EXAMINATION OF CODING REGIONS IN THE *DPY-14* REGIONS OF

CAENORHABDITIS ELEGANS

by

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Abstract

The purpose of this study was to identify and clone the genes rescued by the cosmid T21G5 located in the *dpy-14* region of chromosome I. Mutants in four genes, *let-394, let-534, let-545* and *dpy-14* were rescued by transgenic *C. elegans* carrying T21G5 cosmic DNA created by McKay *et al*, 1993. Using the transgenic of hEx24 created by S. McKay, phenotypic rescue was confirmed. In order to isolate the individual genes corresponding with each of the mutant phenotypes, a set of plasmid subclones was constructed from T21G5 cosmid DNA. Each subclone was injected into wild-type *C. elegans* along with a plasmid carrying the dominant marker, *Rol-6*. Heritable transmission of the *Rol-6* plasmid was detected by the roller phenotype. DNA molecules that share sequence identity could recombine to form an extrachromosomal arrays after injection into the gonad. In order to confirm the presence of the T21G5 plasmid subclone in the transgenic strain, PCR analysis with a set of primers from the Bluescript vector was used. The generation of the plasmid containing transgenics and PCR detection of the cosmid subclones distinct from the *Rol-6* plasmid was successful. However, none of the plasmid transgenics rescued any of the mutants. In fact, three new T21G5 transgenics were generated, and none of these rescued the mutants. A number of controls were performed leading to the conclusion that hEx24 was exceptional in its ability to express and rescue the mutants in the germline. It has been shown that transgenic arrays often do not express well in the germline. An examination of the DNA sequence of T21G5 reveals two genes proposed to exhibit germline specific expression (*gld-1*-like, Ross *et al*, 1995) and (*glh-1*, Bennett *et al*, personal communication). Thus, this cosmid may not normally express well explaining the difficulty in repeating the rescuing ability of hEx24.

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Introduction

The background

The *C. elegans* genome project led the approaches to physical mapping and genomic sequencing in the 1980s. The physical map of the 100-Mb *C. elegans* genome consists of 17,500 cosmids and 3500 yeast artificial chromosomes (YACs). A total of 50 Mb has been sequenced, annotated and submitted to the Genebank. This covered over half sequences of the genome and a large fracftions of the genes. 4255 putative protein coding genes ($\cong 1$ per each 5 kb) were predicted, and approximately 45% have significant similarity to non-*C. elegans* genes (Waterston and Sulston, 1995). The estimation of lethal loci in different regions of the *C. elegans* genome showed that 1 in 3 genes is essential for viability (Miklos and Rubin, 1996).

One approach to understanding what the DNA sequences do is to study the mutational changes that cause the mutant phenotype. The first step towards doing this on a large genomic scale is the correlation of mutant phenotype with DNA coding sequences. Ann Rose laboratory has taken an approach of rescue mutant by the sequenced cosmids as a way of correlating phenotype with DNA sequences. For my thesis study, I have investigated the organization of the cosmid T21G5. From previous study, it has shown the ability to rescue the mutants of *let-394, let-534, let-545*, and *dpy-14*.

The physical map and sequence available from ACEDB can be used to study mutationally defined genes. The precise correlation of the genetic map with the sequence can be achieved by generating transgenic strains carrying sequenced cosmids, rescuing the lethal and visible mutants (Rose, Edgley and Baillie, 1994).

C. elegans is cultured on petri plates containing nematode growth medium (NGM) streaked with *Escherichia coli* OP-50 at 20°C and reproduces with a life cycle of about 3 days (Brenner, 1974). Because of the short time of its life cycle and easy growth and maintenance in the laboratory, *C. elegans* is a good model for genetic study.

C. elegans is a self fertilizing hermaphrodite. Males, arising by sex chromosome non-disjunction, have only one X chromosome. A mutant allele present in the heterozygous state in a hermaphrodite will become homozygous in one quarter of the self progeny, therefore the recovery of recessive alleles is convenient (Brenner, 1974). All the mutants collected from different laboratories around the world are stocked in the CGC center providing the source for studying genetics and functions of genes.

The relative small size of *C. elegans* genome, containing up proximately 100 megabases (10^8) (Sulston, *et al.*, 1992) makes it easy and possible to study the physical map and sequence the whole genome. The physical map of the *C. elegans* genome was established in YACs and cosmids which are available for studying the function and structure of the genes (Sulston, 1988). The increased knowledge from the genome sequence and the more defined physical map will enhance the understanding of gene organization. The defined genetic map helps to organize the physical map, and mutant alleles provide an important source for verifying gene.

The gene can be cloned and studied by transformed lines carrying cosmid arrays in the region of the physical map corresponding with the mutants in the genetic map. The physical map and the cosmid clones are the basis of the genomic sequencing project. The completed sequence of the chromosome III of *C. elegans* provides a large amount of genetic information developed from homology searches. All of the known information about gene function and its organization controlling *C. elegans* development will contribute to a model of developmental biology.

The known complete cell lineage provides a precise description of cellular development of a wild-type animal, in which the relationship between the detailed patterns of cell fate and the ancestral cells in tissues is defined (Sulston, 1988). Based on the pathway of cell lineage, laser ablation can be used to study the effect of mutant alleles on cell fate. The function of genes correlated with the cell fate can be defined. This knowledge of genes correlated with cell fate will

help determine how genes regulate the development of *C. elegans* (Bowerman, *et al.*, 1992).

ACEDB archives genetic information of *C. elegans*. The *Newsletter* twice yearly provides an opportunity to the *C. elegans* community to communicate with each other about their recent results and questions.

The *dpy-14* region

Mutant screening provides a way to study gene function and gene mapping. Ethyl methane sulfonate (EMS) is a common mutagen used for creating mutants. Mapping information is obtained by dividing the resulting mutants into complementation groups, positioning them to different regions of the genome and characterizing their phenotypes (Johnsen and Baillie, 1991). The gene cluster on left chromosome I [LG (I)] has been studied intensively (Rose and Baillie 1980; Howell, *et al.*, 1987). A high resolution genetic map [LG (I)] was established (Howell *et al*, 1987; McDowall, 1990; McKim *et al*, 1992). The middle of the "gene cluster" region defined as the *dpy-14* region was studied intensively, both genetically and molecularly (McKim, *et al.*, 1992; Prasad, *et al.*, 1993; McKay, 1993).

A physical map of *C. elegans* has been constructed (Coulson, *et al.*, 1986). The *C. elegans* genome has been partially digested with the Sau3AI restriction enzyme and cloned into the cosmids pJB8, pHC79 and Lorist as well as the yeast artificial chromosome pYAC4. Due to the selective differences between the cosmid vectors pJB8, pHC79 and Lorist 2, segments of the *C. elegans* genome were cloned into these vectors for simplifying the procedure of testing partially overlapped genomic DNA in different cosmid vectors. The cosmid vectors can hold about 30 kb of *C. elegans* genomic DNA. The cosmid vector sequences are available from the DNA strider 1.0's program. Cosmid clones and YACs were positioned along the chromosome by *in situ* hybridization and aligned by fingerprinting with Southern blot analysis. No gaps remains in the physical map. A physical map of the *dpy-14* region is shown in Figure 1.

A large number of genes in the *dpy-14* region of chromosome I in the genetic map were correlated to the cosmid arrays in this region of the physical map (McDowall, 1990 and McKay,

1993). The four mutants, *let-394, let-534, let-545* and the *dpy-14*, in the dpy-14 region were rescued by the cosmid T21G5 (McKay, 1993).

Verification of subclones in transformed lines

Transformation technology in *C. elegans* is widely used to assess the characterization of the function of a cloned DNA sequence. The technique in *C. elegans* was initiated by Kimble *et al.*, 1982, who developed a procedure for microinjecting the nucleotide sequences into the germline of adult worms. The gene of tRNA from an amber suppressor strain was first used to inject into the non-suppressor strain with a transient suppression expression of the amber suppressor. The microinjection procedure was modified by immobilizing nonanesthetized worms on dried agarose pads under a layer of halocarbon oil (Fire, 1986). The modified procedure simplified microinjection and made possible injecting a large numbers of worms in a short time, when compared with the method of anesthesia of animals (Kimble, *et al.*, 1982). A cloned mutant collagen gene *rol-6* (*su* 1006) was developed as a dominant genetic marker for screening DNA transformants (Kramer *et al*, 1990) and an efficient microinjection procedure was a mixture of pRF-4 (*rol-6*) and pASTRB (*sup-7*) injected into the cytoplasm of the distal gonad provided a modified procedure which helped to increase the recovery frequency of transformants (Mello, *et al.*, 1991).

Southern blot analysis was used for verifying whether a transformant carries the injected DNA. A probe from the plasmid, pASTRB (*sup-7*) (Mello, *et al.*, 1991) was hybridized to DNA from uninjected control and transformed lines, obtained by coinjection of *rol-6* and *sup-7* digested with *EcoRI* and *BamHI*. The transformed lines had a higher intensity of the *sup-7* fragment band than the uninjected animals, because of the extra copies of *sup-7* in the extrachromosomal arrays. This method is labor-intensive and can produce ambiguous results since the fragment sizes can be difficult to resolve.

A method of testing whether the transformant carries three or four cosmids injected at the same time was developed by Clark and Baillie, 1992. The transformed lines were digested with

YK792   cm21g8   cm15b8   YK561   cm14f3
/K1177   cm19f2   cm12d6   cm13h11
   YK861   YK1228   cm9g5   cm06g8

(Y101C9)   (Y30A0)
(Y51F2)   (Y40A4U)
                   (Y27F7)
                   (Y22F2)
(Y50D2)

B0453 *   T10B11
T13C1 *   R11F8
CCCC5   C14A12 *
E02D9   C48B6
BC#S401   F56F2
RW#L198   KB#P5431 *   KR#H26
?D3   C37A2 *   KR#H22   T28A7 *   W03B3
      QQQB5 *   BL#111   F15E3   C15F9 *
   K01B11   K02F2 *   KR#83   C30F12
F08B6   T14D10   KR#LS1

Pick here to go to the genetic map   Pick here to go to the genetic m?

