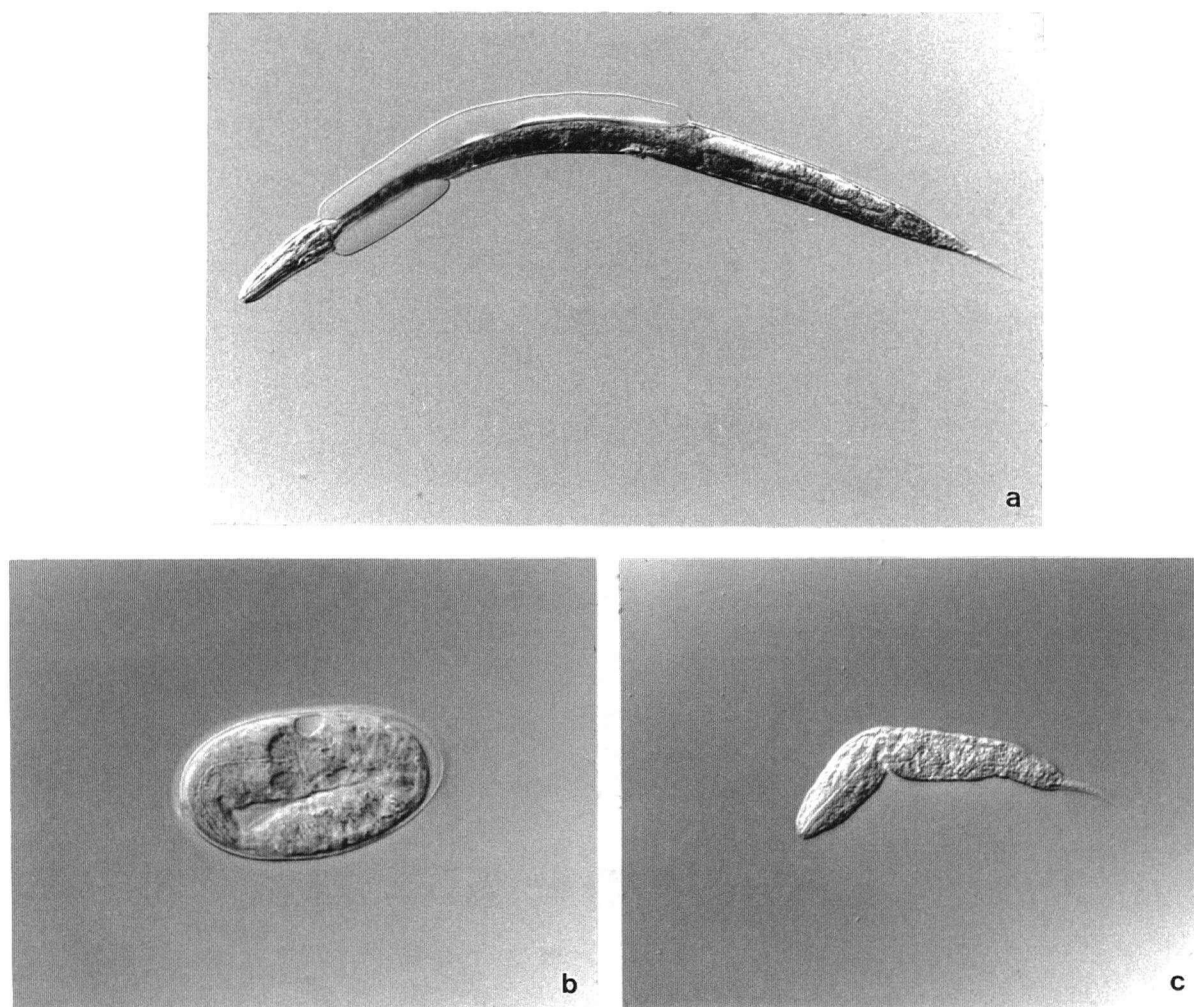
Figure 1.   Mutant phenotypes of *bli-4*

Nomarski photographs (courtesy of C. Thacker) of

a.   Class I *e937* homozygote showing the blistered phenotype at adulthood
b.   Class II *q508* homozygote arresting development in late embryogenesis
c.   Class III *s90* homozygote arresting development in early L1 larval stage. 70% of *s90* homozygotes appear as Class II homozygotes.

represented by the only viable allele, *e937*, as described above. Class II includes a group of twelve embryonic lethal alleles that fail to complement *e937*. Heteroallelic combination of most lethal class II alleles with *e937* result in viable, blistered animals with, interestingly enough, close to complete penetrance of blistering. The implication of this observation is not further discussed in this thesis. Class III contains a single allele, *s90*, which complements *e937* but fails to complement all the class II alleles. By genetic criteria, *s90* is therefore classified as a member of *bli-4* mutants. The majority of *s90* homozygotes are embryonic lethals similar to the class II alleles yet about 30% delay arrest to the early L1 larval stage. The complicated complementation pattern indicates that *bli-4* is a complex locus, while the two main kinds of phenotype (adult blistering and embryonic lethality) displayed by *bli-4* alleles implies a multi-functional role of *bli-4* gene product(s) at different development stages.

Molecular structure of *bli-4*

The molecular structure of *bli-4* was characterized by the efforts of Peters et al. (1992), Srayko (1995), and Thacker et al. (1995). *bli-4* is composed of 21 exons stretched over a region of over 20 kb of genomic sequence (Figure 3). Transpliced to the leader sequence SL1, four gene products arise by alternative splicing of the first 12 exons to specific exons on the 3′ end.

	Class I	Class II	Class III
Class I	Blistered (~80%)		
Class II	Blistered (~100%)	Embryonic Arrest	
Class III	Wild-type	Embryonic Arrest	Embryonic Arrest (~70%)/ L1 Arrest (~30%)

Figure 2. Intracomplementation of *bli-4* alleles.

The percentage within parenthesis represents the approximate population frequency of the phenotype.

Class I: *e937*

Class II: *h42, h199, h254, h384, h427, h520, h670*
              h791, h1010, h1403, q508, h1549

Class III: *s90*

Database searches revealed that BLI-4 shows homology to a group of serine endoproteases, the kex-2/subtilisin family of proprotein convertases (or kexins, to be discussed later). A protease domain based on sequence similarity is recognizable in *bli-4* within exons 5-9, with the conservation of the important catalytic amino acids - Asp-202, His-241, Asn-342, and Ser-415 (Figure 4). In addition, the common region of the four isoforms, termed blisterases A-D, shares the same structural organization common to all members of this particular family of enzymes (Figure 4).

The 3′ exons of individual *bli-4* isoforms encode unique carboxyl termini (C-termini) featured by the presence/absence of special structural domain(s) that are recognizable in their kexin homologues (Figure 4). Both blisterases C and D contain a cysteine-rich region (CRR) also found in, for example, furin, PACE4, and PC5/PC6. In addition, blisterase D contains a transmembrane domain (TMD) that is shared by both Kex2p and furin. Neither of these domains is present in blisterase A or B. The production of multiple gene products from *bli-4* by alternative splicing is consistent with the association of multiple classes of mutant alleles and phenotypes with this locus.
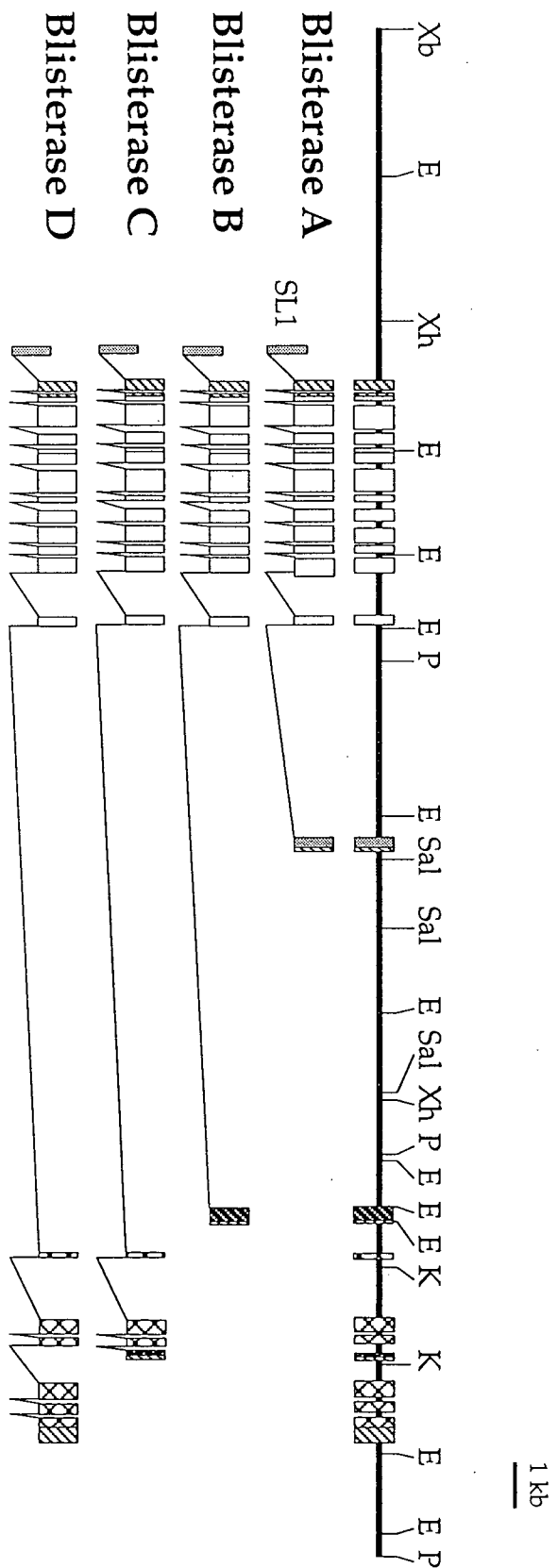
Figure 3. The molecular structure of *bli-4*

Hatched boxes represent untranslated sequences. Open boxes represent exons common to all isoforms. 3' exons specific to each isoform are filled with different patterns.

Restriction enzymes: (Xb) *XbaI*; (Xh) *XhoI*; (P) *PstI*; (Sal) *SalI*; (E) *EcoRI*; (K) *KpnI*; (B) *BamHI*.

9

Figure 4. A schematic comparison of the structures of *bli-4* gene products and other kexin convertases. The vertical bar that cuts through the *bli-4* isoforms represents the site of alternative splicing. On the amino side, BLI-4 possesses all the structural features shared by the kexins: secretion signal peptides are shown stippled; potential autocatalytic cleavage sites are shown as vertical bars on the amino side of the protease domain; protease domains are shown as shaded with the positions of the catalytically important amino acids Asp, His, Asn, and Ser indicated by D, H, N, and S. The numbers within the protease domains represent the percentages of identical residues when compared with BLI-4. Structural divergence on the carboxyl termini of *bli-4* isoforms displays homology to different kexin homologues: the cysteine-rich regions (CRR) are depicted as hatched boxes; transmembrane domains (TMD) are shown as dark boxes. Ovals indicate potential N-linked glycosylations sites. (Figure modified from Thacker et al., 1995)

C. elegans
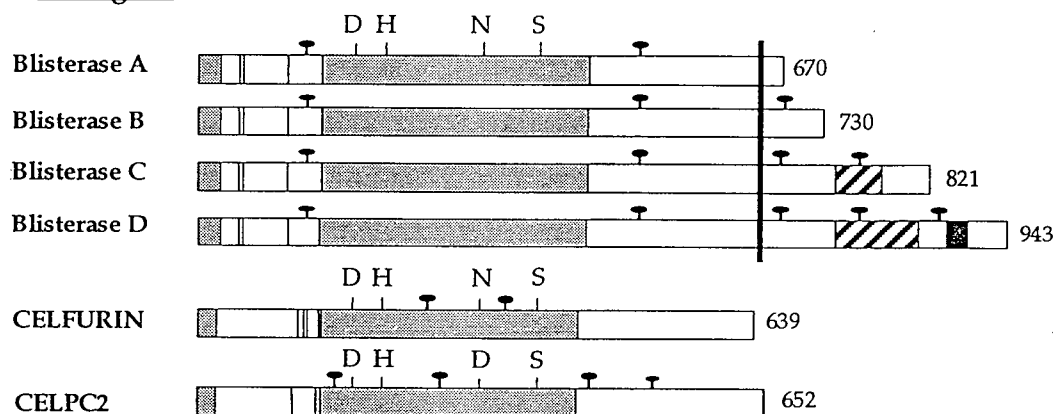
Vertebrates
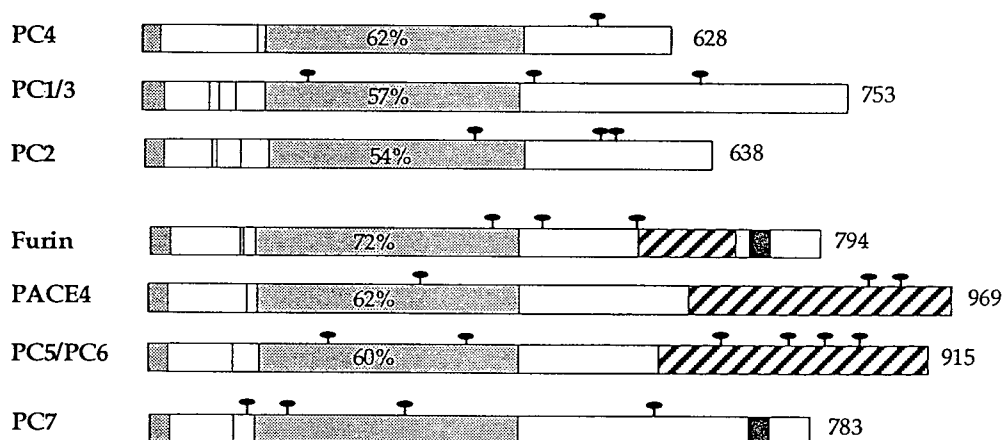
Drosophila
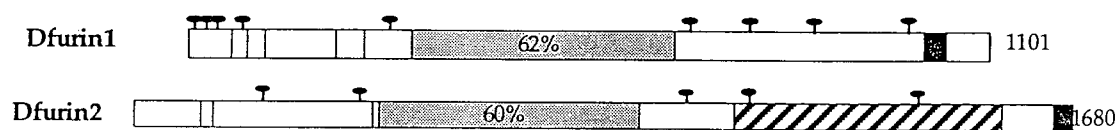
Yeast

Mutational Analysis

Molecular lesions corresponding to the majority of *bli-4* mutants have been mapped and characterized using restriction fragment length polymorphism (RFLP) and polymerase chain reaction (PCR)-based heteroduplex analysis (Srayko, 1995; Thacker et al., 1995, 1996). The original blistering allele, *e937*, results from a 3.5 kb deletion that removes the 3' exon unique to blisterase A (Figure 5). Northern and reverse-transcription-PCR (RT-PCR) analysis confirmed that the blistering phenotype of *e937* results from the specific elimination of the expression of blisterase A (Thacker et al., 1995). Eleven of the class II lethal mutations were found to reside in the common region of the gene and presumably abolish the expression of all isoforms (Figure 5; Thacker and Rose, in preparation). Thus, they represent potential null alleles of *bli-4*. The molecular lesion associated with the class III mutation, *s90*, has yet to be identified.

The kexins

A large number of biologically important molecules are synthesized as inactive precursors and subsequently activated by proteolytic processing. In humans, this includes a host of hormones, neuropeptides, and receptor molecules (Steiner et al., 1993; Dubois et al., 1995; Nachtigal et al., 1996;
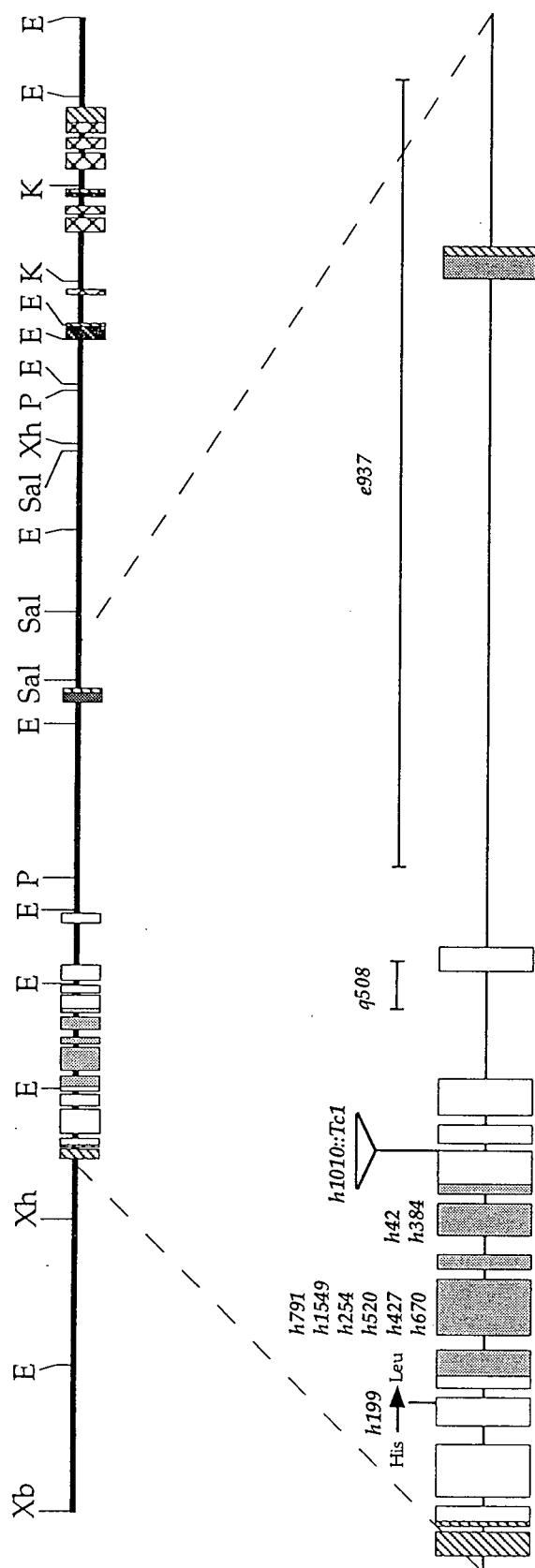
Figure 5 . Location and molecular identities of *bli-4* mutations (information provided by C. Thacker)

Hatched boxes represent untranslated sequences whereas the protease domain is shaded.

q508 and *e937* are deletions. *h1010* is a Tc1 insertion. *h199* is a misense mutation. Other alleles represent nonsense mutations in the protease domain.

Restriction enzymes: (Xb) *XbaI*; (Xh) *XhoI*; (P) *PstI*; (Sal) *SalI*; (E) *EcoRI*; (K) *KpnI*; (B) *BamHI*.

Willnow et al., 1996). Viral coat proteins and bacterial toxins are also physiologically relevant molecules requiring this mode of post-translational modification (Decroly et al., 1996; Gu et al., 1996). The enzymes enforcing this significant process had been long sought after and their identities were recently unveiled. The kex2/subtilisin family of proprotein convertases, or kexins, are serine endoproteases featured by their proteolytic action at dibasic amino acid sites, usually Arg-Arg↓ or Lys-Arg↓ (for reviews, see Barr, 1991; Seidah and Chretien, 1992). The original prototype of kexins is the gene product of the yeast gene kex-2, a Ca^{++}-dependent subtilisin-like protease which is responsible for the maturation of the pro-α-mating factor and the pro-killer toxin in the organism (Fuller et al., 1989a). Based on sequence homology to kex-2, seven members of mammalian kexins have been identified to date. They 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), furin (Roebroek et al., 1986; Fuller et al., 1989b; van den Ouweland et al., 1990; Wise et al., 1990), and PC7 (Seidah et al., 1996). In addition, homologues of mammalian kexins are found in *Drosophila* (Roebroek et al., 1991,1992), *Xenopus* (Braks et al., 1992) and *Hydra* (Chan et al., 1992). In *C. elegans*, homologues of PC2 (Gomez-Saladin et al., 1994), and furin (Thacker and Rose, unpublished finding) have been reported. *bli-4*, as mentioned in the previous section, encodes products homologous to various members of the kexin family (Figure 4).

The different members of kexins have been shown to function in either the constitutive or regulated secretory pathway of the cell, where different modes of post-translational modifications take place before molecules assume their mature structures. On the level of tissue distribution, different expression patterns are exhibited by the seven mammalian kexins, which accordingly have been generalized into two categories. One category exhibits expression restricted to the endocrine and neuroendocrine tissues and therefore assumes functional roles within the regulated secretory pathway. Members of this category include PC1/PC3 and PC2 (Seidah et al,. 1990, 1991; Smeekens and Steiner, 1990; Smeekens et al., 1991.) The other category, including furin and PACE4, displays ubiquitous expression patterns and participates within the constitutive secretory pathway (Roebroek et al., 1986; van den Ouweland et al., 1990; Kiefer et al., 1991). Members of this category also demonstrate a more elaborate cleavage specificity than the usual dibasic motif. They process substrates at a tetra-basic site: Arg-X-Arg/Lys-Arg↓ (where X stands for any amino acid). PC5/6 and the recently identified PC7 also display widespread tissue distribution although the level of expression is predominant in intestinal tissue (PC5/6; Lusson et al., 1993), or in colon and lymphoid-associated tissues (PC7; Seidah et al., 1996). Therefore PC5/6 and PC7 may function in both types of secretory pathway.

