GENETIC CHARACTERIZATION OF THE *CAENORHABDITIS ELEGANS ROL-3(V)* AND *SRL-2(III)* GENES, AND THEIR MOLECULAR LOCALIZATIONS THROUGH CORRELATION OF THE GENETIC AND PHYSICAL MAPS

by

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ABSTRACT

The *Caenorhabditis elegans rol* mutations are members of a general class of mutations which affect gross morphology. The phenotype is thought to be caused by disruption of the nematode cuticle. Worms homozygous for *rol-3(e754)* exhibit an adult specific rotation about their long axis associated with a left-hand twisted cuticle, musculature, gut and ventral nerve cord. Our laboratory has isolated 12 recessive lethal alleles of *rol-3* suggesting that the *rol-3* gene product also performs an essential developmental function.

I generated intergenic recessive suppressors of the temperature sensitive (*ts*) mid-larval lethal phenotype of *rol-3(s1040ts)*. These suppressors define two complementation groups, *srl-1(II)* and *srl-2 (III)*; and, while they suppress the *rol-3(s1040ts)* lethality, they do not suppress the adult specific visible rolling phenotype. Furthermore, there is a complex genetic interaction between the *srl-1* and *srl-2* loci. These results suggest that *srl-1* and *srl-2* may share a common function and possibly constitute members of the same gene family. Mutations in both *srl-1* and *srl-2* produce no obvious hermaphrodite phenotypes in the absence of *rol-3(s1040ts);* however, males homozygous for either *srl-1* or *srl-2* display aberrant tail morphology.

In order to aid in understanding the molecular nature of the *rol-3* and *srl-2* genes an attempt was made to determine the physical location of both *rol-3(V)* and the *srl-2(III)* genes. This was accomplished through correlation of the genetic and physical maps in both the *rol-3* region of LGV and the *srl-2* region of LGIII. The Polymerase Chain Reaction (PCR) was utilized to place the breakpoints of genetically defined deficiencies onto the physical map. This analysis allowed cosmid clones expected to represent the physical maps at both the *rol-3(V)* and *srl-2(III)* regions to be identified.

In the case of LGV, transgenic strains were constructed carrying extrachromosomal arrays composed of clones from the *C. elegans* physical map in the

region thought to surround *rol-3*. These strains were instrumental in positioning mutationally identified genes surrounding *rol-3* onto the physical map, as well as defining the region known to harbor the *rol-3* gene.

Transgenic *C. elegans* strains representing a subset of the *C. elegans* transgenic cosmid library being constructed in Dr. Baillie's laboratory were employed in an attempt to rescue the *srl-2* mutation. I demonstrate that the extrachromosomal arrays carrying either cosmid B0361 or F56C9 were able to rescue mutations at the *srl-2* locus suggesting that *srl-2* resides within an 8.6 kilobase interval defined by the overlap of these cosmid clones.

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GENERAL INTRODUCTION

An appreciation of the molecular processes culminating in a developmentally mature organism, be it prokaryotic or eukaryotic, will ultimately rely upon a thorough understanding of the genome, at the level of both composition and function. Towards this end, the genomes of a number of organisms are currently being sequenced in their entirety. The construction of a comprehensive physical map composed of clones containing contiguous segments of the genome is a prerequisite for a coordinated sequencing project. Organisms which have complete physical maps and whose genomes are currently being, or have been sequenced in their entirety include *Mycoplasma genitalium* (FRASER *et al.*1995), *Haemophilus influenzae* (FLEISCHMANN *et al. 1995*), *Escherichia coli* (KOHARA *et al.* 1987) and *Saccharomyces cerevisiae* (DUJON 1996 and references theirin) The *Caenorhabditis elegans* physical map is essentially complete (COULSON *et al.* 1986; COULSON *et al.* 1988; COULSON *et al.* 1991), and the complete genome sequence is expected by 1999 (WILSON *et al.* 1994, WATERSTON and SULSTON 1995). Other organisms with genomes about to be sequenced in a coordinated fashion upon completion of their physical maps include *Arabidopsis thalinia* (KAISER 1996, ZACHGU 1996), *Drosophila melanogaster* (AJIOKA *et al.* 1991, MERRIAM *et al.* 1991), the Japanese puffer fish, *Fugu ribripes* (TROWER et al. 1996, ELGAR et al. 1996), mouse (DIETRICH *et al.* 1995) and humans (GUYER and COLLINS 1995).