-5   unc-87   rsn-11   dpy-14   let-545
-37   let-398   let-394   sP1   smg-1
-37;rsc T.Barnes,D.Millops-1   U1-glh-1   let-534 Thomas   A.Rose
K.Anders et al   BOTc1 rsc;dat S.Prasad,A.Rose   rsc S.O'Connor et al
     S.Goetinck,R.Waterston   A.Rose;v.nr.dpy-14;KR#LS1 mixt.
     RcB1   J.Boom,M.Smith;Y39A9cy,Y40A4cy+
        POU-1/-2;M.Finney   poly probe:Y50D2,Y27F7,Y39A9
           Ruvkun   A.Rose
             C49D5?   K.Bennett

let-526
spe-11
  dpy-5
  cey-3
   let-393
    let-367
    cey-2
    let-395
    let-530
    let-376
    unc-40
    let-378
    mgl-29
    let-532
   nhr-2
   rtr-1
  ceh-12
  stP124
pgp-2
  let-603
  let-514
   ceh-2
   hP5
   SSG/1
    let-602
    vet-1
     let-611
     let-381
     hli-4
      let-601
       glh-2

           smg-5
           unc-37
           unc-87
           let-398
           ops-1
           rsn-11
           let-394
           glh-1
           dpy-14
           let-534
           sP1
           let-545
           smg-1

                hP9
                sem-4
                kin-14
                unc-14
                hlh-2
                myo-1
                eat-5
                unc-15
                col-7
                unc-13
                spe-4
                goa-1
                lrp-1
                gld-1

                              che-3
                              mau-2

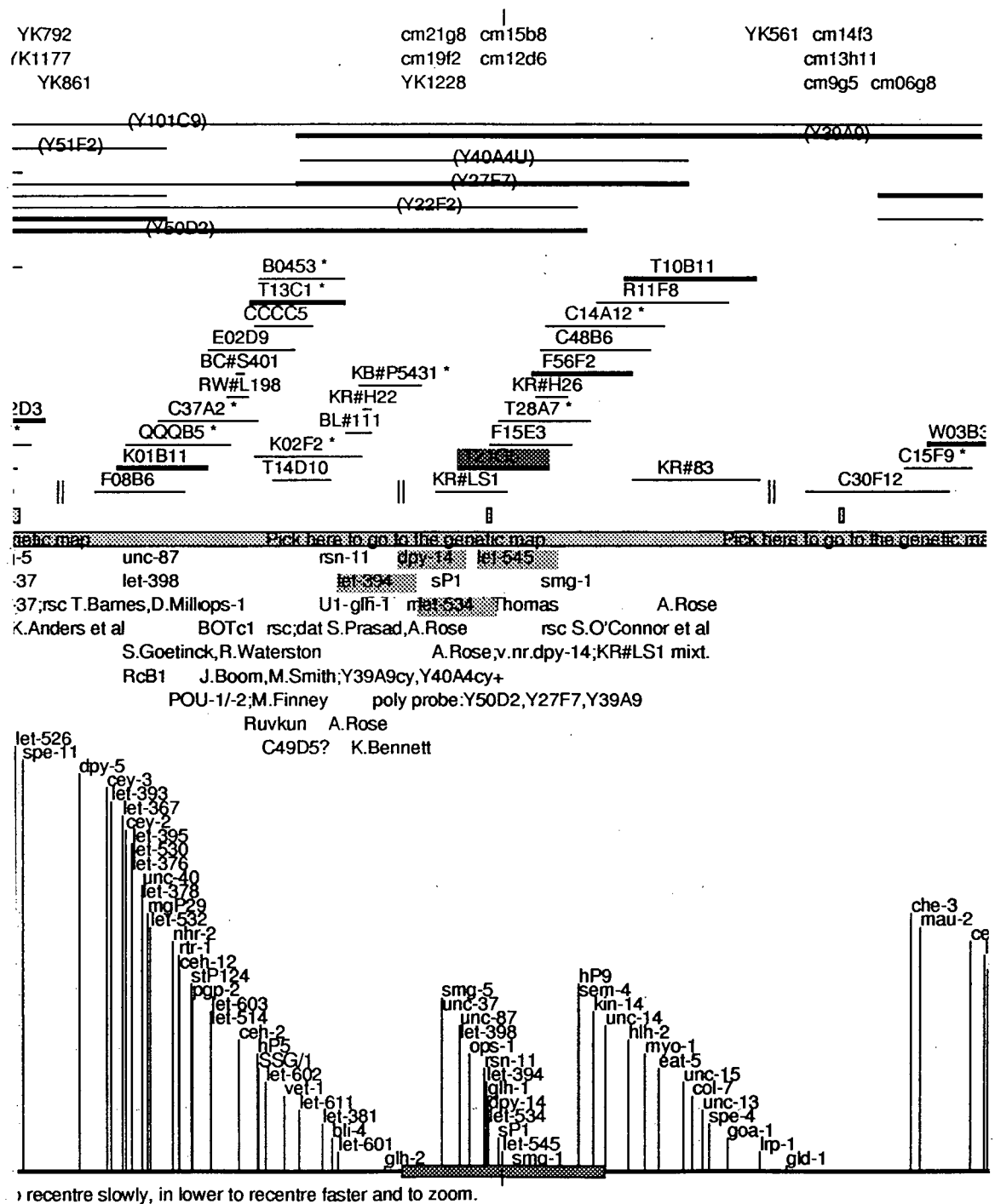) recentre slowly, in lower to recentre faster and to zoom.

Figure 1. A direct print out of the physical map of the *dpy-14* region from ACEDB

an enzyme that does not cut cosmid vector sequences. The result is that the size of the band containing the cosmid vector sequences is different for each cosmid. Vector sequence was used as a probe for Southern blot analysis. Based on the size difference of the DNA fragments from different cosmids, the number of the cosmids successfully transformed equaled the number of bands on the Southern blot. This method clearly shows how many different kinds of cosmid are in the transformant. This method could also be used for testing different plasmid subclones in transformed lines.

In order to have an easy and fast way to test whether transformed lines carry the transforming cosmid, a PCR method was developed (McKay, 1993). Based on sequence differences between the plasmid pRF-4, *rol-6* and cosmid vectors, the presence of the cosmid vector sequences could be used to confirm the presence of the cosmid in the transformed line. The PCR primers were designed from the cosmid vector sequences. The drawback of this assay is that it cannot indicate whether the entire cosmid is present in the transformant not how many different kinds of cosmid are present.

Since the technique of microinjection was invented, it has been applied in different areas, from testing gene function to studying tissue specific gene expression. It has been used as a bridge to link the genetic and physical map together.

The four mutants, *let-394, let-534, let-545* and *dpy-14*, in the *dpy-14* region were rescued by the cosmid T21G5 (McKay, 1993). In order to study the function of these genes, one of my goals was to clone these genes from this cosmid.

In *C. elegans* once injected, DNA forms an extrachromosomal array, heritable but not stable (Mello, *et al.*, 1991). The concentration of DNA injected is critical for forming a heritable, extrachromosomal array. For some genes, the high copy number is toxic to *C. elegans* (Mello, *et al.*, 1991); but for other genes, a high copy number is required for gene expression and function (Blumenthal, *et al.*, 1994). Therefore, the concentration of DNA for each gene needs to be determined independently *in vivo*.

Theoretically, the higher the concentration of test DNA injected, the greater the chance that the extrachromosomal array carries test DNA sequences, when coinjected with a DNA

marker. However, when a high copy number of the gene is toxic, a low copy number is required for germline transformation. When a low copy number of the gene is coinjected with a high copy number of the DNA marker for forming an extrachromosomal array, the extrachromosomal array may not carry the gene of interest (Mello, *et al.*, 1991). Therefore, another of my goal was to develop a method to quickly and easily test whether the DNA subclones were present in the transformed lines.

Materials and Methods

Nematode culture conditions and strains

All *C. elegans* strains used in this study were maintained on petri plates containing nematode growth medium (NGM) streaked with *Escherichia coli* OP-50 at 20°C (Brenner, 1974). The nematode used in this work is the Bristol strain of *C. elegans* and all the mutants were established from the N2 strain treated with ethyl methanesulphonate (EMS) (Brenner, 1974). Nomenclature presented in this thesis is based on the guidelines for *C. elegans* published by Horvitz, *et al.*, 1979, and revised by Riddle, *et al.*, 1987. Abbreviations used are listed in Table 1. Strains used in this study are listed in Table 2. The genetic map information and characterization of lethal mutants used in this study are described in Table 2 of Rose, *et al.*, 1985. The lethal mutant alleles and their arrested stages are indicated in Table 3.

Preparation of cosmid DNA for subcloning

A bacterial strain containing the cosmid was streaked on a LB plate containing an appropriate antibiotic and incubated at 37°C overnight. A single colony was inoculated in 2 ml of LB medium with an appropriate antibiotic, incubated at 37°C overnight with shaking at 220 rpm. DNA preparation was performed using a modified alkaline lysis protocol (Sambrook, *et al.*, 1989). The presence of inserted DNA in the cosmid was verified by restriction enzyme digestion.

Table 1. **Abbreviations**

Abbreviation	Description
dpy	Mutations in these genes cause a shorter body (dumpy) morphology.
Dpy	Phenotype of *dpy* mutations
unc	Mutations in these loci give rise to uncoordinated movement.
Unc	Phenotype of *unc* mutations
let	Mutations in these genes cause *C. elegans* developmental arrest (lethality) at different stages.
rol	Genes whose mutations cause the rotating movement (rolling) of the animal.
h	Alleles, extrachromosomal arrays and chromosomal rearrangements originating from the Rose laboratory
KR	All genetic strains generated in the Rose laboratory designated by these letters
KRp	All oligonucleotide primers designed in Rose laboratory for PCR reactions or sequencing.
pCeh	Plasmid carrying DNA fragment subcloned from *C. elegans* DNA generated from Rose laboratory
*h*Ex	Extrachromosomal arrays in nematode strains originated in Rose laboratory
Dp	Chromosomal duplication
Wt	Wild type *C. elegans*

Table 2. **Strains used in this study**

Strain	Genotype	Origin
BC69	*dpy-14 (e188) unc-13 (e51)* I	Rose A.
KR601	*dpy-5 (e61) let-534 (h260) unc-13 (e450)* I; *s*Dp2 (I; f)	Rose lab.[a]
KR603	*dpy-5 (e61) let-394 (h262) unc-13 (e450)* I; *s*Dp2 (I; f)	Rose lab.[a]
KR1504	*dpy-5 let-545 (h842) unc-13 (e4450)* I; *s*Dp2 (I; f)	Rose lab.[a]
N2	*wild-type*	Brenner, S

a. A.M. Howell, L. Harris, B. Rattray, J. S. Kim, K. McNeil, N. Mawji.

Table 3. **Mutant alleles and their phenotypes**

Gene:	*let-394*	*let-534*	*let-545*	*dpy-14*
Reference alleles:	*h262*	*h260*	*h842*	*e188ts*
Total number of alleles:	[8]	[3]	[2]	[1]
Arrested stage:	early larval	mid larval Unc	adult sterile	1. viable Dpy at 16°C 2. very Dpy at 20°C 3. ts lethal at 25°C

Table 3 was simplified from McKim, *et al.,* 1992.

Restriction enzyme digestion

DNA was digested with restriction enzyme(s) purchased from Pharmacia or GIBCO BRL. Digestions were carried out with the enzyme as indicated in the protocols provided by the manufacturer. The buffer concentration in each reaction was according to the recommendation provided by the company for plasmids or cosmids. Spermidine was added to a final concentration of 5mM in the restriction digestion for *C. elegans* genomic DNA (Sulston and Hodgkin, 1988). Digestions of *C. elegans* genomic DNA were performed with three times more enzyme that used in digestion of plasmids or cosmids for 3-4 hours at 37°C unless specified.

Polymerase Chain Reaction (PCR)

Single worm PCR was performed following the protocol described by Barstead, *et al.*, 1991. Polymerase chain reactions were carried out in a Perkin-Elmer/Cetus PCR machine. The PCR program used was 30 cycles of denaturation (94°C, 30 seconds), annealing (45°C-60°C, depending on the Tm of the primers, 30 seconds), and extension (72°C, 2 minutes). Reactions were performed with Taq polymerase as described by Barstead, *et al.*, 1991.

Agarose gel electrophoresis

Gel electrophoresis was used to determine the DNA fragment sizes from restriction digestion to analyze PCR products and fractionate DNA fragments. Gel concentrations ranged from 0.8%-2% w:v in 0.5X TBE with a final concentration of 0.1ug/ml of ethidium bromide. Voltages did not exceed 5V/cm using 0.5X TBE running buffer. The 1 kb ladder and lambda Hind III marker (GIBCO BRL) were used as DNA size standards. Gels were visualized with ultraviolet illumination at 302 nm (Sambrook, *et al.*, 1989).

Southern blotting analysis

Cosmid DNA and *C. elegans* genomic DNA were digested with restriction enzyme(s) as explained above. DNA fragments were separated by gel electrophoresis together with a DNA standard marker for variable times depending on the sizes of the DNA fragments. After the DNA fragments were separated, gels were depurinated with 0.25N HCl for 5-15 minutes. DNA was transferred to Zeta-Probe GT blotting membranes in 0.4N NaOH with capillary transfer for four to twenty four hours depending on the size of the DNA fragments (Southern, 1975). Blots were neutralized in 2X SSC, air dried for a few minutes and baked in a 80°C incubator with the vacuum on for 30 minutes.