The functional roles of individual kexins appear to be a function of intracellular compartmentalization, which in turn depends on the structural

properties conferred, in particular, by the divergent C-termini. For example, the TMD on the carboxyl tail of furin is responsible for anchorage of the enzyme within the *trans*-Golgi network (TGN). Further studies identified additional trafficking signal sequences adjacent to the TMD that direct cycling of furin between the cell surface and the TGN, thus establishing a dynamic multi-compartment distribution equilibrium for the enzyme (Molloy et al., 1994; Schafer et al., 1995; Jones et al., 1995). This characterization fits with the wide spectrum of substrates found to be processed by furin (Pei and Weiss, 1995). On the other hand, PC1/3 and PC2 were found sequestered to and functioned within secretory vesicles. Studies showed that the C-termini are determinants of the specific routing and storage pattern exhibited by these enzymes (Zhou et al., 1995; Creemers et al., 1996). Putative amphipathic domains were identified on the carboxyl tails of PC1/3 and PC2 which may facilitate such localization (Smeekens et al., 1991).

Functional specificity of *bli-4* isoforms

The four gene products of *bli-4* display structural homology to members of kexin family belonging to both the constitutive and regulated secretory pathways. Blisterases A and B are similar to PC1/3 and PC2 while blisterase C and D are similar to PC5/6, PACE4, and furin. Whether corresponding functional difference exists among the *bli-4* isoforms is an intriguing question. *bli-4* is not the only kexin gene that encodes multiple

isoforms through alternative splicing. Three mammalian kexins, PACE4, PC4, and PC5, also produce isoforms with divergent C-termini from a single gene (Tsuji et al., 1994; Seidah et al., 1992; Nakagawa et al., 1993b). Recent work by De Bie et al. (1997) demonstrated for the first time that the PC5-A and PC5-B isoforms, which differ in the length of the CRR and the presence of TMD (in PC5-B), exhibit different sub-cellular localization patterns. The *dfur-1* gene of *Drosophila* also encodes multiple furin homologues with unique C-termini. The individual dfurin isoforms demonstrate different spatial and temporal expression patterns, suggesting they may play different physiological roles in the organism (De Bie et al., 1995).

bli-4 is the only kexin member identified thus far in a multicellular organism for which mutants have been generated that facilitate functional characterization through genetic analysis. The different kinds of mutant phenotypes directly indicate that *bli-4* plays distinct functional roles at different stages during development. The multitude of gene products further raises the possibility that functional specificity exists among individual *bli-4* isoforms. The blistering phenotype of the viable *e937* allele as a result of the specific elimination of blisterase A activity is suggestive of this possibility and provokes further investigation.

To explore the hypothesis of functional specificity, one strategy we have adopted is to construct individual blisterase subclones to rescue different *bli-4* mutants with the aim to correlate individual isoform function with specific mutant phenotypes. Preliminary data from the study of Srayko (1995)

using this approach indicated potential functional redundancy among the different isoforms. The present work extended this line of study by using individual minigenes specific for single blisterase isoform to rescue mutants representing the three classes of *bli-4* alleles. Various studies reported the loss of substrate specificity when kexin convertases were overexpressed *in vitro* (Walker et al., 1994). With this complication in mind, two different concentrations of the minigenes were injected in order to generate transgenic strains containing different copy numbers of the minigenes. A comparison between the rescuing abilities and patterns exhibited by individual minigenes may then provide us with information on the functional specificity of individual *bli-4* isoforms.

Materials and Methods

Nematode growth conditions and strain designations

All the *C. elegans* strains used in this study were maintained on petri dishes containing nematode growth medium (NGM) streaked with the *Escherichia coli* (*E. coli*) strain, OP50, at 20°C. The wild type strain, N2, is originated from Bristol, England (Brenner, 1974). Nomenclature of strains follows the formulations by Horvitz et al. (1979). Briefly, gene names consist of a three-letter code that usually describes the mutant phenotype or the function of the gene product in combination with a number for identification among other genes that display similar mutant phenotypes or encode similar products. Specific alleles are placed in parenthesis following the gene name. For example, *bli-4(e937)* identifies the original blistering allele of the *bli-4* gene generated by Brenner in 1974. Transgenic animals are designated by the genotype of the parent strain followed by a *hEx* number which specifies the transgenic extrachromosomal array. For example, *bli-4(e937); hEx104* represents an *bli-4(e937)* homozygote containing an extrachromosomal array which, in this example, is made up of the *bli-4* A minigene and the transformation marker pRF4. The generation of transgenic animals is further explained below. Abbreviations used in this report are explained in Table 1.

Table 1. Abbreviations used in the thesis

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

Agarose gel electrophoresis

Agarose gel electrophoresis was used for size-separation of DNA

fragments. Agarose gel was prepared by dissolving agarose (Gibco BRL) in 1×

TBE (Tris-Borate, EDTA) buffer at the desired percentage (g/ml, usually

between 0.6% and 1.5% in this study) for optimal resolution. For DNA

extraction, low-melting point agarose was used with 1× TAE (Tris-Acetate,

EDTA) buffer. Electrophoresis was performed under 70-120 V. Ethidium

bromide was added to the dissolved agarose at 0.5 µg/ml for visualization of

DNA fragments under UV at 300 nm.

Preparation of DNA for subcloning

Plasmid DNA was prepared following the standard alkali-lysis method

(Sambrook et al., 1989). Digested DNA fragment was isolated by excising the

desired band from agarose gel and purified using the Qiaex Gel Extraction Kit

from Qiagen. When electrophoresis was not necessary, DNA in restriction

digests was directly purified by phenol/chloroform extraction. The volume of

the reaction mix was increased to 200 µl and extracted with an equal volume

of phenol, followed by another extraction of the aqueous layer with an equal

volume of a chloroform/isoamly alcohol (24:1) mix. The final DNA solution

was usually concentrated by adding 1/10 volume of 3M sodium acetate pH 5.2,

3 µl of 200 µg/ml dextran, and 2 volumes of 95% ethanol and then

centrifugation at 13200 rpm. The resulting DNA pellet was washed with 70%

ethanol and dissolved in TE (Tris-EDTA pH 8.0).

Restriction digests

A typical restriction digest contained 5 units of restriction enzyme, 1×

reaction buffer (supplied), 1 µg/ml of bovine serum albumin (BSA) and ~100

µg of plasmid DNA. The suppliers of restriction enzymes include New

England Biolabs (NEB), Pharmacia, and Gibco BRL. Restriction digests were

performed at 37°C for 1.5 hours unless specified otherwise by the

manufacturer. Partial digestion was achieved using 0.5 unit of restriction

enzyme for a brief incubation of 5 minutes. The enzyme was quickly

inactivated by heating at temperature suggested by suppliers followed by

phenol/chloroform extraction.

For blunt-end ligation, 5′ overhangs created by restriction digests were

filled in by Klenow fragment (1 unit, Pharmacia, 30 minutes at room

temperature) whereas 3′ overhangs were removed by T4 DNA polymerase

(1 unit, NEB, same conditions) in the presence of 0.5 mM

deoxyribonucleotides (dNTPs).

Ligation and bacterial transformation

DNA ligation was performed by incubating a mix of vector and insert

DNA, 1× ligation buffer, and 200 units of T4 DNA ligase supplied by NEB or

Gibco BRL at 16°C overnight. When the vector DNA contained cohesive

termini, it was first phosphatased to prevent self-ligation by 1 unit of calf

intestinal alkaline phosphatase (CIAP, Gibco BRL) at 37°C for 1 hour. The

enzyme was denatured by heat inactivation at 65°C for 10 minutes and

completely removed by phenol-chloroform extraction.

Plasmids were transformed into competent bacterial cells by the

following procedure: 3 (out of 10) μl of the ligation mix was added to 50 μl of

competent cells in a 15 ml falcon tube and the mix was incubated on ice for 30

minutes. The mix was then heat-shocked at 37°C for 45 seconds. After a 2-

minute incubation on ice, 0.5 ml of SOC medium was added and the culture

was inoculated at 37°C under shaking at 250 rpm for 1 hour. 50-200 ml of the

inoculate was plated on an LB plate coated with ampicillin for selection of

transformants. Most transformations in this study utilized the competent *E.

coli* strain *DH5α* from Gibco BRL. Certain restriction enzymes (e.g. *Bcl* I)

process non-methylated DNA. In this case, the *E. coli* strain *GM48* was used because it was deficient for the *dam* methylase.

Plasmid subcloning

All the constructs manufactured in this study were based on the vector plasmid pBlueScript SK (pBSSK) unless otherwise stated. This vector contains an ampicillin-resistance (amp^R) gene that allows for positive selection of successful transformants. Furthermore, a blue/white color selection is supported. Insertion of a DNA fragment in the multiple cloning site of the vector disrupts the formation of β-galactosidase and therefore produces a blue color in the presence of isopropyl-β-D-thio-galactopyranoside (IPTG) and 5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside (X-gal). This enables easy identification of bacterial colonies containing recombinant plasmids. In most cases, however, appropriate restriction digests were required to discern the identities of engineered constructs because multiple cloning steps were involved.

Polymerase chain reaction (PCR)

Template DNA for PCR in this study was either bacterial plasmid DNA or *C. elegans* genomic DNA from a single animal. 1 ng of plasmid DNA was normally used. To prepare template DNA from an animal, a single young adult nematode was transferred to a 5 μl mix of 1× *Taq* buffer (Promega), 1.5 mM MgCl$_2$, and 60 μg/ml of Proteinase K in a 0.65 ml microfuge tube and frozen at -70°C for 15 minutes. 40 μl of mineral oil was layered on top of the reaction mix before the lysis reaction was carried out at 57°C for 60 minutes, followed by inactivation at 95°C for 15 minutes in a Perkin-Elmer/Cetus PCR thermocycler. PCR was then carried out in the same tube by adding appropriate amounts of required components. The final reaction mix contained 1 unit of *Taq* polymerase or *Pfu* polymerase (Stratagene), 1× *Taq* buffer (Promega), MgCl$_2$ (1.5 - 2.5 mM), primers (200 ng), and dNTPs (200 μM). Amplification was achieved by 30 cycles of denaturation (94°C, 45 seconds), annealing (54°C, depending on primers, 30 seconds), and extension (72°C, 1 minute, or 1.5 minutes when two sets of primers were used), followed by extension at 72°C for 7 minutes.

Cloning of PCR products

Blunt-end ligation was necessary to insert gel-purified PCR products into the *Sma*I site of pBSSK. The vector was not phosphatased because PCR products did not possess 5'-phosphates. When *Taq* polymerase was used for PCR, the purified product was further polished by T4 DNA polymerase as described before ligation was performed. *Pfu* polymerase did not require the treatment. The blue/white selection scheme described above allowed easy identification of bacterial colonies containing ligation products.

Preparation of DNA for germline transformation

The various constructs used for injection were prepared using the Qiagen Miniprep Kit. 1 μl of purified DNA was loaded on an agarose gel alongside with known concentrations of standards to determine its approximate concentration. The minigenes in this study (except pCeh309) were injected at two concentrations to determine the effect of copy numbers on their rescuing abilities. A high-concentration injection mix contained about 50 μg/ml of the minigene construct and the same concentration of the transformation marker, pRF4. A low-concentration mix contained about 1 μg/ml of the minigene construct, 50 μg/ml of pRF4, and the same

concentration of pBSSK to maintain the final concentration at approximately 100 µg/ml.

Germline transformation

DNA was introduced into the nematode by injection into the distal gonadal arm in adult hermaphrodites based on the method of Mello et al.. (1991; Figure 6). The components of the injection mix were described above. The transformation marker pRF4, which contained the *rol-6 (su1006)* gene, was used to confer a dominant rolling phenotype (Rol) to transgenic animals. Once injected, the minigenes and the transformation marker molecules undergo recombination at homologous sequences (e.g. the amp^R gene on pBSSK) to generate concatemeric hybrid molecules in the form of an extrachromosomal array. Arrays are assembled independently and incorporated into the oocytes. Resulting F1 transgenics may therefore contain arrays with different structures. The extrachromosomal array behaves similar to a free duplication such that it is transmitted in a non-Mendelian fashion during cell divisions. Transgenic animals therefore exhibit mosacism of the array. Transgenic F1s are singled and usually 10% of them lead to stable lines. Very rarely, the extrachromosomal array may be randomly integrated into the genome by this technique. The underlying mechanism of such integration event is not well understood.
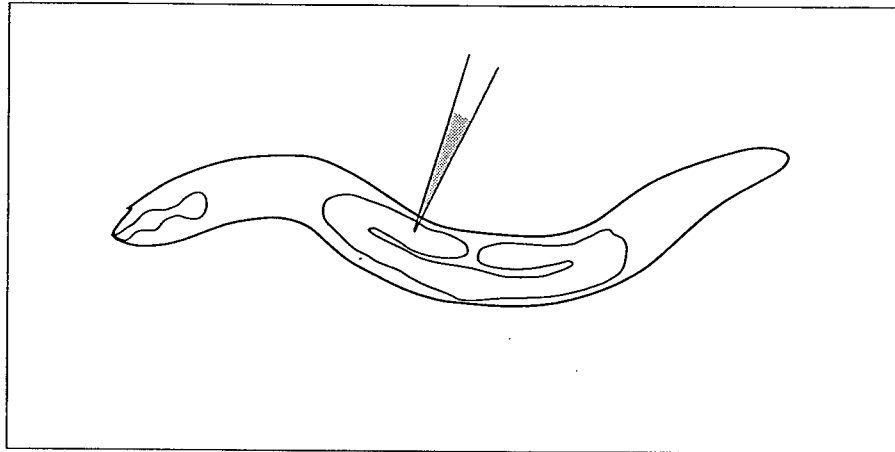
Figure 6. Microjection of plasmid DNA for germline transformation.
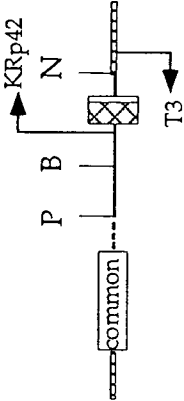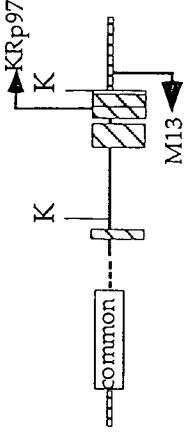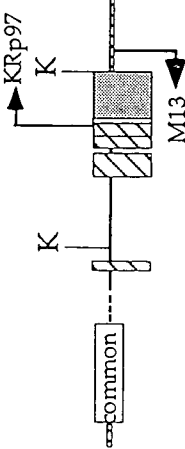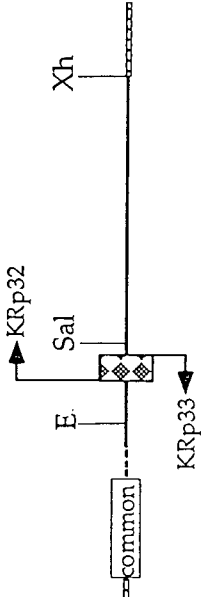
DNA prepared for germline transformation was injected into the distal gonadal arm of young adult hermaphrodites (Mello, et al., 1991)

Putative transgenic lines were confirmed by PCR to detect the presence of the minigene. For pCeh299, pCeh308 and pCeh309, specific *bli-4* 3′ primers in combination with vector primers established the presence of the minigene (Figure 7, 8). For pCeh226, the A minigene, a product produced by primers specific to the blisterase A exon, which is deleted in *e937* mutants (the parent strain used for injection), indicated the presence of the minigene.

Estimation of minigene copy number

An individual transgenic animal was lysed by the Proteinase K treatment as described. The resulting DNA was subjected to PCR amplification using the primers KRp44 and KRp51 to amplify a 886 bp fragment of the common region of *bli-4*, and the primers KRp141 and KRp142 to amplify a 380 bp fragment on the putative *rec-1* gene as a control (Figure 9). The amount of the various components for the PCR are the same as above. 12 cycles of amplification were performed to maintain a linear relationship between the quantity of input template and product. Steps for a single cycle included: denaturation (94°C, 45 seconds), annealing (56°C, 30 seconds), and extension (72°C, 1.5 minutes), followed by a final extension at 72°C for 7 minutes. The PCR products were run on a 1.6% agarose gel and transferred by capillary action to a Zeta-Probe membrane (Bio Rad) while being denatured by

Figure 7. Primers used in PCR to confirm the presence of transgenic arrays in the lines tested for rescue of *bli-4* mutant phenotypes. Only transgenic animals containing minigenes will provide PCR products amplified by the specific pairs of primers listed above. The presence of exon 13, which is specific to blisterase A, as detected by KRp32 and KRp33 indicates the presence of pCeh226, the A minigene, because the animals used for injection are *e937* homozygous. A control set of primers KRp141 (5'-TATGTCCTCAAGCGATGC-3') and KRp142 (5'-TTCCAGCAGAGCCATAGT-3') were included in every PCR to generate a fragment of about 370 bp belonging to the putative *rec-1* gene (C. Wicky, pers. comm.).

Minigene	Primers	Sequences	Product Size
B (pCeh299)	KRp42	5'-ATTTCGACGTGCAATTATCAG-3'	595 bp
	T3	5'-ATTAACCCTCACTAAAGGGA-3'	
C short (pCeh308)	KRp97	5'-CATACTTGTACGAAAGCTGA-3'	442 bp
	M13	5'-TGTAAAACGACGGCCAGT-3'	
C long (pCeh309)	KRp97	5'-CATACTTGTACGAAAGCTGA-3'	1136 bp
	M13	5'-TGTAAAACGACGGCCAGT-3'	
A (pCeh226)	KRp32	5'-ATACTAATCACCATGCGCTA-3'	194 bp
	KRp33	5'-CTCGTAAAAGTATTCATCA-3'	

pBlueScript vector sequence
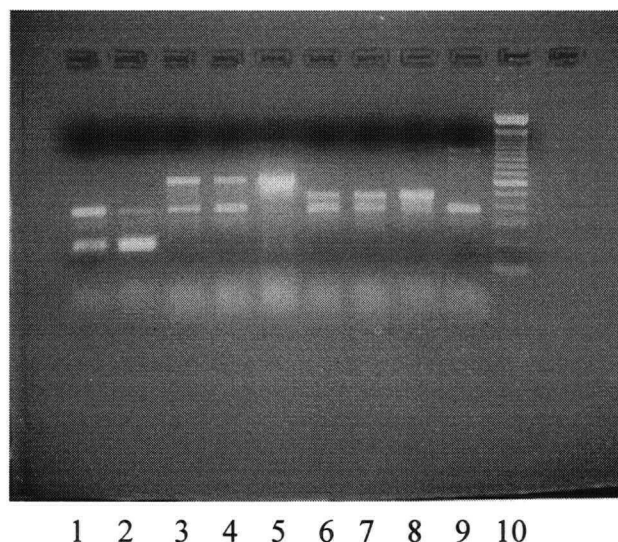
Figure 8.   Use of PCR to verify the presence of minigene constructs in
             transgenic strains

Lanes 1-9 show amplification products obtained using template DNA from
KR3228 (1), KR3231 (2), KR3235 (3), KR3239 (4), KR3233 (5), KR3245 (6), KR3246
(7), KR3241 (8), KR3252 (9). Products obtained using primers specific for
amplification of pCeh226 (A minigene) are shown in lanes 1 and 2, pCeh299
(B minigene) in lanes 3-5, pCeh308 (C short minigene) in lanes 6-8, and
pCeh309 (C long minigene) in lane 9 (see Figure 7 for description of primers
used). The 380 bp product seen in all lanes is the result of amplification using
primers of the putative *rec*-1 gene, included as a positive control. Lane 10
contains a 100 bp DNA ladder (Gibco-BRL).

0.4 M NaOH overnight. The DNA was fixed on the membrane by baking in an oven at 65°C for 1 hour (Sambrook et al., 1989).

PCR products from 30 cycles of amplification were purified using the Qiaex Gel Extraction Kit and used as probes in hybridization. Probes were prepared using the Random Primed Labeling Kit from Boehringer Mannheim. Approximately 30 ng of each PCR product were labeled with ~30 μCi [α^{32}P] dCTP and used for hybridization. The prepared membrane was incubated with a pre-hybridization solution, which contained 2× SSPE, 1% SDS, 0.5% dry milk, 100 μg single sperm salmon (SSS) DNA, at 65°C for 1 hour to minimize unspecific binding. The probes were denatured at 95°C for 5 minutes before hybridization was carried out at 65°C in a plastic bag containing 5 ml of the pre-hybridization solution, the probes, and the membrane with gentle shaking overnight. At the completion of hybridization, the membrane was washed successively in 2 × 15 minute - intervals by the following solutions: 2× SSC, 0.1% SDS → 0.5× SSC, 0.1% SDS → 0.1× SSC, 0.1% SDS at 65°C. Autoradiography was performed after washing. The autoradiograph was scanned and the intensities of individual bands were quantified using the ImageQuant 3.3 software from Molecular Dynamics.