The DNA fragment used as a probe was fractionated in agarose gel in 1X TAE buffer and purified with a Qiagen Gel Extraction kit purchased from the manufacturer. The probe was labeled with the ECL random prime system supplied by Amersham Life Science company. In the ECL random prime system , fluorescein-11-dUTP (Fl -dUTP) is used as the labeling chemical, which partially replaces TTP in the reaction, therefore, the probe is labeled with fluorescein. Later, the fluorescein within DNA will be detected by an anti-fluorescein antibody (Whitehead, 1983). Prehybridization, hybridization and signal detection were performed following the protocol supplied by the manufacturer.

Subcloning of DNA fragments

Cosmid DNA was digested with a restriction enzyme, which was heat inactivated, at the temperature recommended by the manufacturer, after restriction digestion. DNA was extracted with phenol and chloroform after heat inactivation, precipitated with 2 volumes of 95% ethanol, washed in 70% ethanol and dissolved in TE, pH 7.6. The plasmid vector, Bluescript, was digested with restriction enzyme, run on gel with 1X TAE buffer and purified using the Qiagen Gel Extraction kit purification system. The vector was then dephospharylated by using calf intestinal alkaline phosphatase (CIP) (Sambrook, *et al.*, 1989) which was supplied by GIBCO BRL. The ratio of insert DNA fragments to vector for ligation was three to one, using *T4* DNA ligase (GIBCO BRL) at 16°C for overnight (Sambrook, *et al.*, 1989). Ligated DNA was

transformed into DH 5 alpha competent cells (Ullmann, *et al.,* 1967) supplied by GIBCO BRL. Transformed cells were resistant to ampicillin because of the presence of an ampicillin gene on the plasmid. Cells transformed with the plasmid vector without inserted DNA form blue colonies in the presence of the chromogenic substrate 5-bromo-4-chloro-3-indylol-beta-D-galactoside (X-gal) and isopropyl thiogalactoside (IPTG) (Sambrook, *et al.,* 1964). Insertion of a foreign DNA fragment into the polylinker site of the plasmid vector will interrupt the Lac Z alpha gene abolishing alpha-complementation. Therefore, *E. coli* cells carrying the recombinant plasmids produce white colonies. This is a useful tool for screening DNA clones. Plasmids with recombinant DNA are confirmed by restriction analysis of plasmid DNA (Sambrook, *et al.,* 1989).

Cosmid T21G5 was digested with the restriction enzyme of *PstI* and *SacI,* and subcloned into the Bluescript plasmid vector digested with *PstI* and *SacI.* All subclones established are listed in Table 6.

Preparation of DNA for germline transformation

A single bacterial colony was transferred into 50 ml of LB medium containing the appropriate antibiotic in an autoclaved 250 ml flask. The culture was incubated at 37°C with shaking at 220 rpm. The harvested culture was centrifuged 10 minutes at 5000 rpm, and the supernatant fluid was discarded. DNA preparation followed the alkaline lysis protocol (Sambrook, *et al.,* 1989). For germline transformation, the DNA was dissolved in TE (pH 7.6) containing RNaseA (20 ug/ml) and incubated at 37°C for 30 minutes then extracted with phenol and chloroform to eliminate RNaseA, which is toxic to *C. elegans* cells. The concentration of DNA was tested with the Saran wrap method (Sambrook, *et al.,* 1989). The final concentration of subcloned DNA for germline transformation was about 50ng/ul (Mello *et al,* 1991). The plasmid pRF-4 was a gift from C. Mello. This plasmid carries a 4 kb *EcoRI* fragment of *C. elegans* genomic DNA, containing a dominant mutation in the *rol-6 (su1006)* collagen gene (Kramer, *et al.,* 1990), cloned into the Bluescript vector (Stratagene Inc.). The 4 kb *EcoRI* DNA

fragment carrying the *rol-6* collagen gene was subcloned into pGEM -7Zf (+) (kindly provided by R. McMaster) so that the PCR technique could be used to test transgenics for the presence of plasmids other than that carrying the *rol-6* marker.

Germline transformation

A plasmid carrying an inserted DNA fragment was co-injected with *rol-6* (*su1006*) in the pGEM-7Zf (+) vector into the distal arm of both gonads of young adult hermaphrodites using the DNA mixture with the ratio of 50ng/ul of plasmid carrying insert to 50ng/ul of *rol-6* in pGEM-7Zf (+) (Mello, *et al.*, 1991). Single plasmids and overlapping plasmids listed in Table 4 were used for each injection. Approximately ten to twenty percent of F1 roller progeny were stably inherited into the F2 generation. The stable inherited line in the F2 generation was screened from the F1 generation of rollers, and was able to use for rescue analysis of lethal mutations.

Polymerase chain reaction (PCR) analysis of extrachromosomal arrays

PCR was used to test the extrachromosomal arrays in the transgenics. This assay detected the vector used for the recombinant plasmid (McKay, 1993). A single worm PCR was using in the conditions of Barstead, *et al.*, 1991 and Williams, *et al.*, 1992. A set of two primers having unique sequences were designed from the Bluescript vector indicated in Table 5, which do not amplify the pGEM- 7Zf (+) vector. The PCR amplified DNA fragment is 812 base pair (bp). The presence of the 812 bp PCR product indicated that the Bluescript vector, which carries the insert DNA, was present in the transgenics. The PCR reaction was performed using a negative control without DNA template, and a positive control with the Bluescript vector alone.

Complementation test of lethal mutations with plasmid subclones of cosmid T21G5

Heterozygote males carrying *let-394*, *let-534*, *let-545* or *dpy-14 unc-13* were crossed to

roller hermaphrodites carrying plasmid subclones (transgenics *hEx*). After twenty four hours, each mated hermaphrodite roller was transferred to a fresh plate. All of the L4 progeny of hermaphrodite rollers (*dpy-5 let-x unc-13 / + + +; hEx [rol-6] or + + + / + + +; hEx [rol-6]* were picked from the parental plate having more than ten males which indicates successful outcrossing and transferred individually into a fresh plate. Plates segregating the *Dpy Unc* phenotype were scored. Rescue of the mutants was indicated by viable and fertile *Dpy Unc* worms. The rescued *Dpy Unc* worms were maintained on individual plates, and stable rescued strains established. The detailed cross scheme is shown in Figure 2.

Table 4. **Constructions of subclones for microinjection**

Construction of plasmid DNA	Insert DNA fragment in plasmid(s)
pCeh 219 [a]	13.5 kb *PstI*
pCeh 266	13.5 kb *SacI*
pCeh 265	12.5 kb *SacI*
pCeh 260	12.5 kb *PstI*
pCeh 219 & pCeh 265 [b]	13.5 kb *PstI* & 12.5 kb *SacI*
pCeh 265 & pCeh 260 & pCeh 266	12.5 kb *SacI* & 12.5 kb *PstI* & 13.5 kb *SacI*
pCeh 265 & pCeh 260	12.5 kb *SacI* & 12.5 kb *PstI*
pCeh 260 & pCeh 266	12.5 kb *PstI* & 13.5 kb *SacI*

a. All the plasmid(s) were coinjected with *pCeh 267* carrying *rol-6* (*su 1006*) gene in pGEM-7Zf (+) vector.

b. All the double or triple plasmids injected have overlapping region between or among them (figure 3).

Table 5. **Primers for single worm PCR**

Primer	Sequence (5' - 3')	Size of the amplified product	Target gene sequence
KRp 86	GCGATAAGTCGTGTCTTA		
		812 bp	Bluescript[a]
KRp 90	CGATACCGTCGACCTCG		
KRp 46	ATGCTACTGGTCAGTTTTCA		
		1011 bp	*bli-4* gene[b]
KRp47	ATTCTATCCGAATCCTCCGA		

a. Sequence taken from Bluescript vector sequence (Stratagene, La Jolla, Califormia)

b. Antisense strand in intron 12 of *bli-4* (Thacker, personal communication)

dpy-5 let-x *unc-13*; *sDp2* hermaphrodites **X** N2 males

$\downarrow$ pick up wild males F1

dpy-5 let-x *unc-13* / + + + males **X** + / +; hExn [*rol-6*]

$\downarrow$ pick up and self-cross L4 Rol hermaphrodite F2

dpy-5 let-x *unc-13* / + + +; hExn [*rol-6*]   or   + + + / + + +; hExn [*rol-6*]

$\downarrow$ self-cross $\downarrow$ self-cross

Wt, Rol, *dpy-5* let-x *unc-13* / *dpy-5* let-x *unc-13*,   Wt, *Rol*
dpy-5 let-x unc-13 / dpy-5 let-x unc-13; hExn [rol-6]

$\downarrow$ self-cross

dpy-5 let-x *unc-13* / *dpy-5* let-x *unc-13*   or
dpy-5 let-x *unc-13* / *dpy-5* let-x *unc-13*; hExn [*rol-6*]

$\downarrow$ self-cross
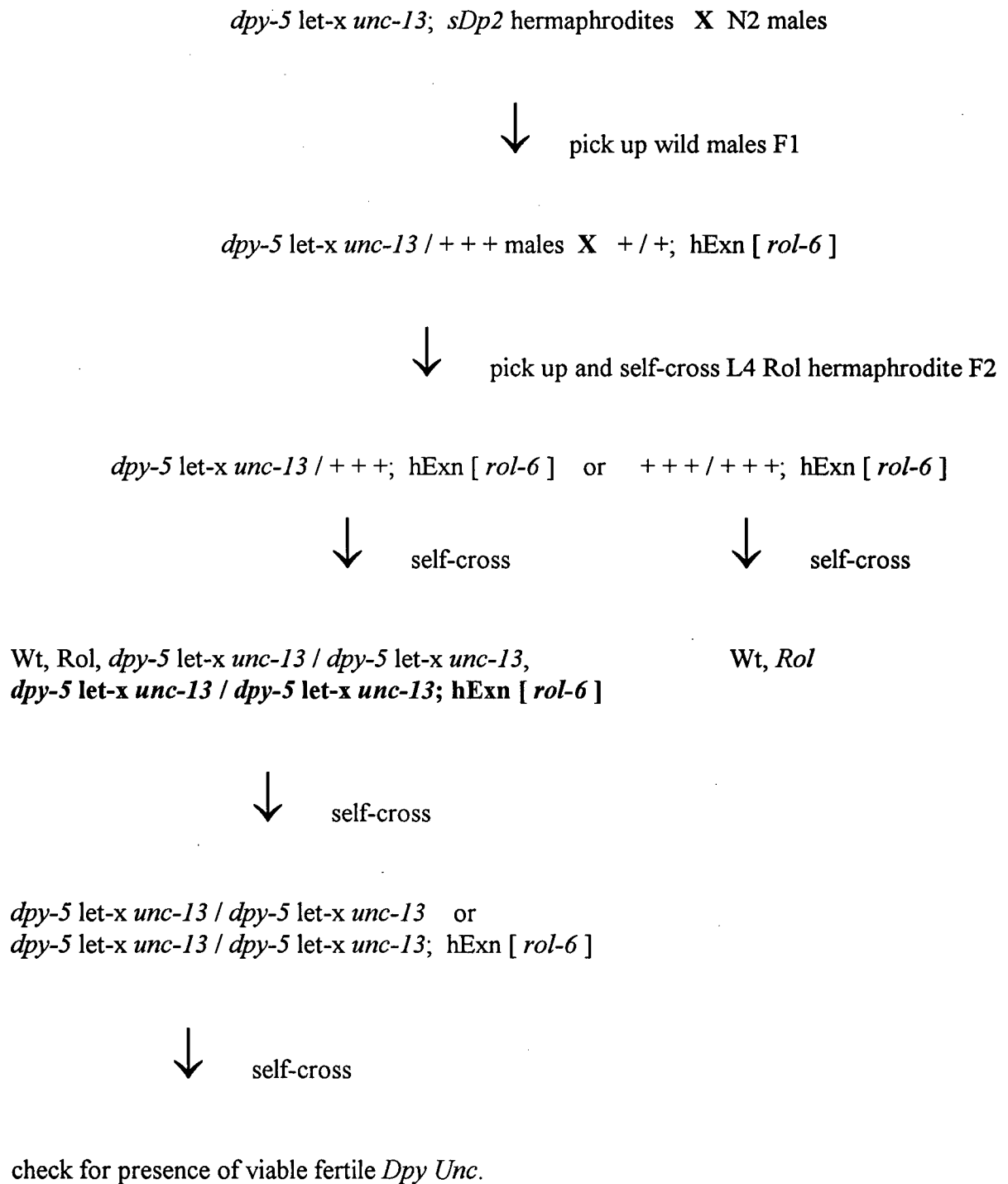
check for presence of viable fertile *Dpy Unc*.