The average intensity of the *bli-4* bands of three transgenic animals for each line was compared to that of three N2 animals in the same
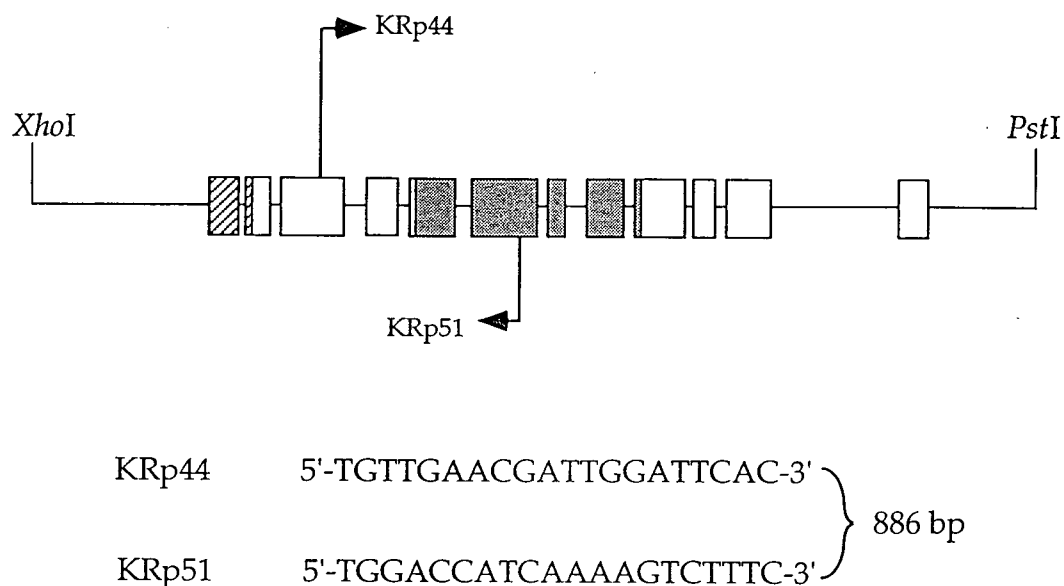
Figure 9.   Location and sequences of primers used for PCR amplification in the estimation of minigene copy number.

The diagram shows the first 12 exons common to all *bli-4* isoforms. The 5' untranslated region (5' UTR) is hatched whereas the protease domain is shaded. The sequences amplified by PCR are present in both the endogenous *bli-4* gene and all the minigene constructs. Therefore the relative amount of PCR products in transgenic animals to the wild-type strain will indicate the copy number of the *bli-4* common region, which estimates the copy number of the actual transgenes.

experiment to provide an estimation of the copy number. Detailed calculations are outlined in **Appendix I**.

Rescue of *bli-4* lethal alleles with transgenic arrays

Extrachromosomal arrays were crossed into *bli-4* lethal-bearing strains based on the scheme depicted in Figure 10. Hermaphrodites of the balanced lethal strains were crossed with N2 males that resulted in F1 progeny (males and hermaphrodites) heterozygous for the lethal mutation and the Dpy and Unc markers. F1 males were then crossed with transgenic rollers generated from DNA transformation as described above. Rol hermaphrodites were isolated from the resulting F2 population and allowed to self-fertilize on individual plates. Plates of F2s who produced arrested embryos indicated the presence of the lethal allele in the F2 parent (even when the extrachromosomal array was integrated, see **Appendix II**). These plates were kept and scored for the following F3 phenotypes: non-Rol, Rol, dead eggs, and Dpy Unc. The extent of rescue was determined by comparing the transmission frequency of the extrachromosomal array and the percentage of rescue (see Table 8 and **Results**).

Figure 10.   Strategy to determine transgenic rescue of *bli-4* lethal alleles

Lethal (*let*) alleles (e.g. *h791, s90*) are balanced by *sDp2*. Heterozygous
F1 males were crossed into Rol strains carrying the extrachromosomal
array. Rol F2 hermaphrodites were picked and set up individually for
scoring. Rol F2 which did not produce any dead progeny were presumed
not to carry the lethal allele and were not scored. (Modified from Srayko,
1995)

Unc   WT

dpy-5   bli-4(let)   unc-13

dpy-5   bli-4(let)   unc-13   ♀   ✕   N2   ♂

——————————⊣ sDp2

dpy-5   bli-4(let)   unc-13

F1   pick wild-type ♂'s
        +   +   +   (may also carry sDp2)

WT   Rol

dpy-5   bli-4(let)   unc-13   +   bli-4(e937)   +

        +   +   +   ♂   ✕   +   bli-4(e937)   +   ♀

                                                          ├—⊥—┤ hEx (includes rol-6)

Bli   WT

dpy-5   bli-4(let)   unc-13   +   +   +

        +   bli-4(e937)   +   +   bli-4(e937)   +

F2
Rol   Rol

dpy-5   bli-4(let)   unc-13   +   +   +

        +   bli-4(e937)   +   +   bli-4(e937)   +

├—⊥—┤ hEx (includes rol-6)   ├—⊥—┤ hEx (includes rol-6)

pick Rol ♀ for scoring F3 progeny
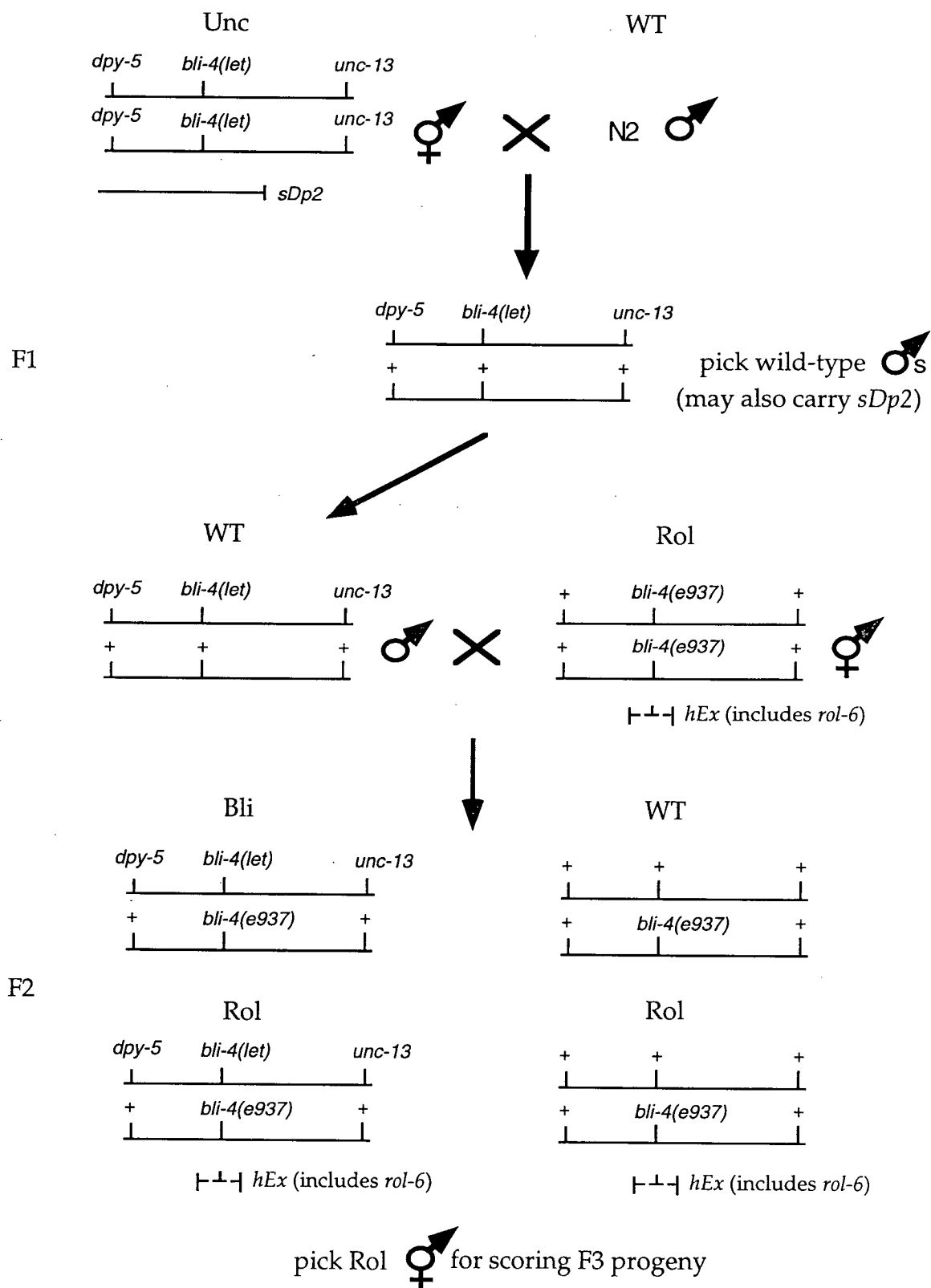
Results

Section I.   Construction of *bli-4* minigene constructs

The strategy to construct minigenes specific for individual *bli-4*

isoforms was based on the previous study by Srayko (1995). Two "parent"

plasmids, pCeh220 and 221, contained a 9 kb DNA fragment corresponding to

the common genomic region of *bli-4* (Figure 11). The 5 kb genomic sequences

5′ of the transcription start site in exon 1 contain the putative promoter

elements for the transcription of *bli-4*. Expression of a fusion of this region

with a reporter *lacZ* gene was detected in selective tissues (hypodermal cells,

the vulva, and the ventral nerve cord) at both the embryonic and adult stages

(Thacker et al., 1995). Further analysis of the promoter region was provided

by Jones (1997). 3′ exons with neighboring intronic sequences specific to

individual blisterases were subcloned and joined to the common region in

the parent plasmids to complete the construction of *bli-4* minigenes.

In this study, minigenes specific for blisterase B (pCeh299), and

blisterase C (pCeh308 and pCeh309) were constructed. pCeh226, constructed

and tested for rescue by Srayko (1995), served as the blisterase A minigene

(Figure 12). Previously, RT-PCR analysis detected a variant blisterase C

transcript putatively resulting from incomplete processing of the last intron

specific to blisterase C (Figure 13; Thacker et al., 1995). Therefore, to eliminate

A.

pCeh220

B.

pCeh221
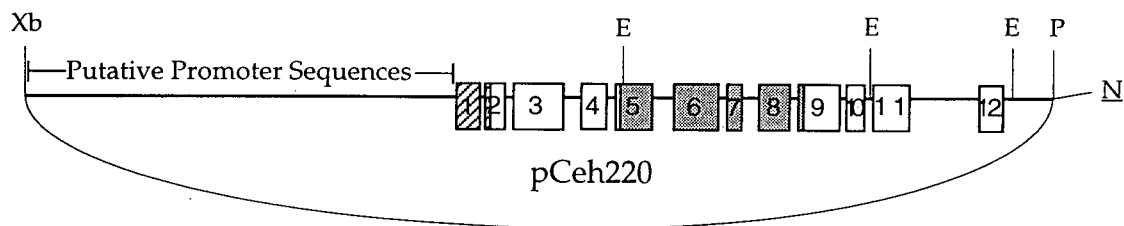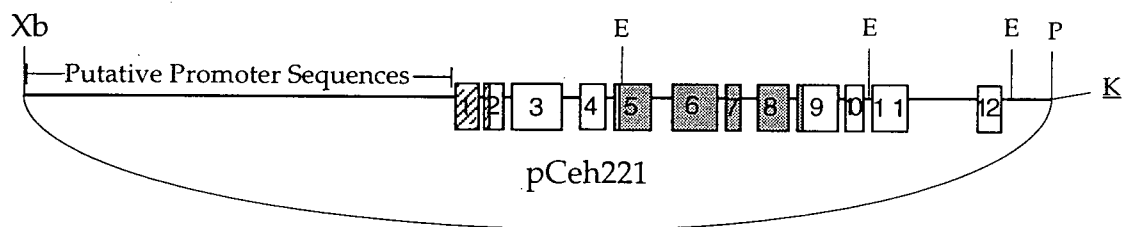
Figure 11. The "parent plasmids" (constructed by C. Thacker) used in the
construction of *bli-4* minigenes in this study. The number within
each box represents the exon number. The 5'-untranslated region
(5' UTR) is hatched whereas the protease domain is shaded.
Underlined restriction sites show the difference in cloning site
available for subcloning downstream exons.

A. pCeh220 was used for the construction of pCeh299, the B minigene

B. pCeh221 was used for the construction of pCeh226 (A minigene, by Srayko),
and pCeh301, which was then utilized for the construction of pCeh308 (C
short minigene) and pCeh309(C long minigene)

Restriction enzymes: (Xb) *Xba*I; (P) *Pst*I; (E) *Eco*RI; (K) *Kpn*I; (N) *Not*I.
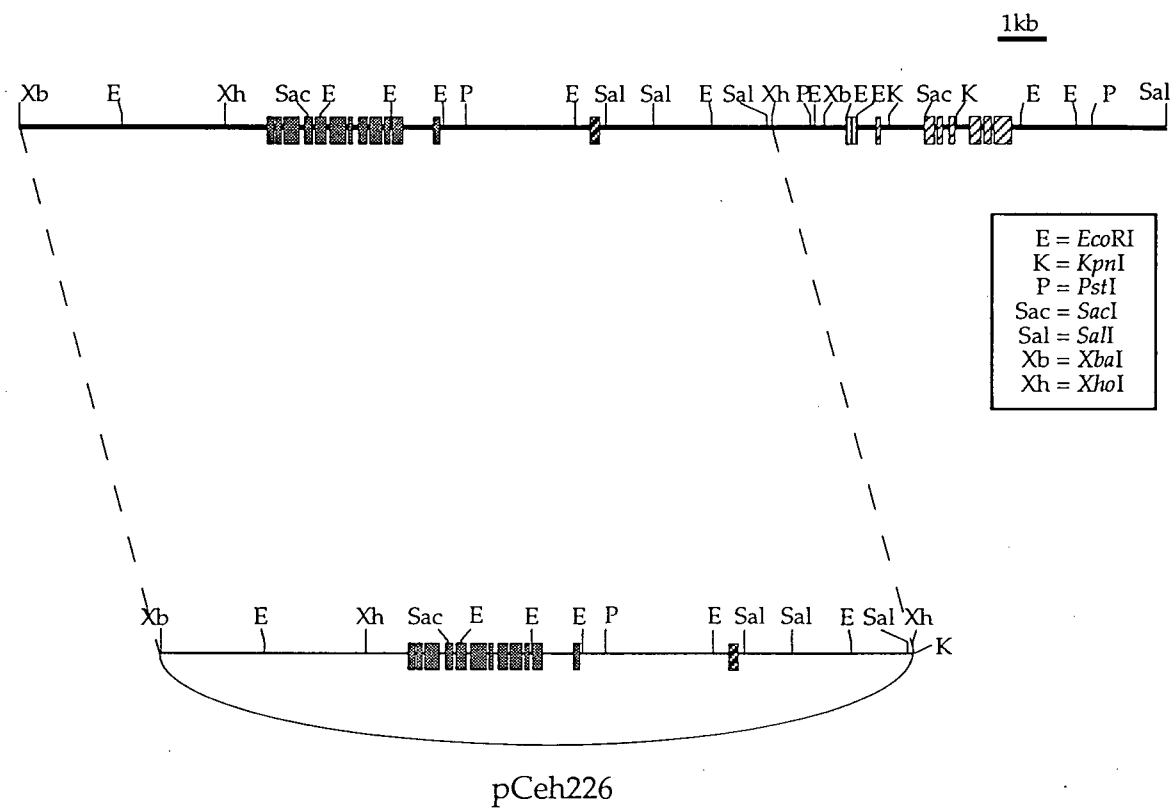
Figure 12. Structure pCeh226, the A minigene in this study (Srayko, 1995)

exon 17   intron 17   exon 18

GCT GAT T gt aag ttt tat aag agt ttc aga ata aaa agt taa tctccag AC GCT GAA

A   D   C   K   F   Y   K   S   F   R   I   K   S   *   Y   A   E
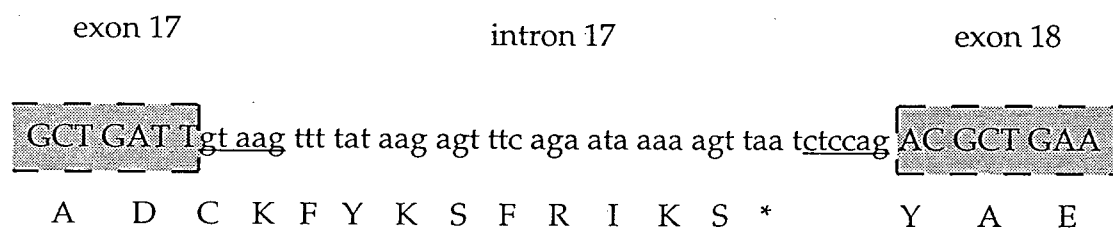
Figure 13.   The putative carboxyl terminus of the variant blisterase C transcript. (Modified from Thacker et al., 1995)

Amino acids potentially encoded within intron 17 are shown. The splice donor and acceptor are underlined.

possible expression of this variant transcript, intron 17 was exluded in both

the blisterase C minigene constructs (Figure 16, 17). Moreover, in light of the

extremely short 3'-untranslated region (3' UTR) of blisterase C (31 nucleotides)

and the technical difficulty of including the small quantity of intronic

sequences immediately downstream of the last blisterase C exon (exon 18), the

unc-54 3' UTR, a commonly utilized 3' element in *C. elegans* gene expression

vectors, was appended to the end of exon 18 in pCeh309 to ensure proper

processing of transcript (Fire et al., 1990; Figure 18.). A detailed construction

scheme for each minigene construct is outlined in Figures 14 to 17.

Section II.   Generation of transgenic strains with high and low copy

numbers of *bli-4* minigenes

Transformation in *C. elegans* is usually performed using a DNA

concentration of about 100 µg/ml. This range of concentration enables the

generation of extrachromosomal arrays of optimal size for efficient

transmission (Mello et al., 1991). In this study, minigenes were injected at

two concentrations, 50 µg/ml and 1 µg/ml, to examine the effect of copy

number on the rescuing ability of each minigene. To ensure the generation

of transmissible extrachromosomal arrays, a constant total DNA

concentration of 100 µg/ml was maintained. Therefore, 50 µg/ml of

pBlueScript, the backbone of each minigene construct, was added in addition

Figure 14. Construction of pCeh299, the B minigene

A.   The substrate clones used

B.   A schematic representation of the cloning steps involved

A

1kb

Xb E Sac E E E P E Sal Sal E Sal P E B E E K Sp K E E P Sal

pCeh221

pCeh232

B = *Bam*HI
E = *Eco*RI
D = *Dra*I
K = *Kpn*I
N = *Not*I
P = *Pst*I
Sac = *Sac*I
Sal = *Sal*I
Sp = *Spe*I
Xb = *Xba*I
Xh = *Xho*I

B

P B D K Sp
N

1. digest with Sp
2. fill in Sp (blunt)
3. digest with B

pCeh232

B/D

P B
N

B D

520 bp

P B
N

pCeh298

P/N

pCeh 221

P/N

P B N

Xb E Xh Sac E E E P N

N

Xb E Xh Sac E E E P B N

pCeh 299 (B minigene)
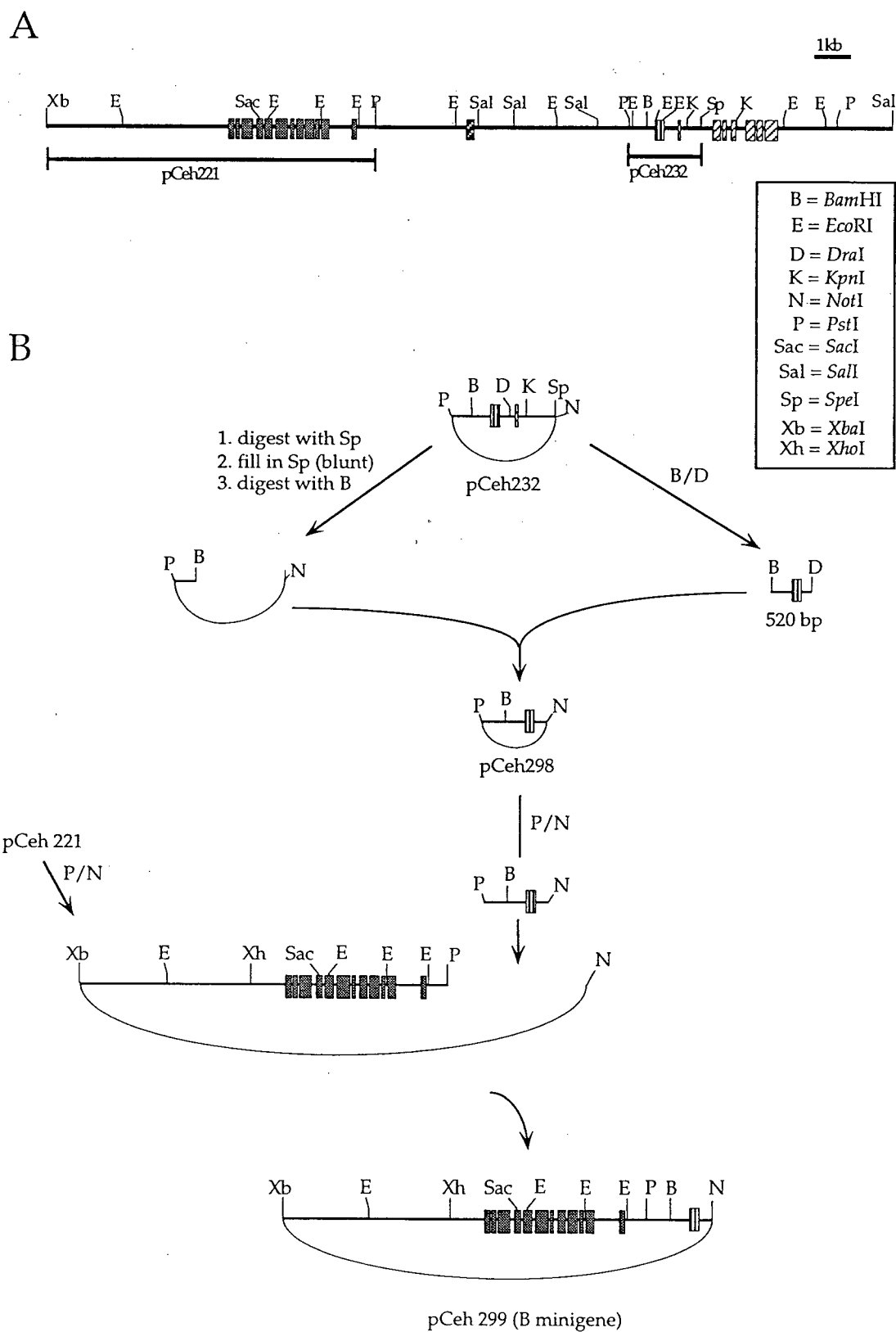
Figure 15. Construction of pCeh301

pCeh301 contains the common region of *bli-4* and the first
exon of blisterases C and D