Figure 2. Protocol of the complementation tests.

Genetic cross-scheme for testing complementation of mutants by the transformed lines. Bold-faced genotype is the class for further complementation tests.

Results

Restriction map of T21G5

A restriction map of cosmid T21G5 was necessary to fine map these mutants. DNA from cosmid T21G5 was digested with either a single restriction enzyme *PstI* or *SacI*, or with two restriction enzymes, *PstI* and *KpnI* or *SacI* and *KpnI* (Figure 3). Because of the large insert in the cosmid and the many restriction sites in the insert, the restriction map cannot easily be drawn from a few single and double digests. Southern blot analysis was used to help determine the position of each DNA fragment. T21G5 contains partial *Sau3A* digested DNA fragment from *C. elegans* genome cloned into the *Hind III* site of the Lorist 2 vector. After the DNA fragment has been cloned into the Lorist 2 vector, it can not be removed from the cloning site of the vector.

Using the restriction sites given for the Lorist 2 vector (DNA strider 1.0, 1993), one end of the inserted DNA fragment has a 13.5 kb *PstI* fragment when cutting with *PstI* or a 12.5 kb *SacI* fragment when cut with *SacI*. There are no *KpnI* sites on these two fragments. This end was verified by hybridizing it with the lorist 2 vector sequences. The cosmid insert DNA has a 2.0 kb *EcoRI* fragment and 3.0 kb *EcoRI* fragment at the other end of the insert DNA derived from a 9.7 kb *PstI* fragment and a 13.5 kb *SacI* fragment when digesting with a double restriction enzymes of *PstI/EcoRI* or *SacI/EcoRI* (Bennett, *et al*, personal communication). A cosmid map of *PstI* digestion could be drawn using the information that the 13.5 kb *SacI* hybridized to the 12.5 kb *PstI* (data not shown), but not the 3.2 kb *PstI* fragment (Figure 4). When analyzing the
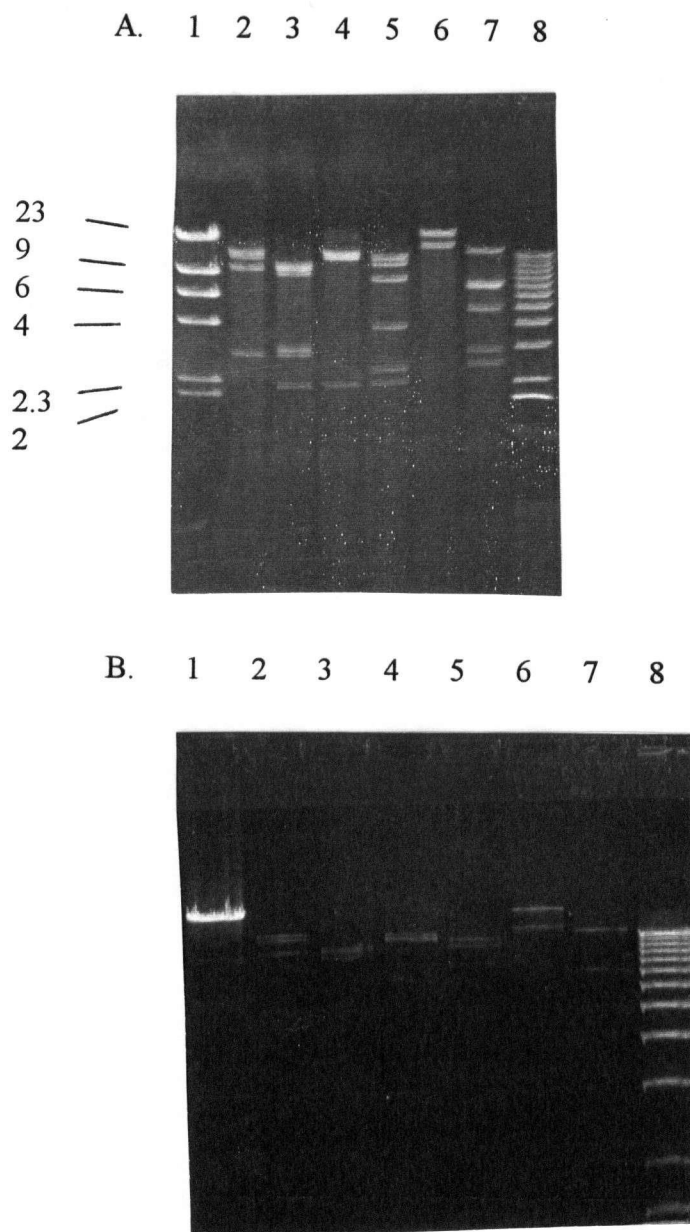
Figure 3. Restriction digests of the cosmid T21G5.

Cosmid DNA was digested with restriction enzyme *PstI* or *SacI* separately or in combination of either *PstI/KpnI* or *SacI/KpnI*. Reaction conditions described in materials and methods.
A. 1.6 ug of cosmid DNA was loaded in each lane and electrophoresed to obtain separation of the small sized DNA fragments. Lane 1: lambda *Hind III* marker; lane 2: *PstI*; lane 3: *PstI/KpnI*; lane 4: *SacI*; lane 5: *SacI/KpnI*; lane 6: *KpnI*; lane 7: *SacI/PstI*; lane 8: 1 kb ladder.
B. 0.5 ug of DNA was loaded in each lane and electrophoresed to and visualize the large sized DNA fragments. The samples were loaded as the same order as that in the A.
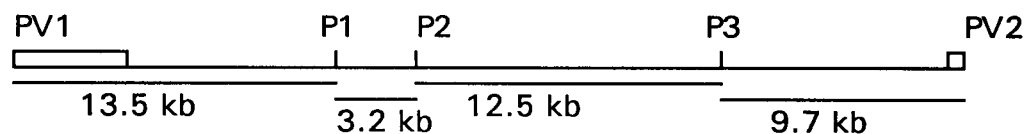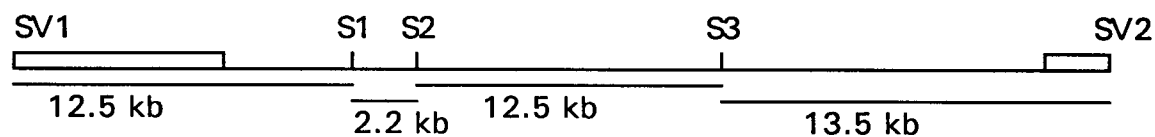
A. PstI:

B. SacI:

Figure 4. Restriction maps of the cosmid T21G5.

A. *PstI* digestion of cosmid T21G5. P represents the *PstI* digestion.
B. *SacI* digestion of cosmid T21G5. S represents the *SacI* digestion.
The blocked areas are the lorist 2 vector sequences.
The vector size for *PstI* map: PV1, 3.8 kb and PV2, 434 bp.
The vector size for *SacI* map: SV1, 5.3 kb and SV2, 357 bp.

patterns of the DNA fragments and the sizes of the fragments with the double digestion of the *PstI* and *SacI*, the cosmid restriction maps of *SacI* or *PstI* digestion were drawn (see Figure 4).

Strategy of subcloning

To obtain large subclones that hopefully would contain complete coding sequences, an infrequently cutting restriction enzyme was chosen. *PstI* and *SacI* were used for subcloning, since they have only three cut sites in the insert DNA, as indicated by the restriction digestion pattern. In total, four *PstI* subclones and four *SacI* subclones were obtained for the mutants rescue study (see Table 6).

Since we have no knowledge of the arrangement of the genes in the cosmid, any restriction site could cut within a coding region, making that gene nonfunctional. In order to overcome this problem, overlapped subclones from the *PstI* or *SacI* digests were designed for generating the transformed lines (Figure 4 and Table 6). For instance, the P3 site of the *PstI* digestion might lie within a gene. To attempt to overcome this problem, a strategy of co-injecting the plasmid containing the 13.5 kb *SacI* fragment with a plasmid containing the 12.5 kb *PstI* fragment was used. The identical insert sequences between the two plasmids could recombine *in vivo* to form a large insert DNA fragment (Mello, *et al.,* 1991). Likewise, the plasmid containing the 12.5 kb *SacI* fragment was coinjected with the plasmid of the 12.5 kb *PstI* fragment, and the plasmid containing the 12.5 kb *SacI* fragment was coinjected with the plasmid of the 13.5 *PstI* fragment. Similarly the plasmids of the 13.5 kb *SacI* fragment and the 12.5 kb *SacI* fragment were coinjected with the plasmid containing the 12.5 kb *PstI* fragment. All of the plasmids used for germline transformation are listed in Table 6.

Table 6. **Subclones of T21G5 and the** *rol-6 (su 1006)* **gene**

Name	Length of insert fragment	Restriction digestion	Vector
pCeh 219	13.5 kb	*PstI*	pBS (KS+)
pCeh 261	3.2 kb	*PstI*	pBS (KS+)
pCeh 260	12.5 kb	*PstI*	pBS (KS+)
pCeh 262	9.7 kb	*PstI*	pBS (KS+)
pCeh 263	12.5 kb (without KpnI cutting site)*SacI*		pBS (KS+)
pCeh 264	2.2 kb	*SacI*	pBS (KS+)
pCeh 265	12.5 kb (with KpnI cutting site)*SacI*		pBS (KS+)
pCeh 266	13.5 kb	*SacI*	pBS (KS+)
pCeh 267	4.0 kb *(rol-6)*	*EcoRI*	pGEM -7Zf (+)

Making transformed lines

In order to be able to select animals carrying the extrachromosomal array for use them in genetic crosses, a dominant marker was coinjected (Mello, *et al.*, 1991). Microinjection was done by injecting the needle tip into the cytoplasm of the distal gonad, which can be visualized as rows on each side of the central grainy core of cytoplasm (see Figure 5). Using Mello's (1991) approach, a high frequency of germ line transformants can be obtained in the progeny of the injected hermaphrodite, when compared with a single injection into the oocyte (Fire, 1986). A cloned mutant collagen gene, *rol-6 (su 1006)* (Kramer *et al.*, 1990) was used as a dominant genetic marker for DNA transformation (Mello, *et al.*, 1991). This gene has the characteristics of being able to form a heritable extrachromosomal array, in which high copy numbers of this gene are not detrimental to the worm, and which readily produces a scorable roller phenotype.

The most frustrating step of performing microinjection is frequent clogging of the needle. To overcome this problem the DNA solution requires sufficient centrifugation (usually 13200 rpm/30 minutes) to precipitate particles. Ten worms were injected at each time. One worm was placed on a 2% agarose pad for each microinjection to prevent it from dehydration. After microinjection, the worm was placed on an individual plate with a drop of M9 buffer. Three days later, the F1 rollers were transferred from these plates which had been incubated in a 20°C incubator, and placed on new plates. The F2 rollers were obtained from those F1 roller plates after three days. The heritable lines established from each injection were used for complementation testing of the mutants. Eight transformed lines were constructed (see Table 7).