A.   The substrate clones used

B.   A schematic representation of the cloning steps involved

A

B

Figure 16. Construction of pCeh308, the C short minigene

A. The substrate clones used

B. A schematic representation of the cloning steps involved

A

Xb E Xh Sac E E E P E Sal Sal E Sal Xh PE Xb B EEK Sp Sac K E E P Sal

1kb

pCeh220

pCeh232

pCeh180   toXh

B = *Bam*HI
Bcl = *Bcl*I
E = *Eco*RI
H = *Hind*III
K = *Kpn*I
N = *Not*I
P = *Pst*I
Sac = *Sac*I
Sal = *Sal*I
Sma = *Sma*I
Sp = *Spe*I
Xb = *Xba*I
Xh = *Xho*I

B

pCeh180 →
1. isolate 953 bp K frag
2. fill in K (blunt)
3. clone in pBSSK (Sma)

H Bcl Sal (pBSSK)
pCeh303

P B K H
pCeh232

insert 990 bp B-H fragment into pBSSK

replace Bcl-Sal fragment with same portion from blisterase C cDNA (pCeh199)

H Bcl H
Sal
pCeh304

1.1 kb H fragment

B K H
K(pBSSK)
pCeh305

H

B K H H
K
pCeh306

isolate 1.2 kb K fragment

K K

pCeh301
K

Xb E Xh Sac E E E B K K

pCeh308 (C short minigene)

Xb E Xh Sac E E E B K K

Figure 17. Construction of pCeh309, the C long minigene

A.   The substrate clones used

The fragment containing the *unc-54* 3' UTR was cloned out

from pPD49.26 (provided by A. Fire)

B.   A schematic representation of the cloning steps involved

A

Xb E Xh Sac E E E P E Sal Sal E Sal Xh P E Xb E E K Sac K E E P Sal

B Sp

1kb

pCeh220

pCeh232

pCeh180 toXh
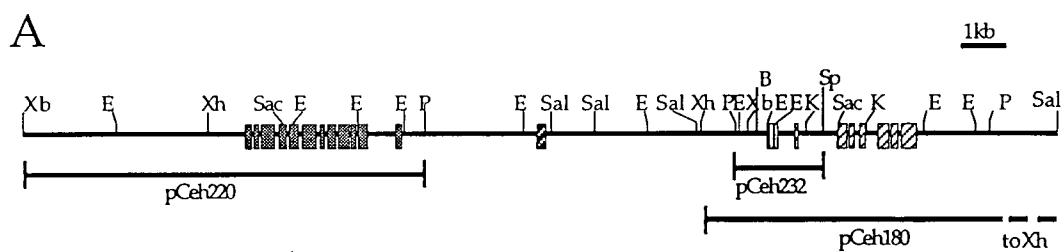
B = *Bam*HI
E = *Eco*RI
RV = *Eco*RV
H = *Hind*III
K = *Kpn*I
P = *Pst*I
Sac = *Sac*I
Sal = *Sal*I
Sp = *Spe*I
Xb = *Xba*I
Xh = *Xho*I

B

B K RV

K

pCeh306

insert *unc-54* 3' UTR

(750 bp RV / Sp (blunted)
fragment) at RV

B K RV K

pCeh307

isolate 1.9 kb
K fragment

pCeh301

K

Xb E Xh Sac E E E B K K

K K

K

pCeh309 (C long minigene)

Xb E Xh Sac E E E B K K

Genomic sequences of blisterase C

pCeh308 (C short)
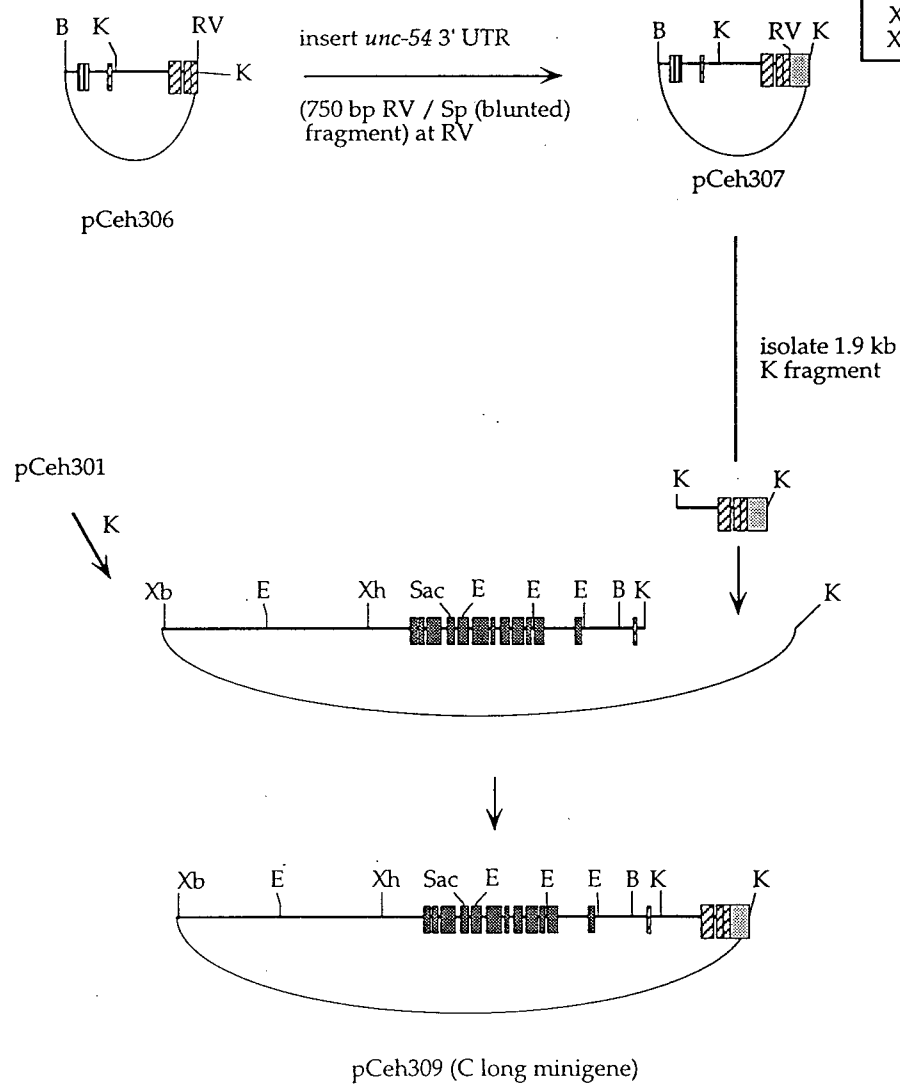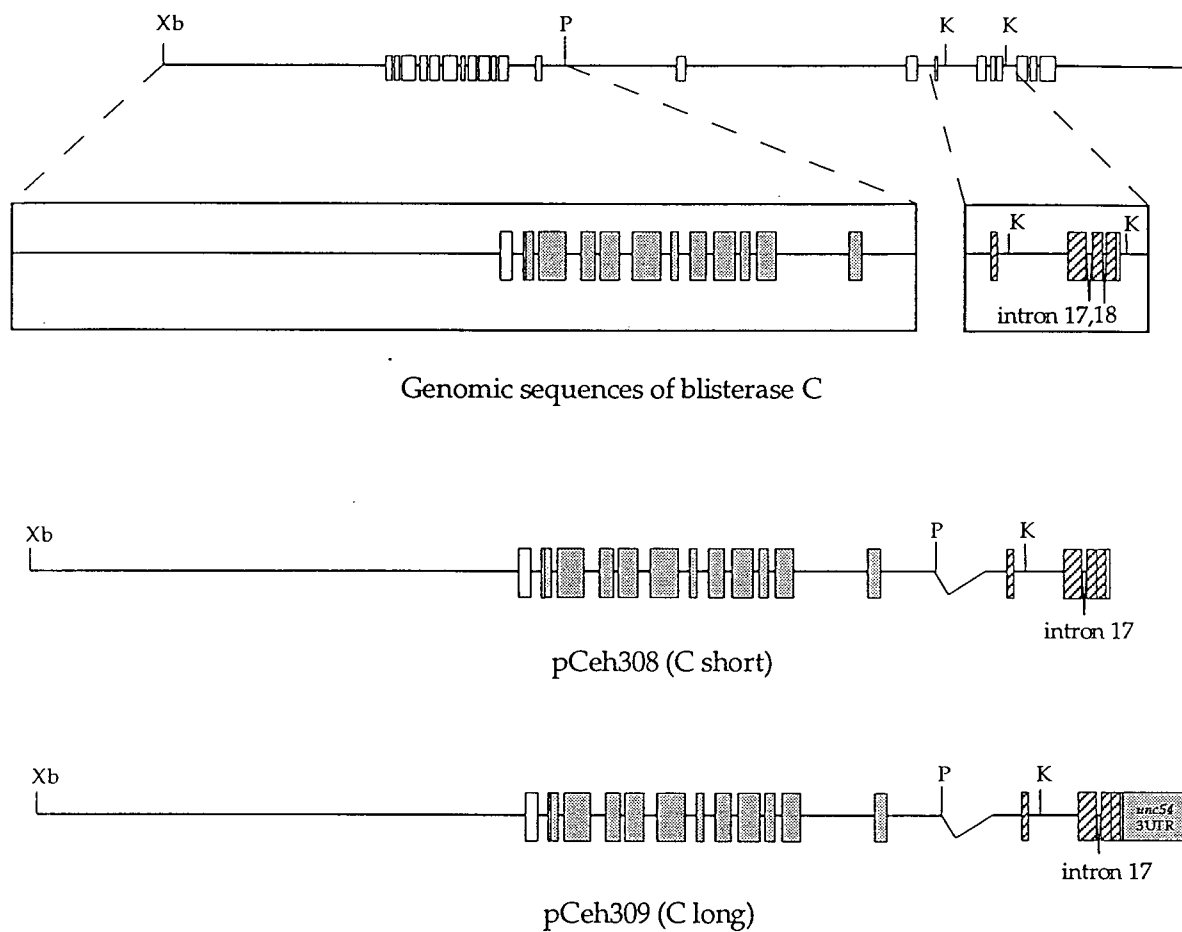
pCeh309 (C long)

Figure 18.   Comparison of the genomic sequences of the 3' end of blisterase C
with pCeh308 (C short), and pCeh309 (C long).

Both pCeh308 and pCeh309 excluded intron 18 to eliminate the potential
production of the C variant transcript (Figure 13).

Hatched boxes represent exons specific to blisterase C.
Restriction enzymes: Xb(*Xba*I); P (*Pst*I); K (*Kpn*I)

to the transformation marker pRF4 (also at 50 µg/ml) when injecting a low concentration of minigene. The injection procedures were kindly performed on *e937* homozygotes by D. Janke (Simon Fraser University). Resulting stable transgenic lines were tested by PCR using specific primers to confirm the presence of minigene constructs (Figure 7, 8). Most of the lines demonstrated similar transmission frequencies (~ 40-50%) of the transgenic arrays and were maintained by selectively transferring rollers. A summary of lines generated by injections of different concentrations of the minigenes is recorded in Table 2.

Section III.   Estimation of copy number of minigene

A semi-quantitative PCR-Southern analysis was used to determine the copy numbers of minigenes in the transgenic strains (Han and Sternberg, 1991). The use of PCR for quantitation analysis relies on the fact that the amount of PCR product is a linear function of input templates when other components (such as primers, Mg^{++}, etc) remain in excess in the initial stage of amplification (Robinson and Simon, 1991). In this study, genomic DNA from single transgenic animals was isolated, and used as the template for PCR. Copy number was measured by the relative abundance of a DNA fragment of the common region of *bli-4* in the transgenic animals to the same fragment in

Table 2. Transgenic strains constructed in this study

Strain	Minigene[a]	Injection concentration of minigene[b]	Segregant	Transmission frequency of Ex. array (%)[c]	Genotype
KR3228	A	High	I	52	*bli-4(e937);hEx104*
KR3229	A	High	II	nd	*bli-4(e937);hEx105*
KR3230	A	High	III	nd	*bli-4(e937);hEx106*
KR3231	A	Low	I	20	*bli-4(e937);hEx107*
KR3232	B	High	I	nd	*bli-4(e937);hEx108*
KR3233	B	High	II	25	*bli-4(e937);hEx109*
KR3234	B	High	III	nd	*bli-4(e937);hEx110*
KR3235	B	Low	I	53	*bli-4(e937);hEx111*
KR3236	B	Low	II	36	*bli-4(e937);hEx112*
KR3237	B	Low	III	37	*bli-4(e937);hEx113*
KR3238	B	Low	IV	24	*bli-4(e937);hEx114*
KR3239	B	Low	V	15	*bli-4(e937);hEx115*
KR3240	CS	High	I	nd	*N2;hEx116*
KR3241	CS	High	II	nd	*N2;hEx117*
KR3242	CS	High	III	nd	*N2;hEx118*
KR3243	CS	High	IV	nd	*N2;hEx119*
KR3244	CS	Low	I	nd	*bli-4(e937);hEx120*
KR3245	CS	Low	II	100	*bli-4(e937);hEx121*

Table 2 (Cont'd)

Strain	Minigene[a]	Injection concentration of minigene[b]	Segregant	Transmission frequency of Ex. array (%)	Genotype
KR3246	CS	Low	III	35	*bli-4(e937);hEx122*
KR3247	CS	Low	IV	24	*bli-4(e937);hEx123*
KR3248	CS	Low	V	68	*bli-4(e937);hEx124*
KR3249	CS	Low	VI	17	*bli-4(e937);hEx125*
KR3250	CS	Low	VII	39	*bli-4(e937);hEx126*
KR3251	CS	Low	VIII	43	*bli-4(e937);hEx127*
KR3252	CL	High	I	42	*bli-4(e937);hEx128*
KR3253	CL	High	II	31	*bli-4(e937);hEx129*
KR3357	CS	-	-	22	*bli-4(e937);hEx117*
KR3333	A	-	-	nd	*dpy-5(e61) bli-4(h791) unc-13(e450);hEx104*
KR3334	B	-	-	nd	*dpy-5(e61) bli-4(h791) unc-13(e450);hEx109*

[a] A = pCeh226; B = pCeh299; CS = pCeh308; CL = pCeh309

[b] High = 50 µg/ml; Low = 1 µg/ml

[c] indicated by percentage of Rol; nd: not determined

N2 animals. A control set of primers that amplify part of the 5′ portion of the putative *rec-1* gene was included in every reaction to compare individual reaction efficiencies (C. Wicky, pers. com.). Judging from the intensities of the control bands of the different reactions, the efficiency of each single reaction appears reasonably uniform to allow cross comparison (Figure 19). Amplification was only allowed for 12 cycles to ensure all reaction components remained in excess and the linear relationship between quantities of templates and products still maintained (**Appendix I**). The resulting PCR product was subject to Southern analysis and the signals were quantified by densitometry.

In general, the data indicated that the transgenic lines generated by injection of high concentration (50 µg/ml) of minigene contained about 10-20 copies of transgenes on average, which is about five-fold higher than those lines generated by injection of low concentration (1 µg/ml) of minigene (Table 3). In particular, the signal from KR3231, obtained from injection of low copy number of pCeh226, the A minigene, could not readily be distinguishable from that of the wild-type control, indicating that the copy number of pCeh226 in this strain was very low (<1 according to our measurement).

Figure 19. Estimation of minigene copy number by PCR/Southern analysis

Panels A-D display Southern blots of PCR products from DNA of a single
animal used for the estimation of minigene copy number in the transgenic
lines tested for rescue of *bli-4* phenoytpes:

Panel	Lane	Strain
A	1, 2, 3	KR3231 (A Low)
A	4, 5, 6	KR3228 (A High)
A	7, 8, 9	N2
B	1, 2	KR3235 (B Low)
B	3, 4, 5	KR3239 (B Low)
B	6, 7, 8	KR3233 (B High)
B	9, 10, 11	KR2868 ("B")
C	1, 2, 3	KR3245 (C Short Low)
C	4, 5, 6	KR3246 (C Short Low)
C	7, 8, 9	KR3357 (C Short High)
C	10, 11, 12	KR3252 (C Long High)
D	1, 2, 3	N2
D	4, 5, 6*	KR3252

The top band in each panel represents the PCR product of *bli-4* seqeunces, whereas
the bottom band represents the PCR product of the putative *rec-1* gene, as a positive
control (see Figure 9 for explanation). The intensity of each band was scanned
and used in the estimation of copy number of minigene (See **Appendix I** for
calculation).

* These three lanes serve to demonstrate the linear relationship between input
  template and resulting products under the reaction conditions used in this
  experiment. Lane 5 contains double the amount of template DNA in lane 4,
  whereas lane 6 contains double the amount in lane 5. See **Appendix I** for the
  evaluation of the linear relationship.
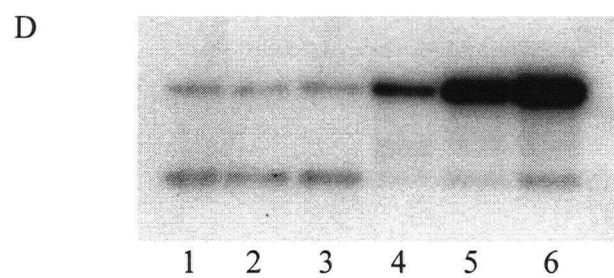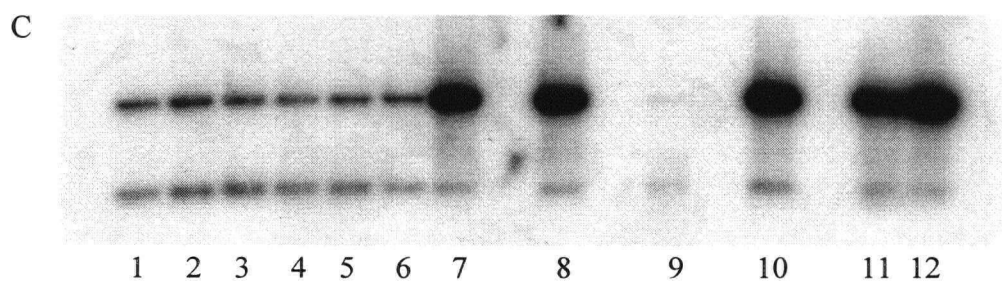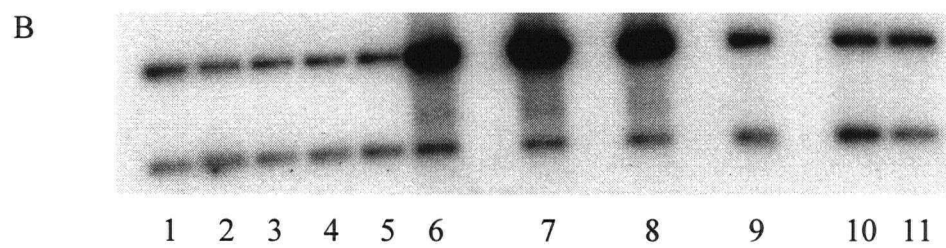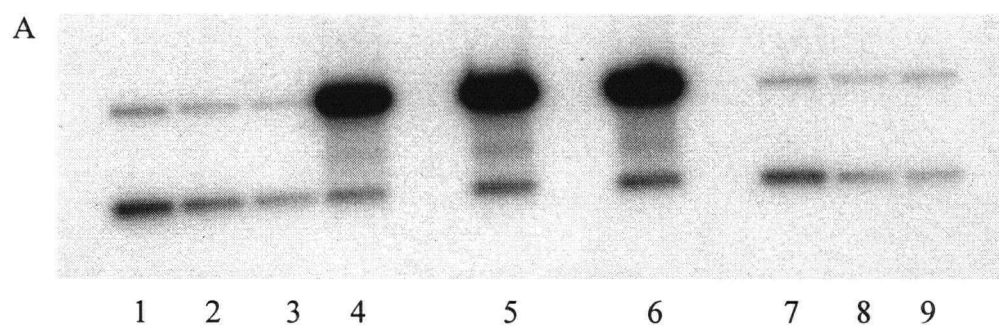
Table 3. Estimation of minigene copy number

Transgenic Line	Transgenic array	Construct/ Minigene	Injection concentration of minigene (High = 50 ng/µl Low = 1 ng/µl)	Copy Number (± 1 SD)
KR3231	hEx107	pCeh226/A	Low	0.7 ± 0.1
KR3235	hEx111	pCeh299/B	Low	2.2 ± 0.9
KR3239	hEx115	pCeh299/B	Low	1.9 ± 0.1
KR3245	hEx121	pCeh308/CS	Low	2.9 ± 0.7
KR3246	hEx122	pCeh308/CS	Low	2.8 ± 1.1
KR3228	hEx104	pCeh226/A	High	16.3 ± 3.6
KR2868*	hEx46	pCeh238/'B'	N/A	3.5 ± 1.0
KR3233	hEx109	pCeh299/B	High	9.7 ± 1.9
KR3357	hEx117	pCeh308/CS	High	20.5 ± 0.7
KR3252	hEx128	pCeh309/CL	High	21.5 ± 4.9

*KR2868 was constructed by Srayko (1995). The injection concentration of pCeh238 was not available.

Section IV.   Rescue of blistering using *bli-4* minigenes

Measure of rescue

The population frequency of blistered animals among transgenic rollers of stable lines was measured to determine the rescuing abilities of individual minigenes. Blistering frequency among rollers was compared to blistering frequency among non-rollers from the same line because both populations possessed identical genetic backgrounds except the presence of extrachromosomal arrays in the former. It is observed that the percentage of blistered animals among rollers was always lower than that among non-rollers within the same line (Tables 4-7). This observation was also true to a control transgenic line, KR2872, which contained only the transformation marker pRF4 (Tables 4), suggesting blistering was partially suppressed by the rolling phenotype (**Appendix III**). In order to control for the effect of the rolling phenotype on blistering, rescue was assessed by comparing the percentage of blistered animals among rollers of each line to the same measurement in KR2872. The rescue result is arbitrarily regarded as positive when <10% blistered roller was observed. When the frequency of blistering

Table 4. Rescue of blistering by *bli-4* minigenes at high copy number*

Construct/ minigene	Transgenic Line	% Blistering among non-Rollers	% Blistering among Rollers
pCeh226/A	KR3228	84.3 (507/601)	0 (0/640)
pCeh299/B	KR3233	60.0 (424/707)	0 (0/236)
pCeh238/'B'[a]	KR2868	73.1 (657/899)	31.6 (136/430)
pCeh308/CS	KR3357	86.1 (827/960)	61.8 (162/262)
pCeh309/CL	KR3252	86.2 (580/673)	0 (0/478)
pCeh309/CL	KR3253	81.6 (231/283)	0 (0/128)
pRF4	KR2872	86.8 (548/631)	70.8 (235/332)
-	CB937	91.1 (246/270)	-

* KR2868 contained an intermediate copy number in comparison to other transgenic lines generated in this study (Table 3). The copy number of KR2872, the control line, was not determined.

[a]pCeh238 is a blisterase B subclone that also includes the first exon specific to blisterases C and D (Figure 20).

Table 5. Rescue of blistering by pCeh226 (A minigene) at low copy number

Transgenic Line	% Blistering among non-Rollers	% Blistering among Rollers
KR3231	92.0 (1058/1150)	67.1 (194/289)

Table 6. Rescue of blistering by pCeh299 (B minigene) at low copy number

Transgenic Line	% Blistering among non-Rollers	% Blistering among Rollers
KR3235	88.9 (335/377)	0 (0/431)
KR3236	87.2 (156/179)	8.9 (9/101)
KR3237	64.6 (62/96)	5 (3/57)
KR3238	75.7 (115/152)	59.6 (28/47)
KR3239	77.3 (586/758)	66.2 (88/133)

Table 7. Rescue of blistering by pCeh308 (C short minigene) at low copy number

Transgenic Line	% Blistering among non-Rollers	% Blistering among Rollers
KR3245*	na	0 (0/1176)
KR3246	89.1 (825/925)	67.2 (334/497)
KR3247	83.6 (286/342)	72.6 (77/106)
KR3348	77.2 (44/57)	41.5 (51/123)
KR3249	86.8 (244/281)	89.7 (52/58)
KR3250	69.4 (193/278)	63.5 (115/181)
KR3251	90.5 (86/95)	87.5 (63/72)

* putative integrated line (100% Rollers)

among rollers is comparable (± 10%) with the control line KR2872, the rescue

is considered negative.

A. **pCeh226, the blisterase A minigene, rescued blistering at high, not**
low, copy number

The plasmid construct pCeh226 was chosen as the blisterase A

minigene in this study because it demonstrated the "best" ability among other

blisterase A subclones to rescue the lethal phenotype in the previous study

(Srayko, 1995). In the present study, KR3228, containing at least 16 copies of

pCeh226, demonstrated complete rescue of blistering as expected (Table 4).

Only one stable line, KR3231, was obtained from injection of pCeh226 at low

concentration. The copy number of pCeh226 in KR3231 was very low and

blistering was not rescued in this strain (Table 5).

B.   pCeh299, the blisterase B minigene, rescued blistering at high copy

number; a low copy number of pCeh299 also rescued blistering in

particular lines

pCeh299 was designed to contain only exon 14, the exon specific to

blisterase B, at the 3′ end. It differs from the subclone constructed by Srayko

(1995), pCeh238, which additionally contained the first blisterase C/D exon,

exon 15 (Figure 20). KR2868, containing pCeh238, demonstrated only a partial

rescue of blistering, in agreement with previous result (Table 4). On the other

hand, complete rescue of blistering was shown by KR3233 with pCeh299

(Table 4). We found that KR2868 only contained about four copies of pCeh238

whereas KR3233 contained about ten copies of pCeh299 (Table 3). The

difference in copy number may therefore account for the difference in rescue

results demonstrated by the two strains.

Five lines were generated from injection of pCeh299 at low

concentration. KR3235 showed complete rescue of blistering while KR3236

and KR3237 showed ~90% rescue. The other two lines were considered

negative for rescue (Table 6). The copy numbers in KR3235 and KR3239 were

determined. The insignificant difference in copy number is unlikely to

contribute to the difference in rescuing performance (Table 3).
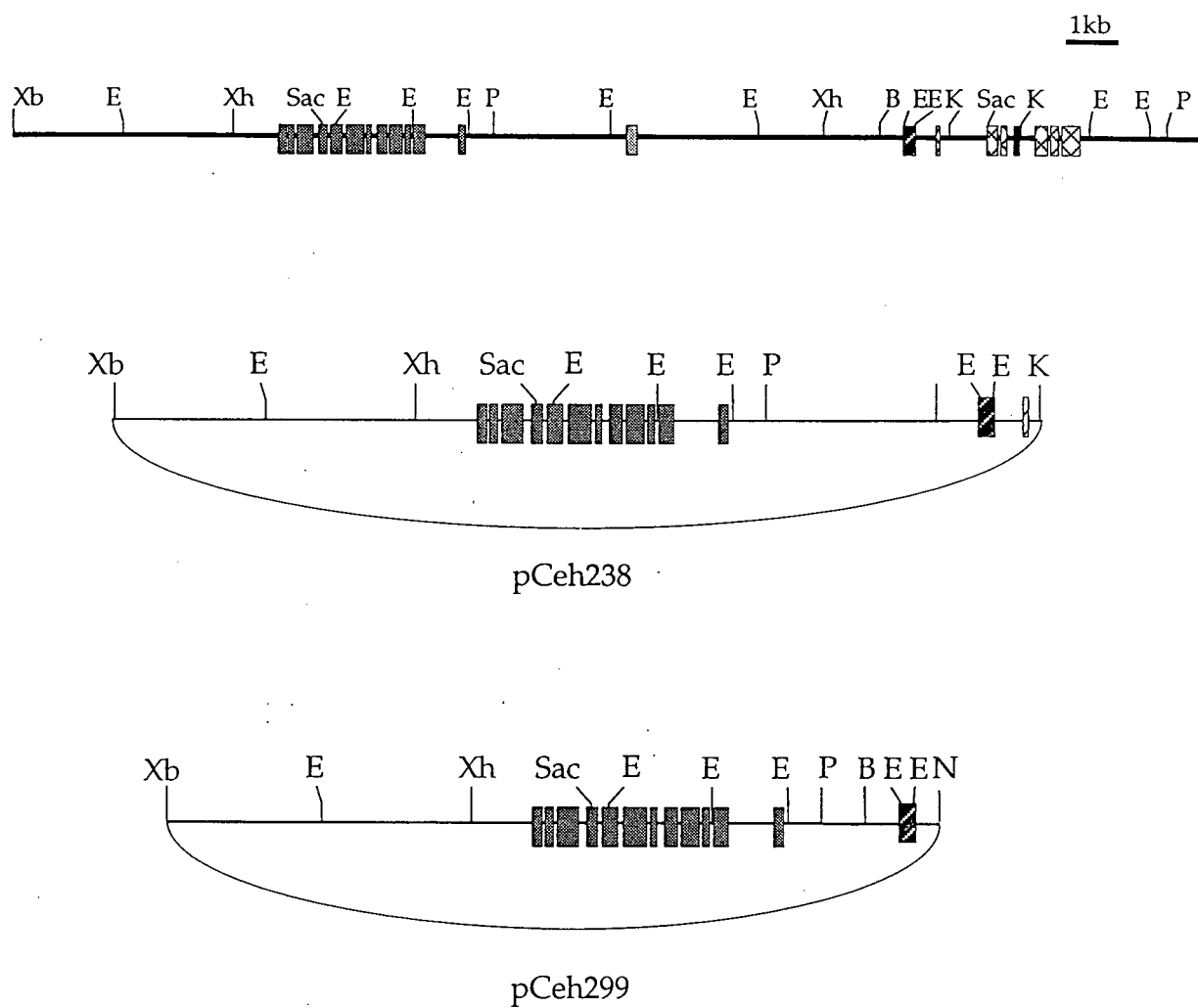
Figure 20 . A comparison of pCeh238 (Srayko, 1995) and pCeh299

Restriction enzymes: (Xb) *Xba*I; (Xh) *Xho*I; (P) *Pst*I; (Sal) *Sal*I; (E) *Eco*RI; (K) *Kpn*I; (B) *Bam*HI; (N) *Not*I.

C. pCeh308, the blisterase C short minigene, did not rescue blistering at high copy number

pCeh308, the C short minigene, was injected at high concentration into N2 animals and introduced into the *e937* background by genetic crosses. The resulting transgenic line KR3357 contained about 20 copies of the minigene but displayed negative rescue of blistering (Table 4).

D. A putative integrated line containing a low copy number of the C short minigene demonstrated complete rescue of blistering

Six of the eight stable lines generated by injection of pCeh308 at low concentration, including KR3246 which contained about three copies of the minigene (Table 3), displayed negative rescue of blistering (Table 7). One line, KR3245, stably produced 100% rollers and completely rescued blistering (Table 7). A rare integration event may have occurred during transformation. KR3245 contained a copy number of the C short comparable to KR3246 (Table 3). The difference in rescue results between these strains indicates pCeh308 is able to rescue blistering when integrated.

E.   pCeh309, the blisterase C long minigene, rescued blistering at high copy number

pCeh309 at a high copy number was found able to completely rescue blistering in KR3252 and KR3253 (Table 4). A comparison of KR3252 with the negative rescue shown by KR3357, which contains a similar copy number of pCeh308, indicates that the *unc-54* 3′ UTR may have enhanced the rescuing ability of the C minigene.

Section V.   Rescue of lethal alleles using *bli-4* minigenes

Measure of rescue

Two lethal *bli-4* alleles were tested for rescue by the minigenes: the class II *h791* allele, and the single class III allele, *s90*. *h791* is a nonsense mutation on Cys-245 (TG<u>T</u>→TG<u>A</u>) in exon 6 (Figure 21). In this mutant, premature termination of translation within the protease domain is predicted, leading to truncated BLI-4. The molecular lesion in *s90* is not identified yet.

Extrachromosomal arrays carrying various minigenes were crossed into lethal strains based on the scheme depicted in Figure 10. To determine the extent of rescue, the percentage of rescued animals was compared to the
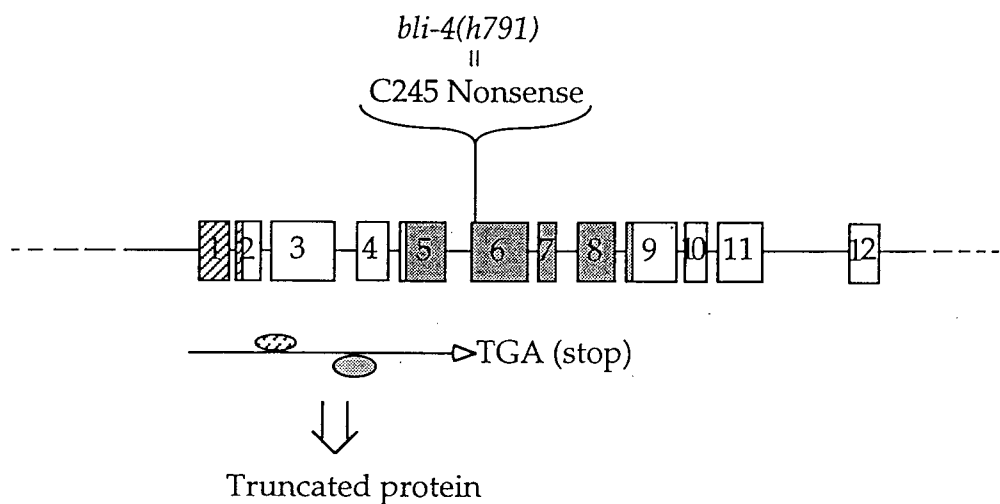
Figure 21. Location and molecular lesion of *h791*.

An EMS-induced transition changed Cys-245 to a premature stop codon
(TG<u>T</u>->TG<u>A</u>), leading to the production of truncated protein. *h791* is
therefore a potential null allele of *bli-4*. (hatched boxes represent the
5' UTR and shaded boxes represent the protease domain)

transmission frequency of the extrachromosomal array (Table 8). A complete rescue was evident when the results from both measurements were similar. The genotype of rescued animals were scrutinized for the production of dead eggs. In addition, the rolling phenotype is epistatic to both the Dpy and Unc phenotype. Therefore, rescued animals were outcrossed with N2 males and the resulting production of Rol progeny verified the presence of extrachromosomal arrays in the putative rescued lines. In particular cases putatively rescued animals were found to perish after being transferred before adulthood. This indicated an incomplete rescue of the lethal allele by the minigene.

A.   pCeh226, the blisterase A minigene, rescued *bli-4* lethal alleles at high copy number

The array *hEx104*, containing a high copy number of pCeh226, rescued both *h791* and *s90*, in agreement with previous experimental results (Srayko, 1995; Table 8A). However, *hEx107*, containing a very low copy number of pCeh226, did not manage to rescue either lethal mutant (Table 8B).

Table 8. Rescue of lethal alleles by *bli-4* minigenes

A.

Ex. array	Construct/ Minigene	Copy number	Lethal mutant	Non-Rol[a]	Rol	Dpy Unc	Dead Egg	% Rol[b]	% Rescue[c]
hEx104	pCeh226/A	High	*s90*	1290	889	225	269	41	41
hEx104	pCeh226/A	High	*h791*	1067	1353	306	252	56	55
hEx109	pCeh299/B	High	*s90*	1155	543	148	200	32	34
hEx109	pCeh299/B	High	*h791*	1224	409	72	279	25	20
hEx128	pCeh309/CL	High	*s90*	1067	773	193	247	42	41[d]

The above shows the arrays that completely or partially rescued the *bli-4* lethal phenotype. Listed is the progeny produced from *dpy-5 bli-4(let) unc-13/+ bli-4(e937) +; hExN*, where *hExN* represents the extrachromosomal array generated by injection (Figure 10). % Rol is compared to % Rescue, and the fecundity of rescued animals is assessed to determine the extent of rescue.

[a] Non-Rol includes both Bli and WT non-rolling animals

[b] % Rol is calculated as Rol/(Rol + Non-Rol), which indicates the transmission frequency of the extrachromosomal array

[c] % Rescue is calculated as Dpy Unc/(Dpy Unc + Dead Egg)

[d] Dpy Unc progeny from this cross perished without producing progeny. Therefore, the rescue is considered partial.

67

Table 8.   Cont'd

B.

Progeny were not scored when only dead eggs but no Dpy Unc progeny, indicating a negative rescue result, were spotted.   A minimum of five animals of the above genotype were examined (indicated by the production of dead eggs) for the non-rescuing arrays.   Arrays tested negative for rescue of both of the lethal alleles, *s90* and *h791* are summarized below:

Ex. array	Construct/Minigene	Copy number
hEx107	pCeh226/A	Low
hEx111	pCeh299/B	Low
hEx115	pCeh299/B	Low
hEx117	pCeh308/CS	High
hEx121	pCeh308/CS	Low
hEx122	pCeh308/CS	Low

B. **pCeh299, the blisterase B minigene rescued *bli-4* lethal alleles at high copy number**

The array *hEx109*, containing a high copy number of pCeh299, was found able to rescue both *h791* and *s90* (Table 8A). Two arrays, which contained similar numbers of low copies of pCeh299 but demonstrated different rescue results of blistering, *hEx111* from KR3235 and *hEx*115 from KR3239 (Tables 3 and 6), both failed to rescue either of the lethal alleles (Table 8B).

C. **pCeh308 and pCeh309, the blisterase C minigenes, failed to rescue *bli-4* lethal alleles**

None of the arrays tested carrying either pCeh308 or pCeh309 at various copy numbers, managed to completely rescue either *h791* or *s90* (Table 8B). Although Dpy Unc animals were observed from the introduction of *hEx128*, which contained a high copy number of pCeh309 (the C long minigene), into *s90*, none of the animals survived and therefore a partial rescue was registered (Table 8A).

Discussion

The *bli-4* gene of *C. elegans* encodes four structurally distinct products by alternative splicing that are homologous to the kexin family of proprotein convertases. In addition to the blistering phenotype of the original allele *e937*, the embryonic or early larval phenotype of potential null alleles suggests *bli-4* plays a role not only in adult morphology but also in the early development of the animal. Based on the strategy adopted by Srayko (1995), this study further dissected the function of individual blisterases by using isoform-specific *bli-4* minigenes to rescue different classes of *bli-4* mutants. Four minigenes individually encoding blisterase A, blisterase B, and blisterase C were tested for rescue at both high and low copy number. The results indicated functional redundancy between blisterases A and B. The relatively inferior rescuing ability of the C minigenes implied a restricted functional role of blisterase C *in vivo*.

High copy number of exogenous blisterases A and B rescued blistering

The present data indicated that both blisterases A and B were able to rescue the blistering phenotype (Table 4). pCeh226, the A minigene, has been shown previously to completely rescue blistering (Srayko, 1995). The

experiment was repeated with the injection concentration monitored at 50 μg/ml in this study, and the positive rescue result was confirmed. As the blistering phenotype is the result of a deletion that causes the removal of the exon specific to blisterase A, the positive rescue result by pCeh226 is expected. pCeh299, the B minigene, also demonstrated complete rescue of blistering at high copy number in *e937* animals. This is not surprising as blisterases A and B bear a high degree of structural similarity. The two isoforms differ in the length of the carboxyl tail in which no special structural features can be identified from sequence analysis. Previously, an RT-PCR experiment, although not fully quantitative, detected an increased level of the transcript of blisterase B in *e937* animals. This suggests that the expression of blisterase B may be elevated to partially compensate for the loss of blisterase A in *e937*, and thus lead to the observed reduced penetrance of the blistering phenotype (Thacker et al., 1995). The rescue of blistering by both pCeh226 and pCeh299 support the notion of potential functional redundancy between blisterases A and B.

pCeh299 and pCeh238 demonstrated different rescue results

Previously, it was reported that a blisterase B subclone, pCeh238, only partially rescued blistering (Srayko, 1995). This contrasts the present positive rescue result demonstrated by pCeh299. pCeh238 differs from pCeh299 by its

incorporation of exon 15, the first blisterase C/D exon, and adjacent intronic sequences (Figure 20). As speculated before, the common region in pCeh238 could possibly be spliced onto exon 15 and hence produced an aberrant transcript, while simultaneously decreasing the production of functional blisterase B transcripts (Srayko, 1995). In the present study, a transgenic line containing pCeh268, KR2868, was picked and the test of blistering was repeated. A partial rescue was recorded, in agreement with the previous result (Table 4). KR2868 was included in the experiment to determine the copy number of transgenes. It was revealed that KR2868 contained a lower copy number of pCeh238 than pCeh299 in KR3233. This, in addition to the proposed undesired splicing event in pCeh238 as described, may explain why only a partial rescue of blistering was observed in KR2868.