A. T21G5 was injected to confirm that it could rescue the four mutants. The concentration of DNA for the injection was 20 ng / ul of T21G5 & 100 ng / ul of pCes 1943 (pRF-4 construct modified to contain a kanR sequence, kindly provided by Baillie's lab). Twelve young adult worms were injected. Forty-four F1 rollers were selected from these twelve injected worms. Four heritable lines were established from the forty-four F1 rollers. After PCR testing, three heritable lines showed lorist 2 vector sequence indicating that the cosmid is in the transgenics (Figure 6).

Table 7 . **Construction of the transformed lines**

Strain	Method	Genotype	clone(s)
KR 3129	Trans.[a]	+ /+ ; hEx 68	pCeh 266
KR 3130	Trans.	+ /+ ; hEx 69	pCeh 266
KR 3131	Trans.	+ / + ; hEx 70	pCeh 266
KR 3153	Trans.	+ / + ; hEx 82	pCeh 265
KR 3139	Trans.	+ /+.; hEx 76	pCeh 260
KR 3151	Trans.	+ / + ; hEx 80	pCeh 260
KR 3152	Trans.	+ /+.; hEx 81	pCeh 260
KR 2987	Trans.	+ / + ; hEx 53	pCeh 219
KR 3154	Trans.	+ / + ; hEx 83	pCeh 266 & pCeh 260
KR 3155	Trans	+ /+.; hEx 84	pCeh266 & pCeh 260
KR 3158	Trans.	+ / + ; hEx 87	pCeh 260 & pCeh 265
KR 3132	Trans.	+ /+.; hEx 71	pCeh 260 & pCeh 265
KR 3142	Trans.	+ /+. ; hEx 79	pCeh 260 & pCeh 265
KR 3156	Trans.	+ /+ ; hEx 85	pCeh 265 & pCeh 219
KR 3157	Trans.	+ / + ; hEx 86	pCeh 265 & pCeh 219
KR 3133	Trans.	+ / + ; hEx 72	pCeh 266 & pCeh 260 & pCeh 265
KR 3134	Trans.	+ /+ ; hEx 73	pCeh 266 & pCeh 260 & pCeh 265

a. Transgenic strains (Trans) were constructed by injecting 50 ng/ul of the plasmid subclone with 50 ng/ul of plasmid pCeh 267, carrying *rol-6 (su 1006)*.
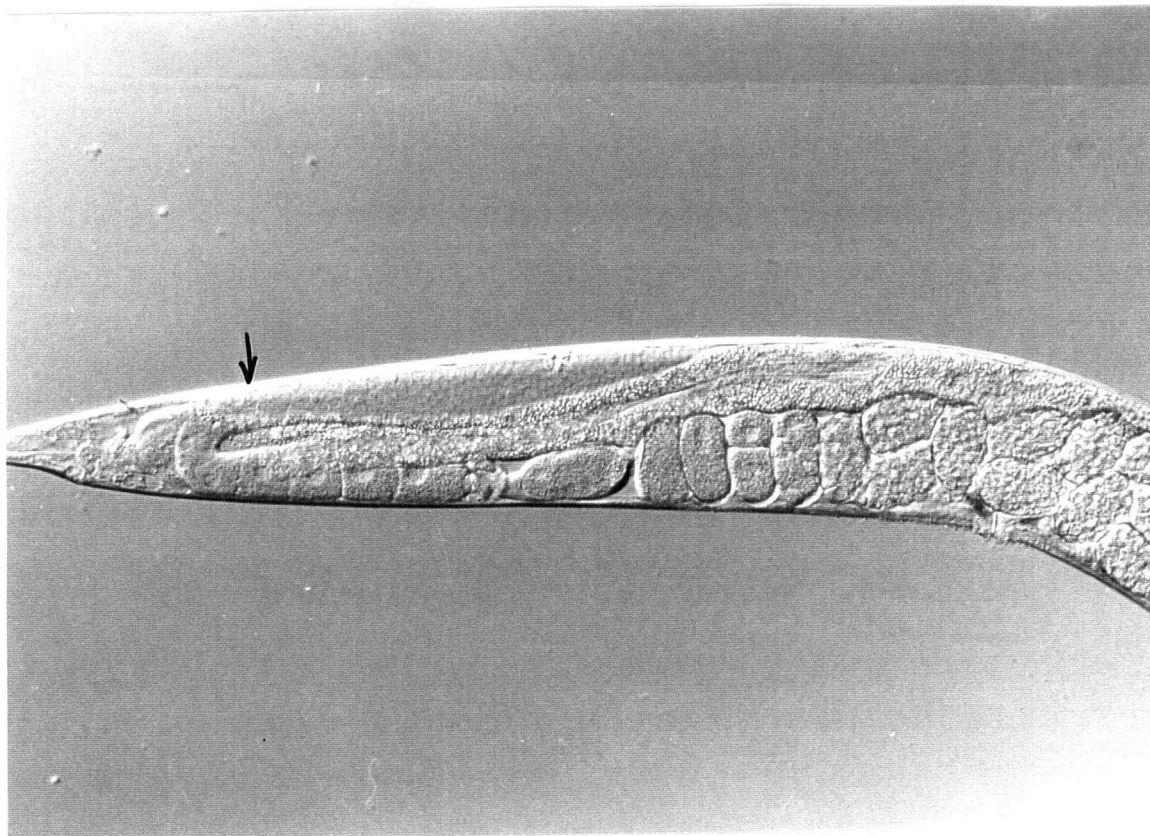
Figure 5. Microinjection position for germline transformation.

The arrow shows the area of the injection site. Photograph courtesy of J. McDowall, University of British Columbia.
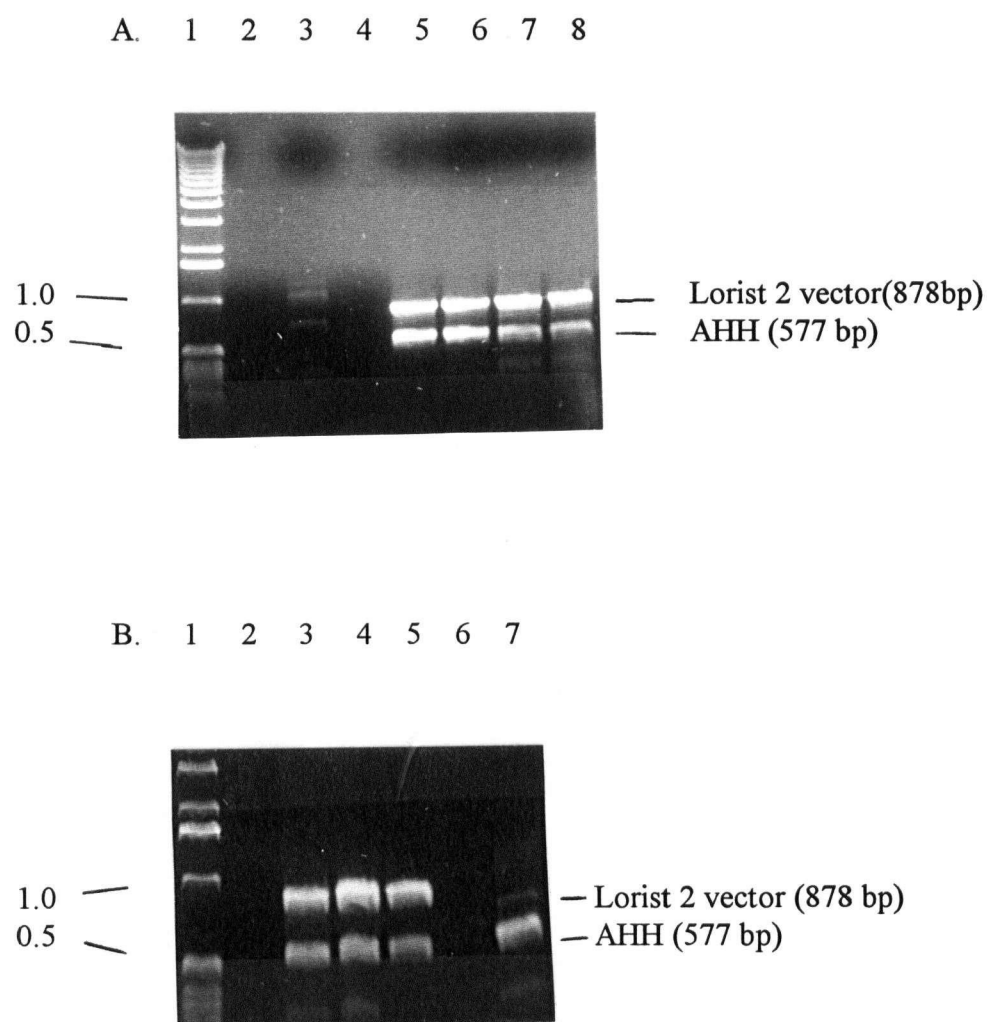
Figure 6. Testing lines transformed with cosmid T21G5 by PCR.

PCR was performed as explained in the method section.
The one set of primers are KRp17 and KRp18 (McKay, 1993) amplifying the lorist 2 vector. The other set of primers are KRp12 and KRp14 (Prasad, *et al.*, 1993) amplifying AHH (S- Adenosyl homocysteine hydrolase).
A. Lane 1: 1 kb ladder; lane 2: blank; lane 3: negative control; lane 4: blank; lane 5: T14D10; lane 6: T14D10 in worm lysis buffer; lane 7: KR 3135; lane 8: KR 3135.
B. Lane 1: 1 kb ladder; lane 2: KR 3159 worm lost in reaction; lane 3: KR 3159; lane 4: KR 3136; lane 5: KR 3136; lane 6: transgenics of T21G5 (4) worm lost in reaction; lane 7: transgenics of T21G5 (4).

B. In order to rescue the mutants with the subclones, microinjection of the subcloned DNA along with the pRF-4 was performed. The final concentration of DNA was 50 ng / ul of pRF-4 & 50 ng / ul of the subclone pCeh219. The transformed line with the pCeh219 having the lorist 2 vector sequence was verified by PCR with primers KRp17 and KRp18 (McKay, 1993) (Figure 7), since a 0.8 kb fragment was observed in the transformed line.

Developing a method to identify the subclones in transformed lines with PCR

A large extrachromosomal array assembled from a high copy number of DNA molecules is required to form a heritable transformed line (Mello, *et al.*, 1991). However, the gene product studied could be toxic to the worms, if high copy numbers of the gene are in the extrachromosomal array. One way of overcoming this problem is to increase the concentration of the pRF-4 (*rol-6*) and decrease the DNA concentration of the gene studied. However, higher concentration of the pRF-4 (*rol-6*) alone may form a large extrachromosomal array without the tested gene. Therefore, it is useful to develop a quick and easy method to test whether the transformed lines are carrying the tested gene, when the low copy numbers of the gene are used in the mixture of the injection solution with the pRF-4 *(rol-6)*.

Bluescript was used for the cosmid subclones. However, the dominant *rol-6* marker is also in the Bluescript vector. Therefore, it was impossible to distinguish between the subclones and the pRF-4 (*rol-6*) based on the presence of the Bluescript sequences in an extrachromosomal array by PCR.