The *unc-54* 3′ UTR improved rescuing ability of pCeh308

pCeh308, the C short minigene, did not rescue blistering at high copy number. However, pCeh309, which is identical to pCeh308 except with the addition of the *unc-54* 3′ UTR, completely rescued blistering at a comparable copy number (Figure 22). The *unc-54* 3′ UTR is commonly employed to serve as the 3′ element for ectopic gene expression in *C. elegans* (Fire et al., 1990). Although *unc-54* encodes the major myosin heavy-chain isoform in the animal, its 3′ UTR was shown not to confer any tissue specificity for gene
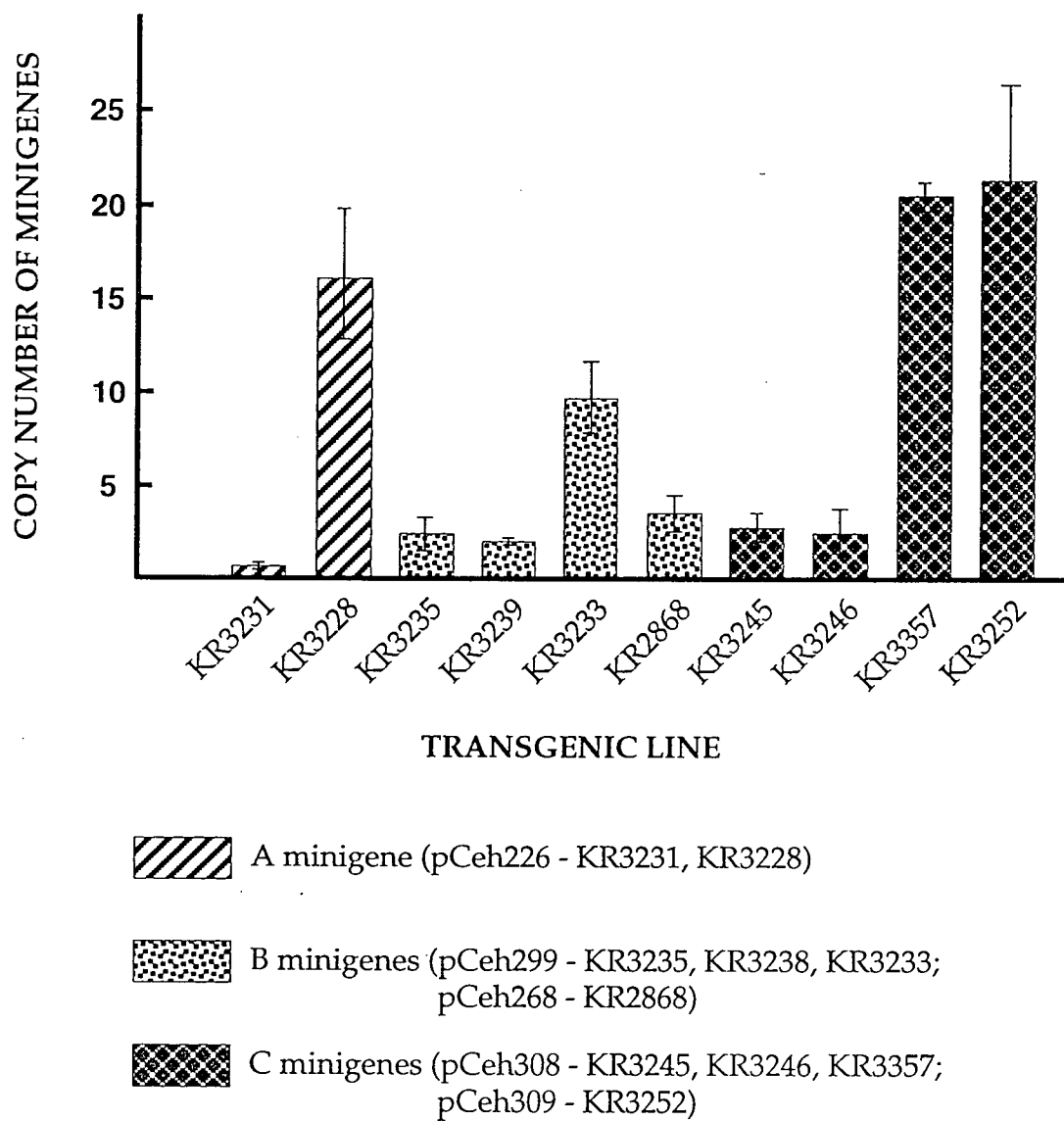
Figure 22.   A comparison of minigene copy number in different transgenic lines

expression (Okkema et al., 1993). Instead, this element permits expression in a wide range of different tissues and cell types specified by the upstream sequences (Fire et al., 1990). It has been known that many 3′ UTRs function to stabilize messenger RNAs (Sachs, 1993). This may be the mechanism by which the *unc-54* 3′ UTR contributes to improving the rescuing ability of pCeh309 over pCeh308.

The natural 3′ UTR of blisterase C is only 31 nucleotides long and is unusually short when compared with other *C. elegans* genes. The poor rescuing ability of pCeh308 may be due to the short length of the 3′ UTR of blisterase C. In agreement with this reasoning, the endogenous amount of blisterase C transcripts was undetectable by Northern analysis (Thacker et al., 1995). Restricted expression of blisterase C indicates that this isoform may have a restricted functional role in the animal.

Extremely low copy number of blisterase A did not rescue blistering

KR3231 was the only stable line generated containing a low copy number of pCeh226, the A minigene. It was expected that an exogenous provision of the genetic material of blisterase A, which has been specifically deleted in the mutant animal, at even a low copy number may be sufficient for rescuing of the blistering phenotype. However, KR3231 did not demonstrate complete rescue of blistering. Nonetheless, the negative result

of KR3231 could be accounted for by the extremely low copy number of

pCeh226 as compared to the copy numbers in other transgenic lines. The copy

number was so low that the corresponding signal could not be differentiated

from that from the non-transgenic control (Figure 22). Therefore, the

comparison of the rescuing ability of pCeh226 as from KR3231 with the other

minigenes at low copy numbers was inconclusive.

Low copy number of blisterase B rescued blistering

A group of five stable lines were generated by injecting a low

concentration of pCeh299. Three of the five lines demonstrated complete or

almost complete rescue blistering while the remaining two were considered

negative for rescue (Table 6). The difference in results could probably not be

caused by a difference in minigene copy number because KR3235, which

demonstrated complete rescue, and KR3239, which displayed substantial

amount of blistered rollers, were found to contain similar copy numbers of

pCeh299 (Figure 22). The difference should also not be the result of a

spontaneous second-site suppressor of blistering in the three rescued lines as

the blistering frequency among non-rollers within these lines remains

normally high (Table 6). We propose that the lines that showed rescue may

contain more functional copies of the minigene (hence more transcripts) on

their extrachromosomal arrays than the lines that were tested negative. This

is possible because extrachromosomal arrays were independently assembled during transformation (Mello et al., 1991). This hypothesis could not be detected by our use of PCR to determine the copy number of transgenes. However, Southern analysis can be applied to detect if structural difference indeed exists between the arrays (Mello et al., 1991). The demonstration of rescue of blistering by a low copy number of pCeh299 in three out of five low copy transgenic lines indicates that blisterase B is likely to function in the assembly or maintenance of the adult cuticle *in vivo*.

Integration improved rescuing ability of pCeh308

A putative integrated line, KR3245, was generated from injecting pCeh308 at low concentration, as evidenced from 100% transmission of the Rol phenotype. Blistering was completely rescued in this line (Table 7). On the other hand, the other six non-integrated lines generated from the same injection event failed to completely rescue blistering (Table 7). Integration of the extrachromosomal array into the genome ensures faithful transmission of the transgenic material during mitotic cell divisions and therefore permits its expression in every tissue of the organism. As a result, expression of the transgene in the correct tissue is presumably secured. As discussed, one possible explanation for the poor rescuing ability of pCeh308 may be that the short 3′ UTR failed to stabilize the blisterase C transcript. Integration of the

minigene may provide correct tissue localization so that even transient expression from pCeh308 may be sufficient for correcting the blistering defect. Alternatively, integration *per se* may stabilize the expression of transgenic array. Furthermore, the site of integration may mediate the expression of the transgene. For example, pCeh308 may be juxtaposed near a collagen gene which is expressed at the adult stage so that co-expression occurs and promotes the function of blisterase C. Genetic mapping of the integration site in KR3245 can be performed to test this hypothesis.

rol-6, the transformation marker, is a potential *bli-4* substrate

A positive rescue result for the blistering phenotype was defined in this report as a complete absence of blistered animals among the transgenic population. However, it is observed from that among the majority of transgenic lines that were considered negative for rescue of blistering, there is a slight decrease of blistering frequency (4-9%) when they are compared to the control line KR2872 (Table 4). This suggests that the different isoforms have at least partial overlapping capability of rescuing blistering. A more noticeable drop in blistering frequency is observed when the comparison is made against non-transgenic, or non-rollers, within the same line. This suggests that the Rol-6 phenotype is a partial suppressor of the Bli-4 phenotype. The *rol-6* gene encodes a cuticle collagen that contains the

hallmark cleavage motif (Arg-X-Arg-Arg↓) for substrates of kexins (Yang et al., 1994). Given *rol-6* as a candidate substrate of *bli-4*, the injection marker pRF4 may contain both temporal and spatial expression signals that interfere with the expression of the adjacent blisterase gene present on the same extrachromosomal array. In future, markers other than cuticular collagens, such as an antisense *unc-4* plasmid, which displays a twitching phenotype, or *unc-22*, which displays an Unc phenotype, could be used for transformation with *bli-4* subclones for precaution against undesired interaction between gene products of co-injected molecules (Mello and Fire, 1995).

Blisterases A, B rescued the lethal alleles *h791* and *s90*

Both pCeh226 and pCeh299, the A and B minigenes, managed to rescue the two lethal alleles *h791* and *s90* completely at high copy number while both the short and long versions of the C minigenes, pCeh308 and pCeh309, and all of the minigenes at low copy numbers failed to do so (Table 8). The A minigene notably demonstrated the best rescuing ability among the four constructs tested in this study - the majority of the rescued animals survived and produced a healthy amount of progeny. On the other hand, only a portion of the animals rescued by the B minigene survived, and they produced less progeny than the animals rescued by the A minigene. This difference may be a result of the lower transmission frequency of the

extrachromosomal array of pCeh299, *hEx109* (Table 8). On the other hand, it may indicate that blisterase A is more specific than blisterase B for the function of *bli-4* at the embryonic stage.

Viable *e937* mutants imply that blisterase A is not specifically essential for development. However, our rescue results indicate that blisterase A is capable of fulfilling the essential role of *bli-4* in development. Preliminary result from RT-PCR on embryos detected expression of blisterase A (Thacker, pers. comm.). This suggests that blisterase A has a functional role at the embryonic stage which can be amplified by high copy number to maintain normal development in the absence of other isoforms.

The rescue by the B minigene also indicates that blisterase B is capable of rescuing *bli-4* lethals when present at high copy number. It is again noted that the positive rescue by pCeh226 contrasts the negative rescue result of pCeh238 reported previously (Srayko, 1995). The difference is probably due to the different structures of the constructs and the different transgene copy numbers between the two strains as discussed above. Blisterase B is not an abundantly expressed *bli-4* isoform as indicated from Northern and RT-PCR analysis using mixed staged worm RNA (Thacker et al., 1995). However, structural similarity between blisterase B and blisterase A may explain the positive rescuing ability of pCeh299.

pCeh309, the C long minigene, partially rescued the class III allele, *s90*. The molecular lesion of *s90* is unknown but its phenotype (30% survive to the L1 larval stage) suggests that *s90* is a weak *bli-4* lethal allele. The

incomplete rescue of a weak embryonic lethal allele demonstrated by the C long minigene indicates that blisterase C is probably not essential for the early development of the animal.

Low copy numbers of blisterases A and B did not rescue the lethal phenotype

That low copy numbers of pCeh226 and pCeh299 did not rescue the lethal alleles may indicate that blisterases A and B are not specific for early development. However, the present data is not convincing enough to support this conclusion. More efforts are required to generate transgenic lines containing higher copy numbers of minigenes. The present DNA concentration (1 µg/ml) used for generating low copy number of transgenes may need to be raised slightly to, for example, 10 µg/ml. This may perhaps provide us with transgenic animals that contain enough copies of, in paritcular, the A minigene to rescue blistering but questionable for the rescue of embryonic lethality.

Blisterase D is likely to play a functional role in early development

Despite the positive rescue results from the A and B minigenes, blisterase D appears the most likely *bli-4* isoform that ensures proper

development inside the embryo. Another construct, pCeh236, which encodes

the blisterases B, C, and D also rescued the embryonic lethal alleles of *bli-4*

(Srayko, 1995; Figure 23). Comparable to blisterase A, blisterase D is also a

highly expressed isoform as shown by Northern analysis (Thacker et al., 1995).

It is, among the four isoforms, most structurally similar to human furin

because of the presence of a TMD. Therefore, we speculate that blisterase D

functions in the constitutive secretory pathway and processes molecules

important to the development of the animal. Although construction of a

minigene for blisterase D was unsuccessful (**Appendix IV**), we predict that

blisterase D would also rescue the *bli-4* lethal phenotype.

BLI-4 is a candidate cuticle-collagen processing enzyme

Various lines of evidence suggest that BLI-4 functions as a cuticle

collagen processing endoprotease in the nematode. The *C. elegans* cuticle is

secreted during the molting process that marks the transition between

different developmental stages of the nematode. Cuticles are made up of

different composition of collagens specific to each developmental stage

(Johnstone, 1994; Kramer, 1994). One of the main functions of the cuticle is to

act as an exoskeleton to model the shape of the animal (Priess et al., 1986).

This functional role is evident in all developmental stages as cuticular defects

lead to a variety of morphological phenotypes including early larval lethality
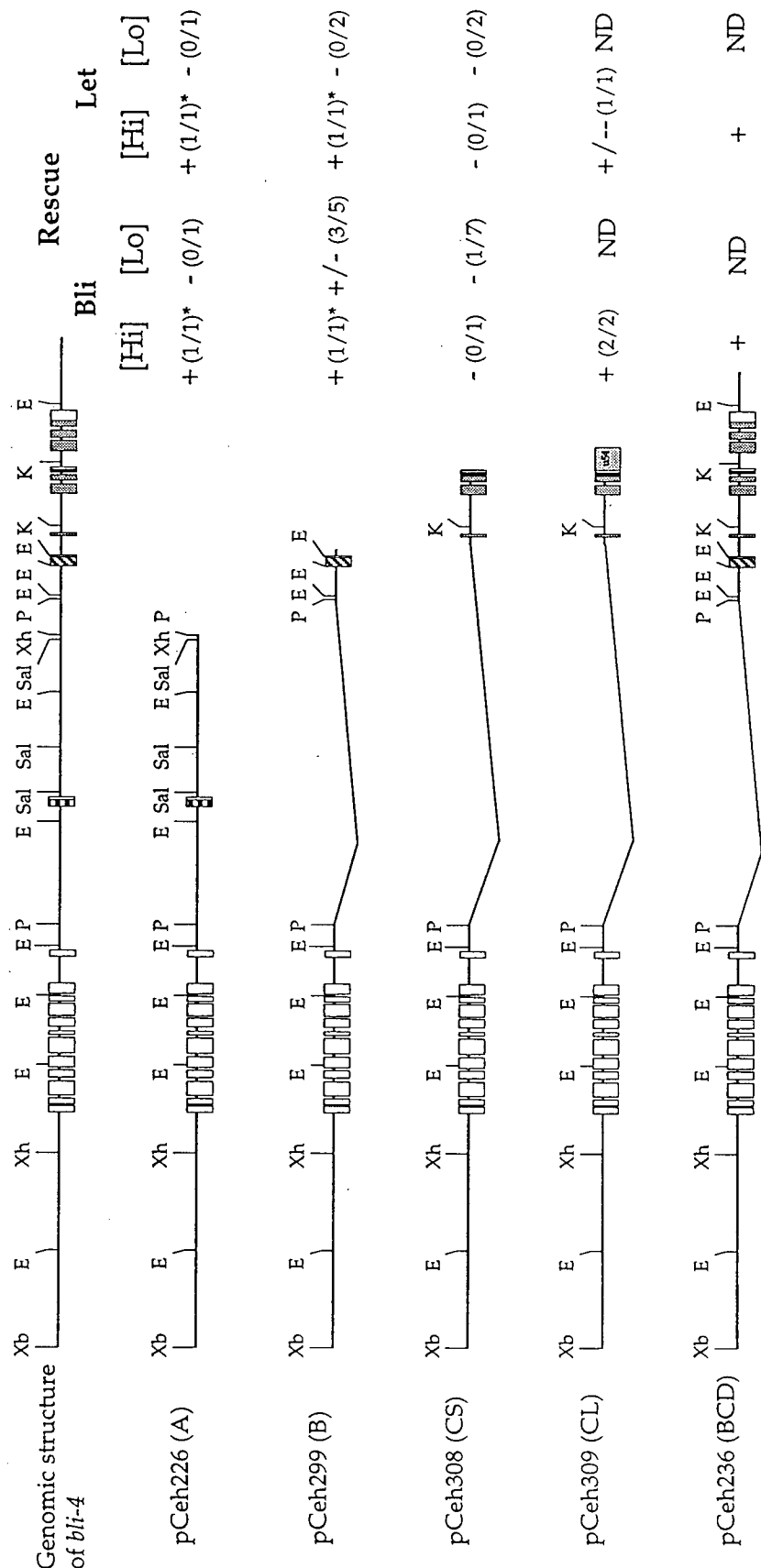
Figure 23. Summary of rescue results by *bli-4* minigenes

+ indicates complete rescue. The ratio in parenthesis indicates the number of lines that displayed rescue (in case of blistering, <10% blistered animals) over the total number of lines scored. +/-- indicates a partial rescue of the lethal allele, *s90* by pCeh309 but negative rescue of the lethal allele, *h791*.

* Although only one line was scored, the remaining segregant lines resulted from the same injection also demonstrated the same positive rescue result by examination

(van der Keyl et al., 1994). Similar to their vertebrate counterparts, the nematode collagens are synthesized as pro-collagen molecules and require limited proteolysis to acquire their mature forms. Many collagen molecules in *C. elegans* possess the hallmark substrate cleavage motif (Arg-X-Arg-Arg↓) recognized by kexin convertases, strongly suggesting *bli-4* may encode the candidate collagen endoproteases (Yang et al., 1994). Expression studies on *bli-4* using a fusion construct of the putative promoter region and a *lac Z* reporter gene detected predominant signals in, among other tissues, the seam cells of the hypodermis, which is involved with cuticle secretion, at both the embryonic and the adult stage (Thacker et al., 1995).

Anatomical studies indicated that during lethargus, the period when new cuticle is deposited, densely packed Golgi bodies and vesicles predominate in the seam cells (Singh et al., 1978). Members of kexin convertases have been shown to localize to the TGN or the secretory vesicles in mammalian cells. The structurally divergent C-termini of kexins provide sorting signals for compartmentalization of the enzymes (Molloy et al., 1994; Jones et al., 1995; Schafer et al., 1995; Zhou et al., 1995; Creemers et al., 1996). It is very likely that the different blisterase isoforms are localized to Golgi bodies and secretory vesicles within the hypodermal seam cells, where processing of collagens occur en route to being secreted. The different *bli-4* isoforms may possess different substrate specificities of collagen molecules, thus giving rise to stage-specific phenotypes, such as blistering in *e937*, when activity of specific *bli-4* isoform is eliminated.

Functional redundancy of *bli-4* isoforms

Our results suggested functional redundancy exists among the different *bli-4* isoforms. Rescue of specific mutant phenotype is not exclusive for any single isoform. Functional redundancy among kexin convertases which are differentially localized on the intracellular level is not a well-understood phenomenon. Despite reports of substrates processed by specific kexin convertases, there are also cases where a single substrate molecule may be processed by more than one kexin that are differently compartmentalized (Keller et al., 1995). C-terminally divergent dfurin isoforms of *Drosophila* exhibited similar catalytic activity or cleavage specificity (De Bie et al., 1995). The question of functional significance of the divergent C-terminus to the overall substrate specificity of kexins is even complicated by the consistent observation of C-terminal truncation during the biosynthesis of many kexin enzymes, leading to constitutive secretion of shortened yet functional molecules (Hatsuzawa et al., 1992). Whether truncated forms of kexins have any functional significance *in vivo* remains to be investigated. In the case of *bli-4*, pCeh221, a subclone which contained only the N-terminal genomic sequences common to all isoforms, reportedly rescued blistering (Srayko, 1995). This showed that aberrant expression of only the protease domain and the N-terminal features could also rescue a specific mutant phenotype

without any localization signals provided by the C-terminal sequences. Finally, the overall structurally similarity of collagen molecules, which are potential *bli-4* substrates as mentioned, may indicate inconspicuous substrate specificity of the different blisterases that may not be easily unveiled by the current methodology (Kramer, 1994).

Other information or experimental approaches that may contribute to our understanding of the individual function of *bli-4* isoforms

Functional specificities of the different blisterase isoforms should be influenced by individual temporal and tissue expression patterns. Individual cuticular collagen genes have been shown to demonstrate distinct temporal expression patterns (Johnstone and Barry, 1996). A temporal expression profile of the different blisterases will contribute to our identification of the specific functional role of individual isoform. Because of the technical difficulty of detecting the scanty amount of blisterases using Northern analysis, a semi-quantitative RT-PCR may be attempted to achieve the goal (Johnstone et al., 1996). Immunostaining using isoform-specific antibody or epitope-tagged minigene available in future will allow us to investigate the tissue distribution of the different isoforms.

Another approach to dissect the functions of *bli-4* gene products is to knock out sequences specific to individual isoforms. This can be achieved by

performing genetic screen using, for example, trimethylpsoralen as the mutagen which tends to produce small deletion mutations (Yandell et al., 1994). Alternatively, antisense RNA against a specific isoform can be injected to achieve a similar goal (Fire et al., 1991). It is expected that knock-outs of blisterases B and C may not produce observable phenotypes due to their possible minor roles and therefore high probability to be functionally compensated in the presence of the other isoforms. However, such experiments can be performed on *e937* animals. For example, a specific deletion of blisterase D in wild-type or *e937* background may provide us with additional information to verify our belief in the important functional role of this isoform.

Conclusion

The present rescue data suggested that both blisterases A and B are more specifically involved in the role played by *bli-4* in the adult cuticle than blisterase C. Blisterase C may have a restricted functional role unspecific to the adult cuticle. Alternatively, the overall expression of blisterase C may be limited by unfavorable sequence features such as the very short 3′ UTR. On the other hand, the positive rescue results from pCeh299 at both high and low copy number despite the fact that blisterase B also contains a very short 3′ UTR (23 nucleotides) demonstrates that blisterase B is potentially specific for the role of *bli-4* within the adult cuticle *in vivo*.

Although *e937* mutant animals are viable, blisterase A is expressed at the embryonic stage and pCeh226, the A minigene, managed to rescue *bli-4* lethal alleles at high copy number. These conflicting observations suggest strongly that functional redundancy exists between blisterase A and other *bli-4* isoforms at the embryonic stage. The positive rescue of lethality by high copy number of blisterase B may be caused by its functional redundancy with blisterase A in light of the structural similarity between the two isoforms. Our rescue results suggest that blisterase C is not involved in embryogenesis. Based on the ability of pCeh236, the BCD minigene, to rescue *bli-4* lethal alleles, its high expression profile, and its structural homology to the

ubiquitous mammalian convertase, furin, we propose that blisterase D may play a role in the early development of the animal and be the major functional redundant partner with blisterase A.

References

Barr P.J. 1991. Mammalian subtilisins: the long-sought dibasic processing endoproteases. *Cell* **66:** 1-3

Braks, J.A.M., K.C.W. Guldemond, M.C.H.M. van Riel, A.J.M. Coenen, and G.J.M. Martens. 1992. Structure and expression of Xenopus prohormone convertase PC2. *FEBS Lett.* **305:** 45-50.

Brenner S. 1974. The genetics of *Caenorhabditis elegans*. *Genetics* **77:** 71-94

Chan S.J., A.A. Oliver, J. LaMendola, A. Grens, H. Bode, and D.F. Steiner. 1992. Conservation of the prohormone convertase gene family in metazoa: analysis of cDNAs encoding a PC3-like protein from hydra. *Proc. Natl. Acad. Sci.* **89:** 6678-6682

Chen J., and A.S. Raikhel. 1996. Subunit cleavage of mosquito pro-vitellogenin by a subtilisin-like convertase. *Proc. Natl. Acad. Sci.* **93:** 6186-6190

Chretien, M. and C.H. Li. 1967. Isolation, purification, and characterization of gamma-lipotropic hormone from sheep ptiuitary glands. *Can. J. Biochem.* **45:** 1164-1174

Creemers J.W.M., E.F. Usac, N.A. Bright, J. Van de Loo, E. Jansen, W.J.M. Van de Ven, and J.C. Hutton. 1996. Identification of a transferable sorting domain for the regulated pathway in the prohormone convertase PC2. *J. Biol. Chem.* **271:** 25284-25291

De Bie I., D. Savaria, A.J.M. Roebroek, R. Day, C. Lazure, W.J.M. Van de Ven, and N.G. Seidah. 1995. Processing specificity and biosynthesis of the *Drosophila melanogaster* converteses dfurin1, dfurin1-CRR, dfurin1-X, and dfurin 2. *J Biol Chem* **270:** 1020-1028

De Bie I., M. Marcinkiewicz, D. Malide, C. Lazure, K. Nakayama, M. Bendayan, and N.G. Seidah. 1996. The isoforms of proprotein convertase PC5 are sorted to different subcellular compartments. *J Cell Biol* **135:** 1261-1275

Decroly E., S. Wouters, C. Di Bello, C. Lazure, J. Ruysschaert, and N.G. Seidah. 1996. Identification of the paired basic convertases implicated in HIV gp160 processing based on in vitro assays and expression in CD4+ cell lines. *J. Biol. Chem.* **271:** 30442-30450

Dubois C.M., M.H. Laprise, F. Blanchette, L.E. Gentry, and R. Leduc. 1995. Processing of transforming growth factor β1 precursor by human furin convertase. *J Biol Chem* **270:** 10618-10624

Fire A., S.W. Harrison, and D. Dixon. 1990. A modular set of *lacZ* fusion vectors for studying gene expression in Caenorhabditis elegans. *Gene* **93:** 189-198

Fire A., D. Albertson, S.W. Harrison, and D.G. Moerman. 1991. Production of antisense RNA leads to effective and specific inhibition of gene expression in *C. elegans* muscle. *Development* **113:** 503-514

Freedman D., R. Pisani, R. Purves, and A. Adhikari. 1991. Statistics. Second Edition. W W Norton and Company, Inc., New York.

Fuller R.S., A. Brake, and J. Thorner. 1989a. Yeast prohormone processing enzyme (KEX2 gene product) is a Ca^{2+} -dependent serine protease. *Proc. Natl. Acad. Sci.* **86:** 1434-1438

Fuller R.S., A. Brake, and J. Thorner. 1989b. Intracellular targetting and structural conservation of a prohormone-processing endoprotease. *Science* **246:** 482-486

Gomez-Saladin E., D.L. Wilson, and I.M. Dickerson. 1994. Isolation and *in situ* localization of a cDNA encoding a Kex2-like prohormone convertase in the nematode *Caenorhabditis elegans. Cell. Mol. Neurobiol.* **14:** 9-25

Gu M., V.M. Gordon, D.J.P. Fitzgerald, and S.H. Leppla. 1996. Furin regulates both the activation of *Pseudomonas* exotoxin A and the quantity of the toxin receptor expressed on target cells. *Infection & Immunity* **64:** 524-527

Han M., P.W. Sternberg. 1991. Analysis of dominant-negative mutations of the *Caenorbabditis elegans let-60 ras* gene. *Genes & Devel.* **5:** 2188-2198

Hatsuzawa K., K. Murakami, and K. Nakayama. 1992. Molecular and enzymatic properties of furin, a Kex-2-like endoprotease involved in precursor cleavage at Arg-X-Lys/Arg-Arg sites. *J Biochem* **11:** 296-301

Horvitz H.R., S. Brenner, J. Hodgkin, and R. Herman. 1979. A uniform genetic nomenclature for the nematode *Caenorhabditis elegans. Mol. Gen. Genet.* **175:** 129-133

Howell, A.M., S.G. Gilmore, R.A. Mancebo, and A.M. Rose. 1987. Genetic analysis of a large autosomal region in *Caenorbabditis elegans* by the use of a free duplication. *Genet. Res.* **49:** 207-213

Johnstone I.L. 1994. The cuticle of the nematode Caenorbabditis elegans: a complex collagen structure. *Bioessays* **16**: 171-178

Johnstone I.L., and J.D. Barry. 1996. Temporal reiteration of a precise gene expression pattern during nematode development. *EMBO J.* **15**: 3633-3639

Jones A.K.B. 1997. Functional analysis of the gene *bli-4* in *Caenorhabditis elegans*. M.Sc. Thesis. University of British Columbia, Vancouver, B.C., Canada

Jones B.G., L.Thomas, S.S. Molloy, C.D. Thulin, M.D. Fry, K.A. Walsh, and G. Thomas. 1995. Intracellular trafficking of furin is modulated by the phoshphorylation state of a casein kinase II site in its cytoplasmic tail. *EMBO J.* **23:** 5869-5883

Kayo T., Y. Sawada, Y. Suzuki, M. Suda, S. Tanaka, Y. Konda, J. Miyazaki, and T. Takeuch. 1996. Proprotein-processing endoprotease furin decreases regulated secretory pathway-specific proteins in the pancreatic β cell line MIN6. *J. Biol. Chem.* **271:** 10731-10737

Keller P., L. Zecca, R. Boukamel, E. Zwicker, S. Gloor, and G. Semenza. 1995. Furin, PC1/3, and/or PC6A process rabbit, but not human, pro-lactase-phlorizin hydrolase to the 180-kDa intermediate. *J. Biol. Chem.* **270:** 25722-25728

Kiefer, M.C., J.E. Tucker, R. Joh, K.E. Landsberg, D. Saltman, and P.J. Barr. 1991. Identification of a second human subtilisin-like protease gene in the *fes/fps* region of chromosome 15. *DNA Cell Biol.* **10:** 757-769

Kramer J.M., R.P. French, E. Park, and J.J. Johnson. 1990. The *Caenorhabditis elegans rol-6* gene, which interacts with the *sqt-1* collagen gene to determine organismal morphology, encodes a collagen. *Mol. Cel. Biol.* **10:** 2081-2089

Kramer J.M. 1994. Structures and functions of collagens in *Caenorhabditis elegans*. *FASEB.* **8:** 330-336

Lusson J., D. Vieau, J. Hamelin, R. Day, M. Chretien, and N.G. Seidah. 1993. cDNA structure of the mouse and rat subtilisin/kexin-like PC5: a candidate proprotein convertase expressed in endocrine and nonendocrine cells. *Proc. Natl. Acad. Sci.* **90:** 6691-6695

Mello C.C., J.M. Kramer, D. Stinchcomb, and V.Ambros. 1991. Efficient gene transfer in *C.elegans*: extrachromosomal maintenance and integration of transforming sequences. *EMBO J.* **10:** 3959-3970

Mello C.C. and A. Fire. 1995. DNA transformation., p451-482, in Epstein H.F. and D.C. Shakes (eds). Methods in Cell Biology, **48.** *Caenorhabditis elegans* Modern biological analysis of an Organism. Academic press.

Molloy S.S., L. Thomas, J.K. VanSlyke, P.E. Stenberg, and G. Thomas. 1994. Intracellular trafficking and activation of the furin proprotein convertase: localization to the TGN and recycling from the cell surface. *EMBO J.***13**: 18-33

Nachtigal M.W. and H.A. Ingraham. 1996. Bioactivtion of Mullerian inhibiting substace during gonadal development by a kex2/subtilisin endoprotease. *Proc. Natl. Acad. Sci* **93**: 7711-7716

Nakayama K., W.-S. Kim, S. Torii, M. Hosaka, T. Nakagawa, J. Ikemizu, T. Baba, and K. Murakami. 1992. Identification of the fourth member of the mammalian endoprotease family homologous to the yeast Kex2 protease. *J. Biol. Chem.* **267**: 5897-5900

Nakayama K., M. Hosaka, S. Torii, T. Watanabe, K. Murakami, and T. Nakagawa. 1993a. Identification and functional expression of a new member of the mammalian Kex2-like processing endoprotease family: its striking structural similarity to PACE4. *J. Biochem.* **113**: 132-135

Nakayama K., K. Murakami, and T. Nakagawa. 1993b. Identification of an isoform with an extremely large Cys-rich region of PC6, a Kex-2-like processing endoprotease. *FEBS Lett* **327**: 165-171

Okkema P.G., S.W. Harrison, V. Plunger, A. Aryana, and A. Fire. 1993. Sequence requirements for myosin gene expression and regulation in *Caenorhabditis elegans. Genetics* **135**: 385-404

Pei D. and S.J. Weiss. 1995. Furin-dependent intracellular activation of the human stromelysin-3 zymogen. *Nature* **375**: 244-247

Peters K.W. 1992. A genetic and molecular characterization of the *bli-4* locus of Caenorhabditis elegans, an essential gene encoding kex-2 like subtilisin-type serine endoproteases. Ph.D. Thesis, University of British Columbia, Vancouver, B.C., Canada.

Peters K., J.M. McDowall, and A.M. Rose. 1991. Mutations in the *bli-4(I)* locus of *Caenorhabditis elegans* disrupt both adult cuticle and early larval development. *Genetics* **129**: 95-102

Priess J.R. and D. I. Hirsh. 1986. *Caenorhabditis elegans* morphogenesis: the role of the cytoskeleton in elongation of the embryo. *Developmental Biology* **117**: 156-173

Robinson M.O., and M.I. Simon. 1991. Determining transcript number using the polymerase chain reaction: Pgk-2, mP2, and PGK-2 transgene mRNA level during spermatogenesis. *Nucleic Acids Res.* **19:** 1557-1562

Roebroek A.J.M., J.A. Schalken, J.A.M. Leunissen, C. Onnekink, H.P.J. Bloemers, and W.J.M. Van de Ven. 1986. Evolutionary conserved close linkage of the c-fes/fps proto-oncogene and genetic sequences encoding a receptor-like protein. *EMBO J.* **5:** 2197-2202

Roebroek A.J.M., I.G.L. Pauli, Y. Zhang, and W.J.M. Van de Ven. 1991. cDNA sequence of a *Drosophila melanogaster* gene, Dfur1, encoding a protein structurally related to the subtilisin-like proprotein processing enzyme furin. *FEBS Lett.* **289:** 133-137

Roebroek A.J.M., J.W.M. Creemers, I.G.L. Pauli, U. Kurzik-Dumke, M. Rentrop, E.A.F. Gateff, J.A.M. Leunissen, and W.J.M. Van de Ven. 1992. Cloning and functional expression of Dfurin2, a subtilisin-like proprotein processing enzyme of *Drosophila melanogaster* with multiple repeats of a cysteine motif. *J. Biol. Chem.* **267:** 17208-17215

Roebroek A.J.M., J.W.M. Creemers, I.G.L. Pauli, T. Bogaert, and W.J.M. Van de Ven. 1993. Generation of structural and functional diversity in furin-like proteins in Drosophila melanogaster by alternative splicing of the Dfur1 gene. *EMBO J.* **12:** 1853-1879

Sachs A.B. 1993. Messenger RNA degradation in eukaryotes. *Cell* **74:** 413-421

Sambrook J., E.F. Fritsch, and T. Maniatis. 1989. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Schafer W., A. Stroh, S. Berghofer, J. Seiler, M. Vey, M. Kruse, H.F. Kern, H. Klenk, and W. Garten. 1995. Two independent targeting signals in the cytoplasmic domain determine trans-Golgi network localization and endosomal trafficking of the proprotein convertase furin. *EMBO J.* **14:** 2424-2435

Seidah N.G., L. Gaspar, P. Mion, M. Marcinkiewicz, M. Mbikay, and M. Chretien. 1990. cDNA sequence of two distinct pituitary proteins homologous to Kex2 and furin gene products: tissue-specific mRNAs encoding candidates for pro-hormone processing proteinases. *DNA Cell Biol.* **9:** 415-424

Seidah N.G., M. Marcinkiewicz, S. Benjannet, L. Gaspar, G. Beaubien, M.G. Mattei, C. Lazure, M. Mbikay, and M. Chretien. 1991. Cloning and primary sequence of a mouse candidate prohormone convertase PC1 homologous to PC2, furin,and Kex2: distinct chromosomal localization and messenger RNA

distribution in the brain and pituitary compared to PC2. *Mol. Endocrinol.* **5:** 111-122

Seidah N.G. and M. Chretien. 1992. Proprotein and prohormone convertases of the subtilisin family. *T.E.M.* **3:** 133-140

Seidah N.G., R. Day, J. Hamelin, A. Gaspar, M.W. Collard, and M. Chretien. 1992. Testicular expression of PC4 in the rat: molecular diversity of a novel germ cell-specific kex2/subtilisin-like proprotein convertase. *Mol. Endocrinol.* **6:** 1559-1570

Seidah N.G., J. Hamelin, M. Mamarbachi, W. Dong, H. Tadros, M. Mbikay, M. Chretien, and R. Day. 1996. cDNA structure, tissue distribution, and chromosomal localization of rat PC7, a novel mammalian proprotein convertase closest to yeast kexin-like proteinases. *Proc. Natl. Acad. Sci.* **93:** 3388-3393

Singh R.N. and J.E. Sulston. 1978. Some observations on moulting in *Caenorhabditis elegans*. *Nematogica*. **24:** 63-71

Smeekens S.P. and D.F. Steiner. 1990. Identification of a human insulinoma cDNA encoding a novel mammalian protein structurally related to the yeast dibasic processing protease Kex2. *J. Biol. Chem.* **265:** 2997-3000

Smeekens S.P., A.S. Avruch, J. LaMendola, S.J. Chan, and D.F. Steiner. 1991. Identification of a cDNA encoding a second putative prohormone convertase related to PC2 in AtT20 cells and islets of Langerhans. *Proc. Natl. Acad. Sci.* **88:** 340-344

Srayko M. 1995. A structure-function analysis of the complex gene, *bli-4*, in *Caenorhabditis elegans*. M.Sc. Thesis, University of British Columbia, Vancouver, B.C., Canada.

Steiner D.F., N. Fox, S.P. Smeekens, S. Ohagi, G. Westermark and S.J. Chan. 1993. New molecular prospectives in islet hormone biosynthesis. *Biochem Soc Trans* **21:** 139-142

Thacker C., K. Peters, M. Srayko, and A.M. Rose. 1995. The *bli-4* locus of *Caenorhabditis elegans* encodes structurally distinct kex2/subtilisin-like endoproteases essential for early development and adult morphology. *Genes & Devel.* **9:** 956-971

Tsuji A., K. Higashine, C. Hine, K. Mori, Y. Tamai, H. Nagamune, and Y. Matsuda. 1994. Identification of novel cDNAs encoding human kexin-like protease, PACE4 isoforms. *Biochem Biophys Res Comm* **200:** 943-950

van den Ouweland A.M., H.L. van Duijnhoven, G.D. Keizer, L.C. Dorssers, and W.J.M. Van de Ven. 1990. Structural homology between the human *fur* gene product and the subtilisin-like protease encoded by yeast KEX2. *Nucleic Acid Res.* **18**: 664

van der Keyl H., H. Kim, R. Espey, C. V. Oke, and M.K. Edwards. 1994. *Caenorhabditis elegans sqt-3* mutants have mutations in the *col-1* collagen gene. *Dev Dyn* **201**: 86-94

Vey M., W. Schafer, S. Berghofer, H. Klenk, and W. Garten. 1994. Maturation of the trans-Golgi network protease furin: compartmentalization of propeptide removal, substrate cleavage and COOH-terminal truncation. *J. Cell. Biol.* **127**: 1829-1842

Walker J.A., S.S. Molloy, G. Thomas, T. Sakaguchi, T. Yoshida, T.M. Chambers, and Y. Kawaoka. 1994. Sequence specificity of furin, a proprotein-processing endoprotease, for the hemagglutinin of a virulent avian influenza virus. *J. Virol.* **68**: 1213-1218

Willnow T.E., J.M. Moehring, N.M. Inocencio, T.J. Moehring, and J. Herz. 1996. The low-density-liproprotein receptor-related protein (LRP) is processed by furin *in vivo* and *in vitro*. *Biochem J.* **313**: 71-76

Wise R.J., P.J. Barr, P.A. Wong, M.C. Kiefer, A.J. Brake, and R.J. Kaufman. 1990. Expression of a human proprotein processing enzyme: Correct cleavage of the von Willebrand factor precursor at a paired basic amino acid site. *Proc. Natl. Acad. Sci.* **87**: 9378-9382

Yandell, M.D., L.G. Edgar, W.B. Wood. 1994. Trimethylpsoralen induces small deletion mutations in *Caenorhabditis elegans*. *Proc. Natl. Acad. Sci.* **91**: 1381-1385

Yang J., and J.M. Kramer. 1994. *In Vitro* Mutagenesis of *Caenorhabditis elegans* cuticle collagens identifies a potential subtilisin-like protease cleavage site and demonstrates that carboxyl domain disulfide bonding is required for normal function but not assembly. *Mol. Cell. Biol.* **14**: 2722-2730

Zhou A., L. Paquet, and R.E. Mains. 1995. Structural elements that direct specific processing of different mammalian subtilisin-like prohormone convertases. *J. Biol. Chem.* **270**: 21509-21516

Appendix I

An estimation of the copy number of minigene is obtained by
calculating the ratio of the intensity of the *bli-4* PCR product in the transgenic
animal to that of the wild-type animal (see Figure 9 for explanation). To adjust
for the difference in the amount of template DNA and the efficiency between
reactions, the intensity of the *bli-4* band from the wild-type animal was first
normalized by the difference in intensity of the control bands from the two animals.
For example, to calculate the copy number of pCeh309 (the C long minigene), the
intensity of band 4 in Figure A1 was first normalized by dividing into the ratio of
the intensity of band 10 to band 7 of the transgenic animal. The copy number is
then computed as the ratio of the intensity of band 1 to the normalized intensity of
band 4.