In order to use PCR to confirm the presence of vector sequences from the subclones in an extrachromosomal array, the inserts and the *rol-6* marker should be prepared in different vectors. Since the Bluescript vector has unique sequences at the polylinker sites that the pGEM-7Zf (+)
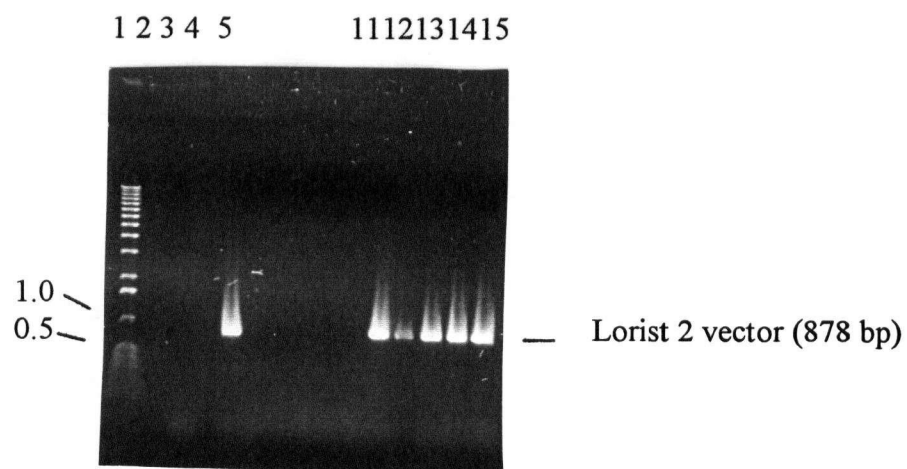
Figure 7. Testing the pCeh219 transformed line by PCR.

pCeh219 carrying part of lorist 2 vector sequence (Figure 3). The primers used for PCR were KRp17 and KRp18 amplifying the lorist 2 vector (McKay, 1993). Lane 1: 1 kb ladder; lane 2: blank; lane 3: negative control; lane 4: blank; lane 5: T14D10; lane 11-15: KR 2987.

does not have, primers in the polylinker sites of Bluescript could now be used to test the presence of Bluescript vector sequences. Therefore, the *rol-6* gene was needed to recloned into pGEM-7Zf (+) (Figure 8).

After searching the data base of vector sequences between Bluescript and pGEM-7Zf(+), the sequences complementary to the KS primer in Bluescript was chosen to be one of the primers, named KRp90, which was not present in the pGEM-7Zf (+) vector. The other primer, named KRp86, came from vector sequences present on both vectors (Table 5).

PCR was performed with pGEM-7Zf (+) vector, rol-6 subcloned in pGEM-7Zf (+) as a negative control, and Bluescript vector as a positive control (Figure 9). The PCR results indicated that the set of primers (KRp86 and KRp90) amplify only the Bluescript vector, but not the pGEM-7Zf (+) vector or the *rol-6* in pGEM-7Zf (+). Therefore, the set primers (KRp86 and KRp90) could be used to test for presence of subclones in the transformed lines.

Demonstrating the transformed lines contain the subclones together with the *rol-6* marker

Based on the result shown in Figure 9, the primers designed from Bluescript vector can be used to test whether the transformed lines carry the subclones in the extrachromosomal array. Occasionally, a negative result is obtained because the worm is lost in the reaction. To detect this problem, another set of primers from the *C. elegans* genome was used to confirm that the worm was in the reaction.

A single worm was used for each reaction. Two sets of primers were amplified at the same time. One set of primers was KRp86 and KRp90 for amplifying the Bluescript vector in an extrachromosomal array, the other set was KRp46 and KRp47 for amplifying intron 12 of the *bli-4* gene in *C. elegans* genome (Thacker, personal communication) as a positive control. The annealing Tm temperature for these two sets of the primers was 45°C. The products of the PCR reaction were loaded on 0.8% agarose gel with 0.5X TBE buffer.
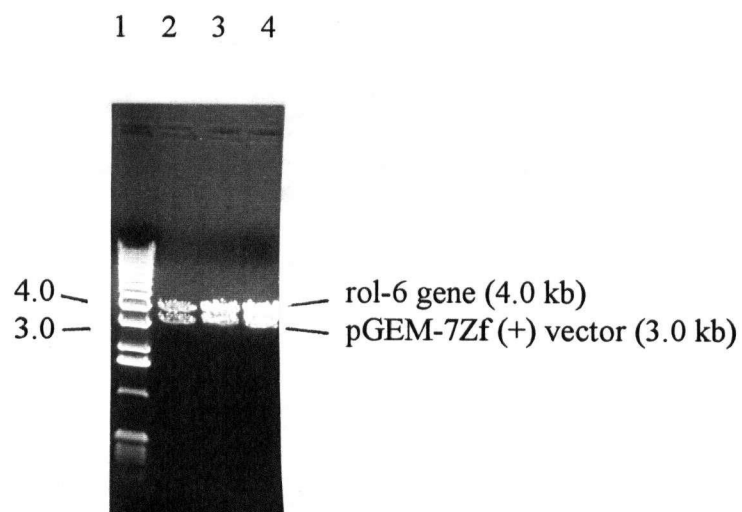
Figure 8. Subclone of the *rol-6* gene in pGEM-7Zf (+).

Subcloning was done following the conditions explained in the method section.
The subclone was digested with *EcoRI*. The *rol-6* gene is about 4 kb. lane 1: 1 kb ladder marker;
lane 2-4: *rol-6* gene subclone *EcoRI* digested at different DNA concentration.
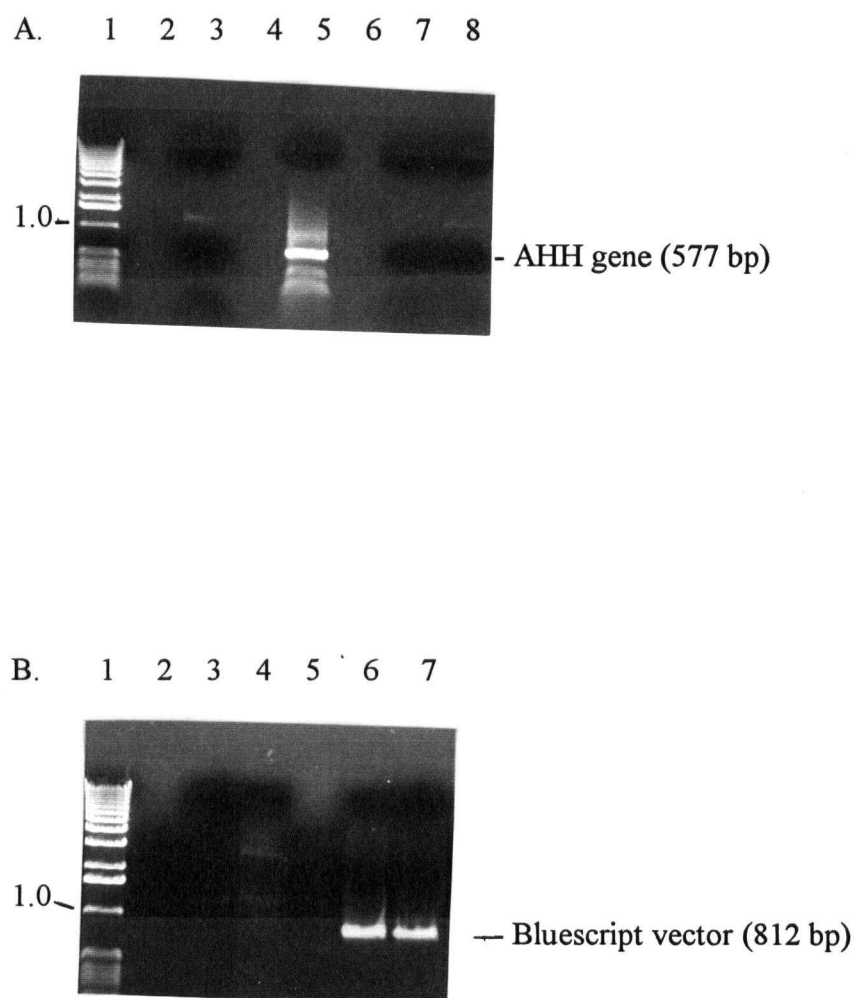
Figure 9. Testing the primers of KRp86 and KRp90 with PCR.

PCR was performed in the condition explained in the method section.
A. Lane 1: 1 kb ladder; lane 2: blank; lane 3: negative control; lane 4: blank; lane 5: cosmid T14D10 (KRp12 and KRp 14); lane 6: blank; lane 7: *rol-6* gene in pGEM-7Zf (+); lane 8: *rol-6* in pGEM-7Zf (+).
B. Lane 1: 1 kb ladder; lane 2: blank; lane 3: pGEM-7Zf (+); lane 4: pGEM-7Zf (+); lane 5: blank; lane 6: Bluescript vector; lane 7: Bluescript vector.

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

1.0 —

— antisense of bli-4 gene (1011 bp)
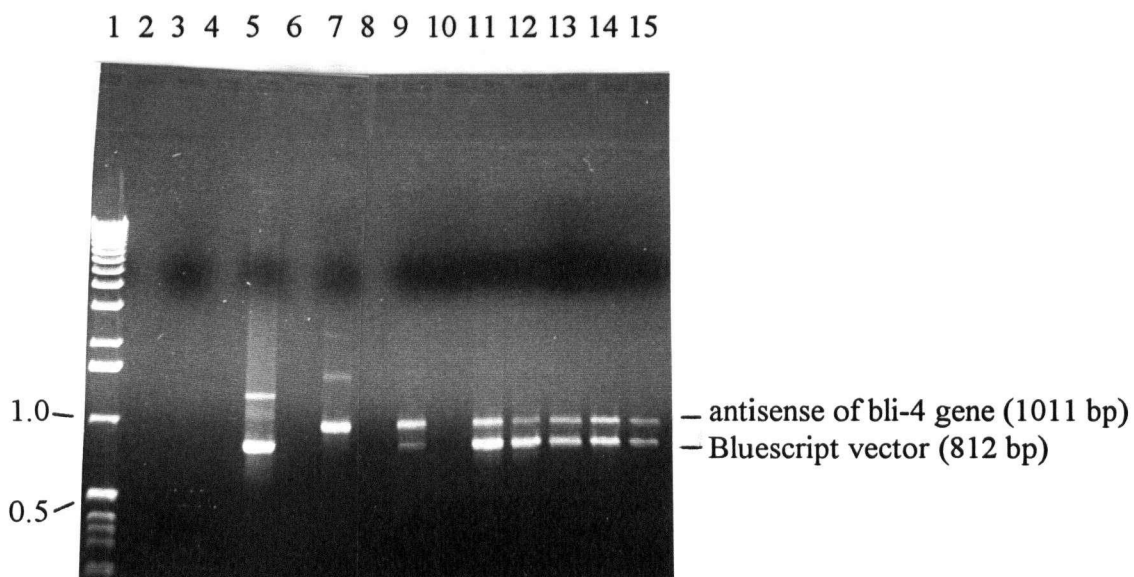— Bluescript vector (812 bp)

0.5 —

Figure 10. Testing subclone transformed lines by PCR.

The final concentration of DNA for these subclone's microinjection were 50 ng / ul of subclones
& 50 ng / ul of pCeh267.
Lane 1: 1 kb ladder; lane 2: blank; lane 3: negative control; lane 4: blank; lane 5: Bluescript
vector; lane 6: blank; lane 7: N2; lane 8: blank; lane 9: KR 3158; lane 10: worm lost in
reaction; lane 11: KR 3154; lane 12: KR KR 3131; lane 13: KR 3133; lane 14: KR 3157; lane
15: KR 3135.

The result showed that the band size of Bluescript vector was about 800 bp whereas a band of 1.0 kb in size was obtained from the antisense of the *bli-4* gene of the *C. elegans* genome (Figure 10). These results indicated the transformed lines tested with PCR carried the vector sequences of Bluescript in an extrachromosomal array. Therefore, I infer that the whole subclones were present in the extrachromosomal array.

Complementation test for rescuing mutant alleles in transformed lines

To test if the subclone was functioning (ie., genes were expressed) in the extrachromosomal array or to correlate the subclone with the mutant phenotype, mutants rescued by cosmid T21G5 were used for testing the subclones for the same rescue ability.