Sample calculation

Normalized intensity of band 4

$$= \text{intensity of band4} \times \frac{\text{intensity of band 7}}{\text{intensity of band 10}}$$

$$= 4.937 \times \frac{6.955}{7.396}$$

$$= 4.494$$

Copy number

$$= \frac{\text{intensity of band 1}}{\text{normalized intensity of band 4}}$$

$$= \frac{84.49}{4.494}$$

$$= 18.8$$
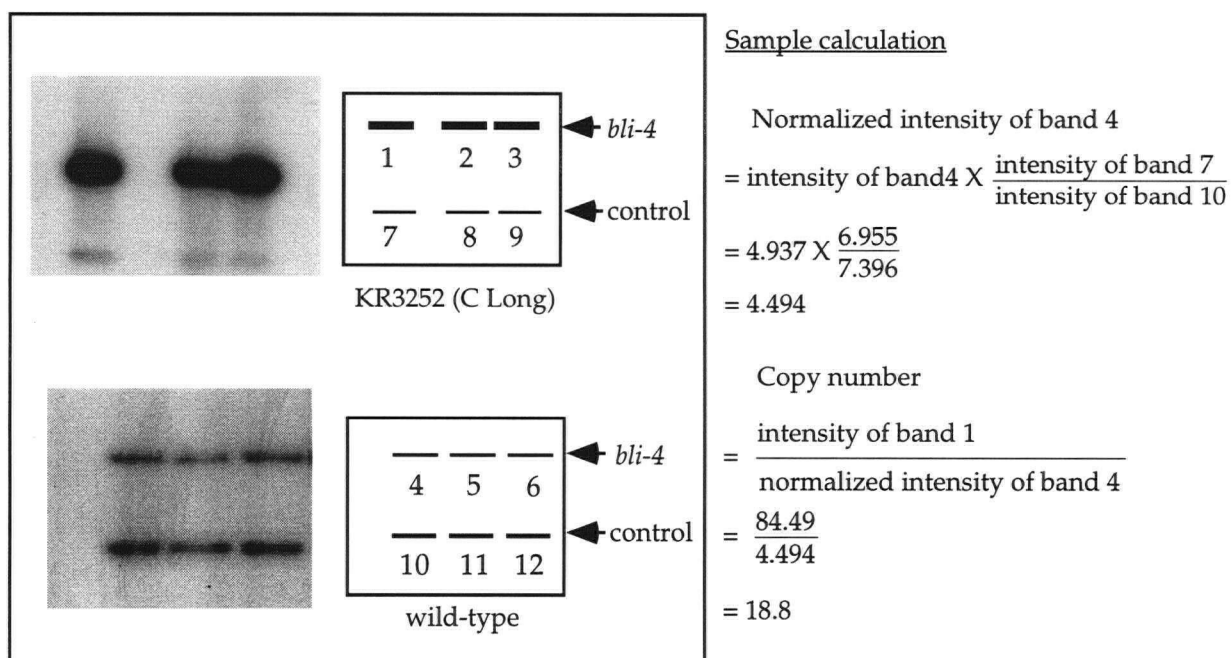
Figure A1

Normalization was repeated against the three wild-type animals for each transgenic animal and the average copy number was taken. The same procedure was repeated for 2-3 transgenic animals and the average was reported as the copy number for the transgenic strain (Table 3).

The use of PCR to estimate the copy number of minigene relies on the linear relationship between template molecules and products in the initial stage of amplification. We performed a control in an attempt to demonstrate the linear relationship within the PCR conditions used in this experiment (Figure A2). A linear relationship can be observed in the first two lanes as the intensity of the signal doubled when the amount of DNA loaded was increased in a similar proportion. However, the reaction (top band) appeared saturated in the third lane. Most of the high-copy signals of the *bli-4* bands appeared to be within the linear range except KR3231 which contained a high copy number of pCeh226, the A minigene. Therefore our estimation should reflect a minimum value of the actual copy number in this strain.

| *bli-4* | 26.34 | 77.63 | 98.8 |
| control | 0.80 | 2.39 | 6.54 |

intensities of the corresponding bands in the picture above
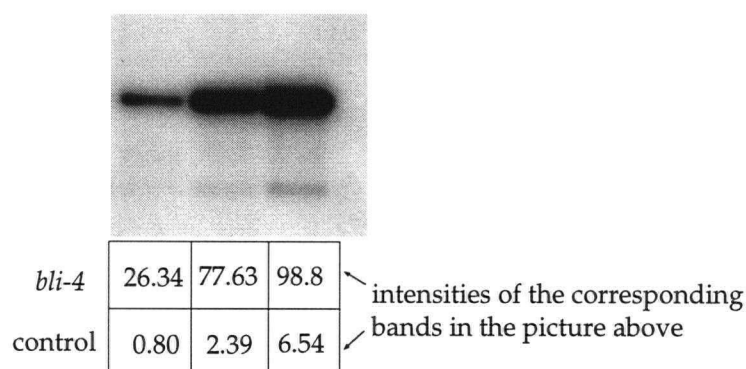
Figure A2

Table A1. Densitometry values corresponding to Figure 19.

Panel.Lane	Strain	Intensity	
		Top band	Bottom band
A.1	KR3231 (A Low)	6.0	19.1
A.2	KR3231 (A Low)	3.2	10.9
A.3	KR3231 (A Low)	2.6	6.5
A.4	KR3228 (A High)	99.8	9.7
A.5	KR3228 (A High)	109.1	14.2
A.6	KR3228 (A High)	107.0	15.8
A.7	N2	4.9	14.3
A.8	N2	3.5	6.1
A.9	N2	3.6	4.6
B.1	KR3235 (B Low)	12.2	8.6
B.2	KR3235 (B Low)	9.2	12.0
B.3	KR3239 (B Low)	9.0	9.1
B.4	KR3239 (B Low)	9.4	9.6
B.5	KR3239 (B Low)	11.2	12.5
B.6	KR3233 (B High)	72.3	18.7
B.7	KR3233 (B High)	87.7	15.1
B.8	KR3233 (B High)	72.0	14.3
B.9	KR2868 ("B")	22.9	10.8
B.10	KR2868 ("B")	20.5	17.3
B.11	KR2868 ("B")	19.1	9.8
C.1	KR3245 (C Short Low)	10.9	5.0
C.2	KR3245 (C Short Low)	17.0	8.0
C.3	KR3245 (C Short Low)	12.0	9.1
C.4	KR3246 (C Short Low)	9.6	7.0
C.5	KR3246 (C Short Low)	12.1	8.5
C.6	KR3246 (C Short Low)	13.7	5.1
C.7	KR3357 (C Short High)	67.2	5.0
C.8	KR3357 (C Short High)	71.2	5.5
C.9	KR3357 (C Short High)	-	-
C.10	KR3252 (C Long High)	84.5	7.0
C.11	KR3252 (C Long High)	70.6	6.0
C.12	KR3252 (C Long High)	76.2	4.4
D.1	N2	4.9	7.4
D.2	N2	3.8	6.5
D.3	N2	5.2	7.6
D.4	KR3252 (C Long High)	26.3	0.8
D.5	KR3252 (C Long High)	77.6	2.4
D.6	KR3252 (C Long High)	98.8	6.6

Appendix II

If the extrachromosomal array is integrated (e.g. in KR3245), dead eggs will still be observed in F3 (Figure 10) regardless of positive or negative rescue:

A. If the array (*hEx*) is integrated in chromosomes other than chromosome I (where *bli-4* resides):

F1
$$\frac{dpy\text{-}5\ \ bli\text{-}4(let)\ \ unc\text{-}13}{+\qquad+\qquad+}\ \ ;\ \ \frac{+}{+}\ \ ;\ \ \frac{bli\text{-}4(e937)\quad+}{bli\text{-}4(e937)\quad+}\ \ ;\ \ \frac{hEx}{hEx}\quad\male \qquad \times \qquad \frac{+\qquad+}{+\qquad+}\ \ ;\ \ \frac{bli\text{-}4(e937)\quad+}{bli\text{-}4(e937)\quad+}\ \ ;\ \ \frac{hEx}{hEx}\quad\female$$

F2
$$\frac{+\qquad bli\text{-}4(e937)\qquad+}{dpy\text{-}5\ \ bli\text{-}4(let)\ \ unc\text{-}13}\ \ ;\ \ \frac{hEx}{+}\quad\female\circlearrowleft \qquad\text{or}\qquad \frac{+\qquad bli\text{-}4(e937)\qquad+}{+\qquad+\qquad+}\ \ ;\ \ \frac{hEx}{+}$$

(will not give dead progeny)

F3	$\dfrac{+\ bli\text{-}4(e937)\ +}{+\ bli\text{-}4(e937)\ +}\ ;\ hEx$	$\dfrac{+\ bli\text{-}4(e937)\ +\ ;\ +}{+\ bli\text{-}4(e937)\ +\ ;\ hEx}$	$\dfrac{dpy\text{-}5\ bli\text{-}4(let)\ unc\text{-}13\ ;\ hEx}{+\ bli\text{-}4(e937)\ +\ ;\ +}$	$\dfrac{dpy\text{-}5\ bli\text{-}4(let)\ unc\text{-}13\ ;\ +}{+\ bli\text{-}4(e937)\ +\ ;\ +}$
$\dfrac{+\ bli\text{-}4(e937)\ +}{+\ bli\text{-}4(e937)\ +}\ ;\ \dfrac{hEx}{hEx}$	$\dfrac{+\ bli\text{-}4(e937)\ +}{+\ bli\text{-}4(e937)\ +}\ ;\ \dfrac{hEx}{hEx}$ Bli Rol	$\dfrac{+\ bli\text{-}4(e937)\ +}{+\ bli\text{-}4(e937)\ +}\ ;\ \dfrac{hEx}{+}$ Bli/WT Rol	$\dfrac{dpy\text{-}5\ bli\text{-}4(let)\ unc\text{-}13}{+\ bli\text{-}4(e937)\ +}\ ;\ \dfrac{hEx}{+}$ Bli/WT Rol	$\dfrac{dpy\text{-}5\ bli\text{-}4(let)\ unc\text{-}13}{+\ bli\text{-}4(e937)\ +}\ ;\ \dfrac{hEx}{+}$ Bli/WT Rol
$\dfrac{+\ bli\text{-}4(e937)\ +}{+\ bli\text{-}4(e937)\ +}\ ;\ \dfrac{hEx}{+}$	$\dfrac{+\ bli\text{-}4(e937)\ +}{+\ bli\text{-}4(e937)\ +}\ ;\ \dfrac{hEx}{+}$ Bli Rol	$\dfrac{+\ bli\text{-}4(e937)\ +}{+\ bli\text{-}4(e937)\ +}\ ;\ \dfrac{+}{+}$ Bli	$\dfrac{dpy\text{-}5\ bli\text{-}4(let)\ unc\text{-}13}{+\ bli\text{-}4(e937)\ +}\ ;\ \dfrac{+}{+}$ Bli/WT	$\dfrac{dpy\text{-}5\ bli\text{-}4(let)\ unc\text{-}13}{+\ bli\text{-}4(e937)\ +}\ ;\ \dfrac{+}{+}$ Bli/WT
$\dfrac{dpy\text{-}5\ bli\text{-}4(let)\ unc\text{-}13}{+\ bli\text{-}4(e937)\ +}\ ;\ \dfrac{hEx}{+}$ Bli/WT Rol	$\dfrac{+\ bli\text{-}4(e937)\ +}{dpy\text{-}5\ bli\text{-}4(let)\ unc\text{-}13}\ ;\ \dfrac{hEx}{+}$ Bli/WT Rol	$\dfrac{+\ bli\text{-}4(e937)\ +}{dpy\text{-}5\ bli\text{-}4(let)\ unc\text{-}13}\ ;\ \dfrac{hEx}{+}$ Bli/WT Rol	$\dfrac{dpy\text{-}5\ bli\text{-}4(let)\ unc\text{-}13}{dpy\text{-}5\ bli\text{-}4(let)\ unc\text{-}13}\ ;\ \dfrac{hEx}{+}$ Dead or Dpy Unc	$\dfrac{dpy\text{-}5\ bli\text{-}4(let)\ unc\text{-}13}{dpy\text{-}5\ bli\text{-}4(let)\ unc\text{-}13}\ ;\ \dfrac{hEx}{+}$ Dead or Dpy Unc
$\dfrac{dpy\text{-}5\ bli\text{-}4(let)\ unc\text{-}13}{+\ bli\text{-}4(e937)\ +}\ ;\ \dfrac{+}{+}$ Bli/WT Rol	$\dfrac{+\ bli\text{-}4(e937)\ +}{dpy\text{-}5\ bli\text{-}4(let)\ unc\text{-}13}\ ;\ \dfrac{hEx}{+}$ Bli/WT	$\dfrac{+\ bli\text{-}4(e937)\ +}{dpy\text{-}5\ bli\text{-}4(let)\ unc\text{-}13}\ ;\ \dfrac{+}{+}$ Bli/WT	$\dfrac{dpy\text{-}5\ bli\text{-}4(let)\ unc\text{-}13}{dpy\text{-}5\ bli\text{-}4(let)\ unc\text{-}13}\ ;\ \dfrac{+}{+}$ Dead or Dpy Unc	$\dfrac{dpy\text{-}5\ bli\text{-}4(let)\ unc\text{-}13}{dpy\text{-}5\ bli\text{-}4(let)\ unc\text{-}13}\ ;\ \dfrac{+}{+}$ Dead

- at least 1/16 of F3 will be dead eggs

B. If the array (*hEx*) is integrated into chromosome I (where *bli-4* resides):

F1
$$\frac{dpy\text{-}5 \quad bli\text{-}4(let) \quad unc\text{-}13 \quad +}{+ \qquad + \qquad\qquad + \qquad +} \; \male \quad \times \quad \frac{bli\text{-}4(e937) \quad + \; hEx}{bli\text{-}4(e937) \quad + \; hEx} \; \female$$

F2
$$\frac{dpy\text{-}5 \quad bli\text{-}4(let) \quad unc\text{-}13 \quad +}{+ \qquad bli\text{-}4(e937) \qquad + \quad hEx} \; \female \;\circlearrowleft \quad \text{or} \quad \frac{+ \qquad + \qquad +}{+ \quad bli\text{-}4(e937) \quad + \; hEx} \; \female$$
$$\text{(will not give dead progeny)}$$

F3
$$\frac{dpy\text{-}5 \quad bli\text{-}4(let) \quad unc\text{-}13 \quad +}{dpy\text{-}5 \quad bli\text{-}4(let) \quad unc\text{-}13 \quad +} \quad \text{or} \quad \frac{bli\text{-}4(e937) \quad + \; hEx}{bli\text{-}4(e937) \quad + \; hEx}$$

Dead   Rol
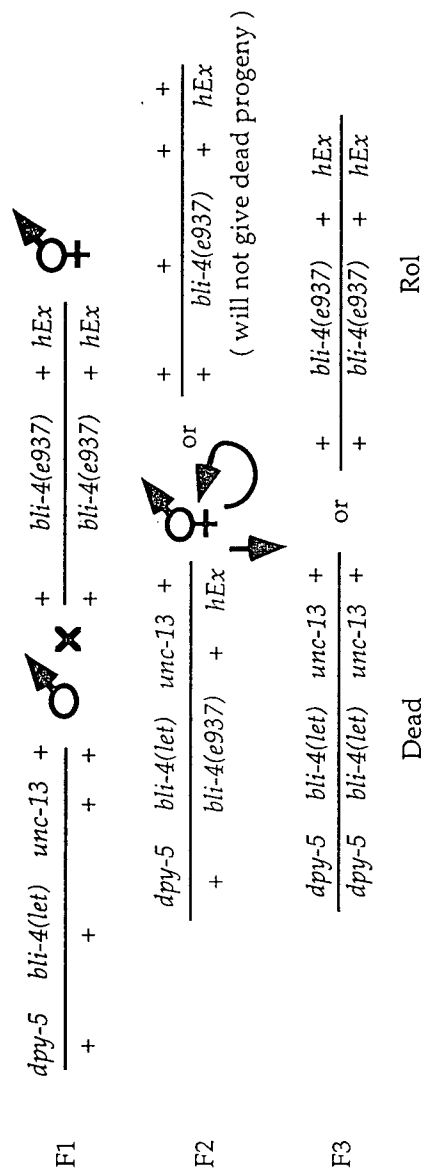
- Half of F3 will be dead eggs

Since less than half of F3 were found to be dead eggs, and no Dpy Unc was observed when KR3245 was tested against the *bli-4* lethal alleles, it was concluded that the array was not integrated into chromosome I and that pCeh308, the C Short minigene, was not able to rescue the lethal phenotype even when it was integrated.

Appendix III

The Rol-6 phenotype partially suppresses the Bli-4 phenotype

The difference in the frequency of blistering in non-Rol and Rol of KR2872, the transgenic line which contained pRF-4, the injection marker was tested for statistical significance using the two-sample z-test (Freedman et al., 1991). The null hypothesis in this test is that the two frequencies are the same and the difference only represents chance variation. The alternative hypothesis states that the difference is real. The z-score is computed as the ratio of the difference between the frequencies to the root-mean square of the standard errors (SE) of the frequencies. SE is computed using the formula: $\{[p(1-p)]/n\}^{1/2}$, where p = the percentage of blistering and n = the total number of animals scored (Freedman et al., 1991). The *P*-value represents the probability the recorded difference is observed if the null hypothesis is true i.e. by chance variation. A *P*-value of 0.01 (1%) is normally used as the criteria for rejecting the null hypothesis, i.e. the difference is statistically significant.

Using the data from Table 4, *P*<0.0001 for the difference in blistering frequency between the non-Rol and the Rol populations of KR2872. For comparison, *P*<0.025 for the difference in blistering frequency between the non-Rol population of KR2872 and CB937, the original *bli-4(e937)* strain. Therefore, the Rol-6 phenotype partially suppresses the Bli-4 phenotype.

Appendix IV

The construction of a minigene specific to blisterase D was attempted. One of the cloning schemes is shown in Figure A3. All of the cloning steps were completed except the final ligation of the D-specific exons with the common region (pCeh301). This step was repeatedly tried but the final construct was not able to be obtained.

Figure A3.   Construction of blisterase D minigene

A.   Substrate clones used for cloning

B.   A schematic representation of the cloning steps

A

1kb

Xb   E   Xh Sac E   E   E P   E Sal Sal   E Sal Xh P E Xb E E K   Sac K   E   E   P   Sal

B   Sp

pCeh180   toXh
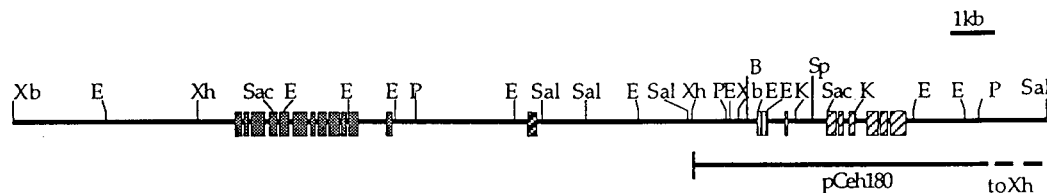
B = *Bam*HI
Bcl = *Bcl*I
E = *Eco*RI
RV = *Eco*RV
H = *Hind*III
K = *Kpn*I
N = *Not*I
P = *Pst*I
Sac = *Sac*I
Sal = *Sal*I
Sma = *Sma*I
Sp = *Spe*I
Xb = *Xba*I
Xh = *Xho*I

B

Sp   H
pCeh300

1. digest with Sp
2. fill in Sp (blunt)

3. digest with H
4. clone into BSSK (Sal (blunted), H)

H   K
Sma
pCeh310

1. H
2. Sma

pCeh180

1. digest with P
2. fill in P (blunt)
3. digest with H
4. isolate 4 Kb fragment

pCeh 196
(blisterase D cDNA)

replace Bcl-Bgl fragment
of pCeh311 with same
portion from pCeh 196

K   H   Bcl   Bgl
pCeh311

K   H Bcl   Bgl   RV
pCeh312

B   K   Bcl   RV   K
pCeh306

replace Bcl-RV of pCeh306
fragment with same portion
from pCeh312

B   K   Bcl   RV   K
pCeh314

K

K   Bcl   RV   K

pCeh301

K

Xb   E   Xh Sac E   E   E   B K

K

Xb   E   Xh Sac E   E   E   B K   K

D minigene