A. Complementation test of the mutants with the transformed lines of *hEx24*

The transformed line, hEx24 carrying cosmid T21G5, constructed by McKay (1993), was used for genetic crosses to confirm the rescue of the four mutants. The procedure used followed the protocol shown in Figure 2. Each of the four mutant strains was maintained with the balancer *sDp2* to prevent crossing over between the lethal and the visible markers (*Dpy* and *Unc*). Four hermaphrodites from each strain were crossed to six N2 males for twenty-four hours. After mating, the hermaphrodites were transferred to individual plates. Heterozygous males from this cross were mated to hEx24 rollers, which carry the extrachromosomal array. Twenty four hours later, rollers were transferred to individual plates. Each plate was examined for the presence of males indicating outcrossing. Plates having more than ten males were kept for further analysis of *Dpy Unc* progeny. Five or ten *Dpy Unc* hermaphrodites were picked from F2 plates to a fresh plate for progeny testing. Fertile *Dpy Unc* were diagnostic of rescue (see Table 8).

In order to confirm whether the transformant of hEx24 carries the cosmid T21G5, Southern blot analysis was performed. DNA was extracted from the transformed line of hEx24, digested with KpnI which does not cut the Lorist 2 vector sequence, electrophoresed and transferred to the nylon blot. The part of Lorist 2 vector sequences amplified from PCR reaction

was used as a probe. The Southern blot analysis showed that the hybridizing band was 23 kb, which is one of the bands of the T21G5 digested with *KpnI* (data not shown). This result indicated that the transformant of hEx24 carried the cosmid T21G5.

B. Complementation test of mutants with transformed lines of cosmid T21G5

To confirm that the cosmid used for subcloning has the same rescue activity as the cosmid in *hEx24*, T21G5 was injected into young adult hermaphrodites. Three heritable transformed lines were established for tests of the four mutants. The complementation test results showed that after crossing the transformed lines with heterozygous mutant males, all of the *dpy unc* progeny from the crosses were still arrested at the same stage as the *dpy unc* mutants in the control plates (Table 9). This indicated that the transformed lines I made with the cosmid T21G5 did not rescue the four mutants.

To test the cosmid T21G5 used for making the transformed lines, DNA was digested with either *KpnI* or *PstI*, electrophoresed, and run together with a sample of control cosmid T21G5 newly arrived from the cosmid center. The pattern of cosmid T21G5 digested with either *KpnI* or *PstI* is the same as the control cosmid. This result indicated that the cosmid used for making transformed lines was not different from the control sample (see Figure 12 and Figure 13).

C. Complementation test of the mutants with transformed lines of the subclones

The heritable transformed lines established with subclones were complementation tested for rescuing the four mutants. The procedure used for the complementation test was the same as the procedure shown in Figure 2. The result of the complementation test was that the *dpy unc* progeny from the crosses were still the same as those in the control plate (Table 10). This indicated that the transformed lines with the subclones from cosmid T21G5 did not rescue any of the mutants.
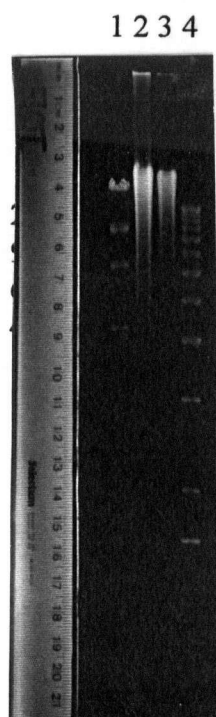
Figure 11. Southern blot of hEx 21 and hEx 24 transformants.

Genomic DNA of the transformants of hEx21 and hEx24 were prepared from transformed populations and digested with KpnI which did not cut the cosmid vector sequences (see Methods section). lane 1: 1ug of the lambda Hind III maker; lane 2: 10 ug of the transformant of hEx21; lane 3: 10 ug of the transformant of hEx24; lane 4: 1ug of the 1 kb ladder maker. The DNA was transferred into Zeta-Probe GT blotting membrane and baked 30 minutes at 80°C with vacuum.
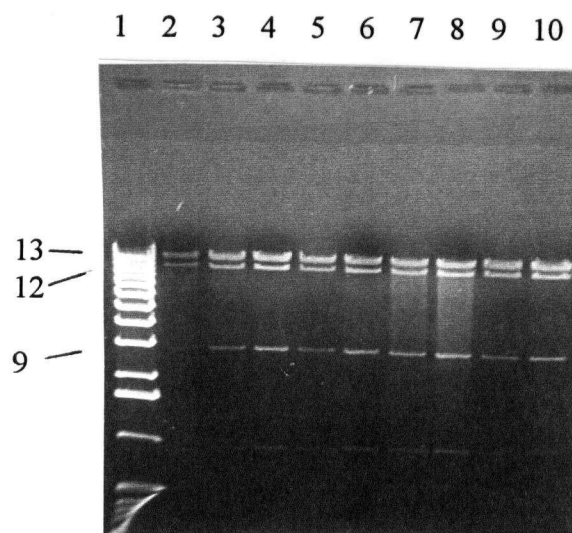
Figure 12. Comparison of cosmid T21G5 used for making transformants with the cosmid T21G5 from the cosmid centre.

Cosmid T21G5 stock from the cosmid centre was streaked on a kanamycin plate. Four single colonies were inoculated into 10 ml of LB medium with kanamycin and incubated for overnight at 37°C. Four DNA preparations from the four cell cultures were done (see the Methods section). DNA from the minipreparation was digested with *PstI*, and the cosmid DNA I used for making the transformants was also digested with *PstI*. All of the digestion samples were run on an agarose gel with the 1 kb ladder marker. lane2: the cosmid used for making transformants; lane 3-10: DNA prepared and digested with *PstI* from the cosmid T21G5 stock from the cosmid centre.
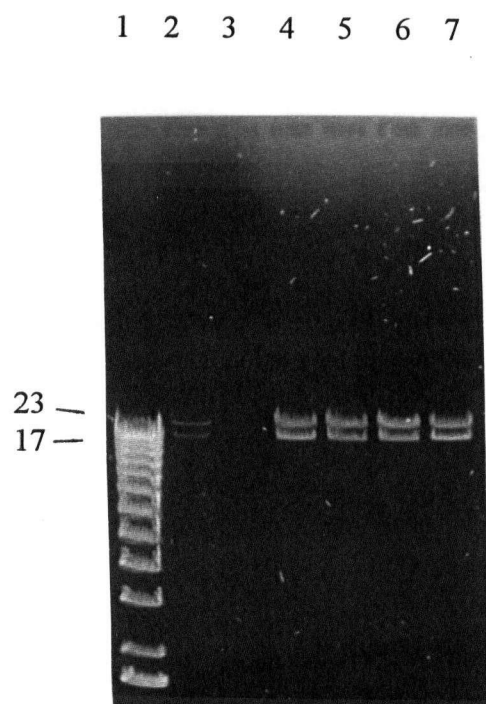
Figure 13. Comparison of cosmid T21G5 I used for making transformants with the cosmid
T21G5 from the cosmid centre.

DNA was digested with *KpnI*. lane 2: DNA digested with *KpnI* from the cosmid used for making
transformants; lane 4-7: DNA digested with *KpnI* from the cosmid T21G5 stock from the cosmid
centre.

Table 8. **hEx24 rescues the mutants of** *let-394, let-534, let-545* **and** *dpy-14*

	let-394	*let-534*	*let-545*	*dpy-14*
hEx 24	+	+	+	+

1. The phenotype of the rescued *let-394* is adult Dpy Unc and gave progeny at 20°C.

2. The phenotype of the rescued *let-534* is adult Dpy Unc and gave progeny at 20°C.

3. The phenotype of the rescued *let-545* gave Dpy Unc progeny at 20°C.

4. The phenotype of the rescued *dpy-14* is Unc-13 appeared in the *dpy-14* plate.

Table 9. **Rescuing the mutants of** *let-394*, *let-534*, *let-545* **and** *dpy-14* **with transformed lines carrying the cosmid T21G5**

	let-394	*let-534*	*let-545*	*dpy-14*
KR 3135 (hEx 74)	-	-	-	-
KR 3159 (hEx 88)	n.d.	-	n.d.	n.d.
KR 3136 (hEx 75)	n.d.	-	-	n.d.

The lethal phenotype in complementation test plates looked the same as that in the control plate.

Table 10. **Rescue of the mutants with the subclones**

Name of transgenics	let-394	let-534	let-545	dpy-14
KR 3131	-	-	-	-
KR 3153	n.d.	n.d.	n.d.	n.d.
KR 3151	-	-^{a}	-^{a}	-^{a}
KR 2987	-	-	-	-
KR 3154	-	-	-	-
KR 3158	-	-	-	n.d.
KR 3157	n.d.	n.d.	n.d.	n.d.
KR 3133	-	-	-	n.d.

- : fail to rescue

n.d. : not done

a. transgenic strains were not tested by PCR or Southern.

Discussion

The genome sequencing project

The genome of *Caenorhabditis elegans* is being sequenced by groups led by Dr. John Sulston, Cambridge England and Dr. Bob Waterston, St. Louis, MO using available cosmids assembled into the physical map of *C. elegans* (Coulson, *et al*, 1991). Currently, 50 million base pairs have been sequenced, and the genome is expected to be finished by 1998. Proteins encoded by the DNA are being predicted by GENEFINDER (written by P. Green) and the *C. elegans* genome is estimated to contain approximately 14,000 genes. A number of approaches are being taken to determine the functions of these genes, one of which is mutational analysis. Two laboratories have been taking a comprehensive approach to mutating genes essential to the growth and survival of *C. elegans* (Rose and Baillie). The region of the gene cluster on the left arm of chromosome I [LG (I)] has been studied intensively (Rose and Baillie, 1980; Howell, *et al*, 1987). A high resolution genetic map was established in this region (Howell, 1989; McKim and Rose, 1992; McDowall, 1990).

The physical map of *C. elegans* can be correlated with the genetic map using genetic crosses involving transgenic strains carrying cosmids from regions where the genes were mapped. A high resolution genetic map has been generated by the Genetic Toolkit project. The total number of cosmids presently available as transgenic strains is 153 which are being generated through a grant from the Canadian Genome Analysis and Technology Program to Ann Rose and

David Baillie (Janke, personal communication). This resource will enable the alignment map of the physical map with the genetic map and provide a tool to study gene functions.

In addition, a number of individual efforts to generate a series of transgenic strains to rescue mutant phenotypes in the *hDp16/hDp19* region of chromosome I and the *unc-22* IV region of *Caenorhabditis elegans* have been carried out (McDowall, 1990; McKay, 1993; Clark and Baillie, 1992). On average about half the transgenic strains generated rescue mutants, and of those that rescue, on average, about one mutant per cosmid is rescued. A few transgenic strains rescue more than one gene. For example, hEx24 carrying cosmid T21G5 rescues mutants of *let-394, let-534, let-545*, and *dpy-14* (McKay, 1993).

The goal of this thesis was to correlate coding regions in the cosmid T21G5 with four rescued mutants of *let-394, let-534, let-545*, and *dpy-14*, and to describe the organization of these genes in this cosmid.

The approach that was taken was restriction mapping of the cosmid T21G5 with two restriction enzymes *PstI* and *SacI*. The overlapped fragments were subcloned into a plasmid vector and chosen for making transgenic strains along with a subclone containing a semi-dominant allele of the *rol-6* gene (Kramer, *et al*, 1990).

The transgenic strains were made from the subclones of the cosmid T21G5 along with the *rol-6* gene subcloned in pGEM-7Zf(+) vector. The DNA concentration for making transgenic strains was a 1:1 ratio of 50ng/ul of the subclone to 50ng/ul of the *rol-6* gene clone. All transgenic strains were screened for exhibition of the *Rol-6* phenotype and all the transgenic strains with *Rol-6* phenotype were tested with PCR to confirm the phenotypic presence of subclones in the extrachromosomal array together with the subclone of the *rol-6* plasmid. The verified transgenic strains were used for genetic crosses to describe the organization of these genes in the cosmid.

Analysis of results of mutant rescue with T21G5 in different transgenic strains

The *let-394, let-534, let-545,* and *dpy-14* strains were rescued by the hEx24 carrying cosmid T21G5 as documented by McKay, 1993. The result of Southern blot analysis showed that the strain hEx24 carries the cosmid T21G5.

Normally in Southern blot analysis when testing whether the transgenic strains carry the cosmids, strong bands should be seen, which correspond to the bands seen in the isolated cosmid after cutting with a restriction enzyme that does not cut the vector sequences, as well as other bands which arise because of recombination events which occur between the vectors (Clark and Baillie, 1992).

However, the result of Southern blot analysis of hEx24 is different from the result illustrated by Clark and Baillie, 1992. The band is quite faint compared with that from the lamda DNA marker (1 ug) used as an internal control (data not shown), although a lot of hEx24 genomic DNA and vector probe from PCR amplification of Lorist 2 were used and the exposure time of 12 hours is quite long (usually for ECL labeling the exposure time is from 5 - 60 minutes). The precise copy number of the cosmid T21G5 in the extrachromosomal array in the strain of hEx 24 could be tested by DNA densitometry.

Three new transgenics carrying T21G5 were generated for testing mutant rescue. The mutant rescue results with these three new transgenic strains were negative (see Table 9). The DNA concentration used for making the transgenics was a ratio of 5:1 with the *Rol-6* plasmid to cosmid T21G5 as used for making the transgenic strain hEx24 (McKay, 1993). The heritable transgenic strains were screened by the appearance of the *rol-6* phenotype. The three new transgenic strains were PCR tested confirming the appearance of the cosmid T21G5 vector in the extrachromosomal array. The PCR result showed that the three transgenic strains carried the cosmid vector sequences. However, Southern blot analysis was not performed with the three new transgenic strains and the arrangement of the cosmid T21G5 in the extrachromosomal array cannot be predicted from the PCR result. The copy number of cosmid T21G5 in the extrachromosomal array was not tested.

The question is raised concerning why cosmid T21G5 has different behaviors after transformation of different transgenic strains. First, the nature of the gene in the

extrachromosomal arrays of the cosmid T21G5 could affect mutant rescue. So far, there are two genes found from the cosmid T21G5 by searching the genome sequence from ACEDB. One is the *gld-1* like gene (Ross, *et al*, 1995) gene and the other is *glh-1* gene (Bennett, *et al*, personal communication). Both of these genes are expressed in the germline (Ross, *et al*, 1995 and Bennett, personal communication). Second, germline expression might not occur from the extrachromosomal array, because its structure might be viewed by the animal as foreign heterochromatin (Kelly, personal communication). Third, the copy number of genes in the extrachromosomal array may affect gene expression (Kelly, personal communication).

In the transgenic strain hEx24, all of the mutants were rescued. Southern analysis showed that the copy number of the cosmid T21G5 in the extrachromosomal array was very low giving a very faint band (data not shown). This result may explain why the strain hEx24 rescued all of mutants.

However, none of the three new transgenic strains I created rescued any of the mutants. The cosmid used to make the transgenic strains was tested by comparing with cosmid T21G5 from the cosmid center (see Figure12 and Figure13). The concentration of DNA mixture of *Rol-6* and cosmid T21G5 for making the transgenics was 80 ng/ul to 20 ng/ul. This standard transformation scheme results in the formation of repetitive (head-to-tail) arrays with several hundred tandem interspersed copies of the injected plasmids and cosmids (Mello, *et al*, 1991). The DNA sequences of the cosmid T21G5 and *rol-6* plasmids in the extrachromosomal array may not express in the germline because of the formation of repetitive arrays viewed by the animals as foreign heterochromatin (Kelly, personal communication).

Cloning and studying of the genes in the cosmid T21G5 and their expression

The cosmid T21G5 was cut with the restriction enzymes PstI and SacI in order to obtain an overlapped region between two or three plasmids to prevent gene disruption by these restriction sites (see Figure 4 and Table 4). The genome sequence of T21G5 and the gene positions on T21G5 were not known at that time. In order to obtain an intact gene sequence, two or three plasmids with overlapped regions were injected together with the *rol-6* marker to make

the transgenic strains. However, it is hard to predict how recombination of the two or three plasmids might have happened between the overlapped cosmid DNA sequences or the sequences of the plasmid vectors. If recombination happened between the two vector sequences rather than the overlapped cosmid DNA sequences, the broken sites of the genomic sequences in the cosmid have no way to be compensated for by the overlapped genomic DNA sequences, and the gene disrupted by the restriction sites may not be functional. Therefore, the transgenic strains made from this broken gene may not rescue any of the mutants. Theoretically, the larger the overlapped region, the more readily the overlapped cosmid DNA recombines.

The genome sequencing project has now sequenced cosmid T21G5 (see Appendix 2.). The restriction fragments of the cosmid and their positions are known through searching ACEDB (Waterston and Sulston, 1995). After searching the restriction fragments of cosmid T21G5 cut by *PstI* and *SacI*, restriction maps were drawn with the help of this knowledge shown in Figure 4 (see Appendix 1.). This demonstrated that the fragments on the restriction map of Figure 4 matched the restriction fragments of the genome sequences shown on that region from the search of ACEDB.

Two genes, *gld-1* like (Ross, *et al*, 1995) and *glh-1* (Bennett, personal communication) were found after searching the ACEDB done by Ann Rose. The *gld-1* gene is a tumour suppressor gene required for oocyte development in *C. elegans*. Mutants of *gld-1* are defective in germline development (Ross, *et al*, 1995).

The position of the *gld-1* like and *glh-1* genes are known from ACEDB (see Appendix 1.). The *gld-1* like gene was broken by the one of the *PstI* and *SacI* sites. The *glh-1* gene is at the end of cosmid T21G5 (Bennett, personal communication). The broken genes in the subclones may explain why the subclones did not rescue the mutants, because these genes cannot be functional.

The high DNA concentration of the subclones used for making transgenic strains may be another reason why the mutants were not rescued by the transgenic strains. The DNA concentration used for making the transgenic strain was a 1: 1 ratio of 50ng/ul of *rol-6* plasmid to 50ng/ul of subclone DNA that is of cosmid T21G5 (Mello, *et al*, 1991). This favours the formation of a large extrachromosomal array that is stably inherited during the meiosis and mitosis

in the germline. The two genes *gld-1* like and *glh-1* are expressed in the germline (Ross, *et al*, 1995 and Bennett, personal communication). When these genes were in the extrachromosomal array of the transgenic strains, they might not be expressed in the germline since the extrachromosomal array may be viewed as foreign (Kelly, personal communication).

We should use other approaches to study the genes correlating to the mutants. This thesis result may implicate that the transgenic method is a good way to study gene functions correlating to the mutants, but it may not apply to all the genes.

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Appendix 1.

A.

B.
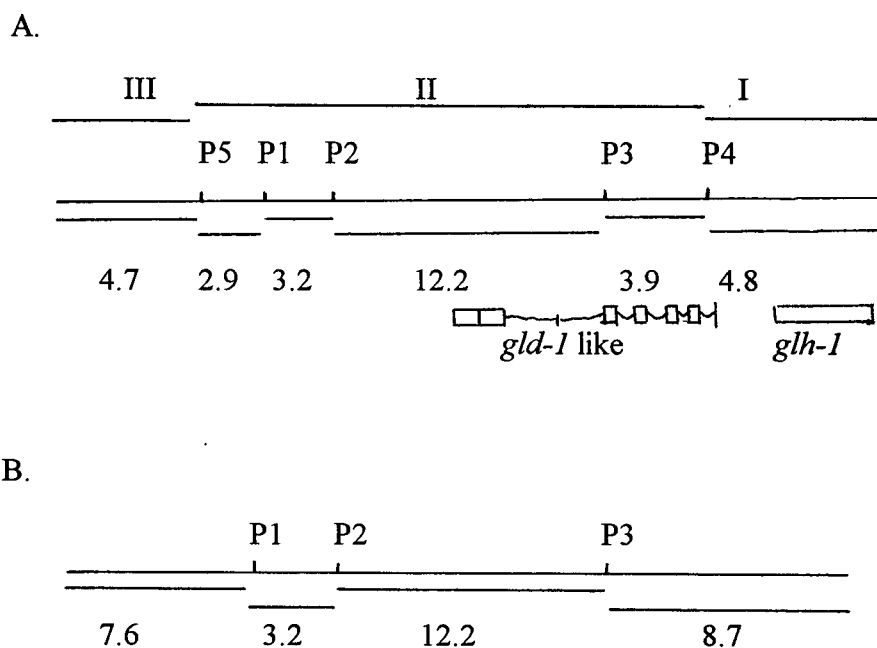
Figure 14. Restriction map of T21G5 with *PstI*.

A. Restriction map of T21G5 with *PstI*.
The restriction map derived from the genome sequence of ACeDB.
Section I: the *PstI* fragment in part I.
Section II: the *PstI* fragments in part II.
Section III: the *PstI* fragment in part III.
Two genes (*gld-1* like and *glh-1*) were found on the T21G5 from ACeDB.The position of the genes were shown on the map.
B. Restriction map of T21G5 with *PstI*.
The restriction map was derived from the combination of the restriction fragments from genome sequence of ACeDB and Figure 4.
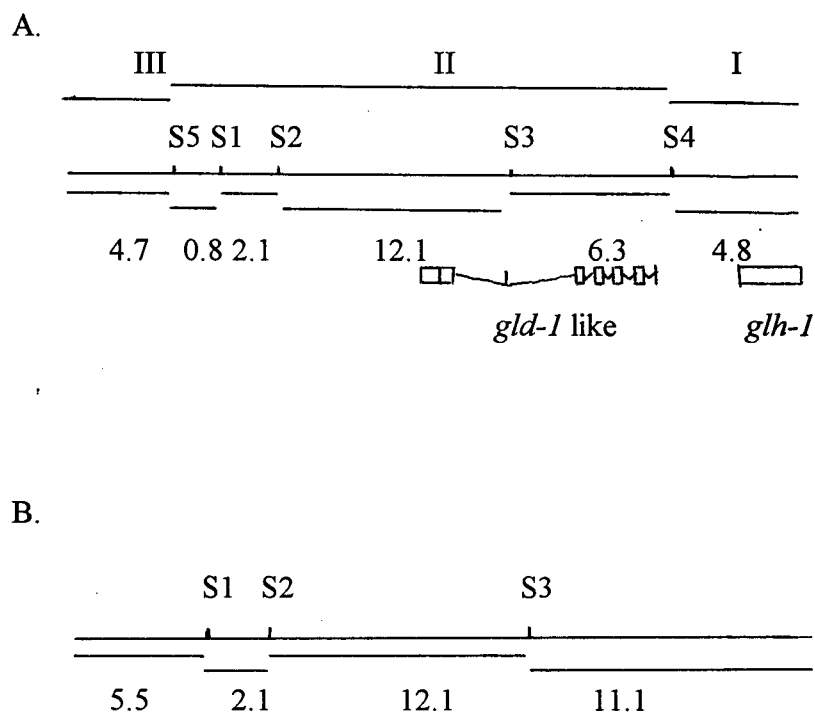
A.

B.

Figure 15. Restriction map of T21G5 with *SacI*.

A. Restriction map of T21G5 with SacI.
The restriction map derived from the genome sequence of ACeDB.
Section I: the *SacI* fragment in part I.
Section II: the *SacI* fragments in part II.
Section III: the *SacI* fragment in part III.
B. Restriction map of T21G5 with *SacI*.
The restriction map was derived from the combination of the restriction fragments from genome sequence of ACeDB and Figure 4.

Appendix 2.

The DNA sequence of T21G5 can be obtained from ACeDB through the web site. ftp: // genome. wust1.edu/pub/gsc1/sequence/st.louis/elegans/shotgun/1/T21G5.ftp.