The nematode *Caenorhabditis elegans* is an excellent system in which to study the genome at a functional level. Perhaps the most attractive features of the worm labeling it for studies of this type are its relatively modest genome size and it's anatomical simplicity (BRENNER 1974). The *C. elegans* hermaphrodite is composed of only 959 somatic cells, yet it possesses all of the basic differentiated tissue types of multicellular eukaryotes. Currently, *C. elegans* represents the only metazoan for which the entire cell lineage throughout its life cycle is known (SULSTON and HORVITZ 1977, 1981; SULSTON *et al.* 1983). Furthermore, the anatomy and connectivity of all

synaptic junctions is known (WHITE, SOUTHGATE, THOMPSON and BRENNER, 1986) *C. elegans* has two sexes, a self fertilizing hermaphrodite and a male. The existence of a male sexual form facilitates genetic studies by promoting genome variability in successive generations, while the hermaphrodite mode of reproduction permits easy maintenance of genetic lines, and will drive novel gene variants to homozygosity in a single generation. The average brood size of a self-fertilized *C. elegans* hermaphrodite is 350, while outcrossed individuals may yield in excess of 1,000 progeny. This fecundity, dual modes of reproduction, small size and short generation time (3 and one half days from egg to adult at 20 °C) combine to make *C. elegans* a powerful model organism for genetic studies. *C. elegans* is transparent thus allowing single cells to be observed with Nomarski optics. This transparent nature, and the presence of a complete and invariant lineage map promotes the study of development, and indeed, a number of labs world wide are actively investigating the genetics of development and the process of cell to cell communication influencing cell fate decisions.

C. elegans has the smallest genome known for any metazoan, with a haploid genome size estimated at 100 million base pairs (bp) (SULSTON and BRENNER, 1974; COULSON *et al.*, 1991). It is organized into 6 chromosome pairs, 5 autosome pairs (I - V) and one pair of sex chromosomes (X). Hermaphrodites are diploid for the X chromosome, whereas males are haploid of the X chromosome. A *C. elegans* physical map has been constructed and is composed of overlapping cosmid and YAC (Yeast Artificial Chromosome) clones. 17,500 cosmid clones have been assembled into 700 contigs covering approximately 80 % of the genome. The generation and analysis of a further 3,500 YACs has greatly improved genome coverage. Hybridization of the cosmids to the YACs has resulted in a genome map upon which only seven gaps remain to be filled.

C. elegans has been widely exploited as a model organism for the study of genetics. This has resulted in the identification of over 1,400 genes by mutagenesis, and in

the generation and characterization of 143 reported duplications (*Dp*) and 289 reported genetic deficiencies (*Df*), or deletions (HODGKIN, DURBIN and MARTINELLI *personal communication*). Both duplications and deficiencies are valuable in their ability to rapidly position new or unmapped mutations to a genetically defined chromosomal interval. A number of rearrangements have been employed as genetic balancers. These are of two general types. The first type reduces or eliminates recombination between homologous chromosomes in an animal heterozygous for the rearrangement. These include a single inversion *hIn(I)* (ZETKA and ROSE 1992); translocations including *eT1(III;V)* (ROSENBLUTH and BAILLIE 1981) *hT1(I;V)* (McKIM HOWELL and ROSE. 1988), *hT2(I;III) and hT3(I;X)* (McKIM PETERS and ROSE 1993); and, a number of rearrangements of unknown structure such as *mnC1(II)* (HERMAN 1978) and *qC1(III)* (AUSTIN and KIMBLE 1989). The second type of balancer provides an extrachromosomal or integrated piece of DNA. These include large duplications like *sDp2(I;f)* (ROSE, BAILLIE and CURRAN 1984) and *sDp3(III;f)* (ROSENBLUTH *et al.* 1985).

C. elegans is amenable to transgenic experimentation. Microinjection of cloned DNA is possible (MELLO *et al.* 1991), and is used routinely by many *C. elegans* researchers. Microinjected DNA does not normally integrate into the chromosome, but rather forms a multi-copy extrachromosomal array which can be heritably transmitted. The relative ease with which transgenic *C. elegans* can be produced has revolutionized the way *C. elegans* genes can be located among existing clones. Rather than having to rely on the co-segregation of polymorphisms with a gene of interest in order to expedite cloning, accurate genetic mapping relative to markers previously anchored to the physical map is often sufficient to determine a physical position within a few hundred kilobases of DNA. Once this is determined, candidate cosmid and YAC clones can be identified and used to generate extrachromosomal arrays employed in marker rescue experiments aimed at repairing the mutant phenotype. If successful, experiments of this type usually map the

physical position of the gene to within a 40 kb interval. Transgenic techniques are also the basis for over-expression studies and synthetic knockout via antisense methods. The ease and rapidity with which transgenic *C. elegans* can be produced promises an excellent system for *in vivo* experimentation to address the function of known genes.

In the yeast *Saccharomyces cerevisiae*, the method of targeted gene disruption has been instrumental in assaying the effects of interrupting sequence identified genes in *vivo* (OLIVER *et al.* 1992; OUELLETE *et al.* 1993). A similar but more cumbersome technology is available in *C. elegans*. This technique is a two step, PCR based system aimed at the rapid detection of a Tc1 transposon insertion into a gene or other chromosomal location of known sequence (ZWAAL *et al.* 1993). Although technically unlike gene replacement in yeast and mice, the method of ZWAAL *et al.* (1993) has been used successfully for the isolation of knockout mutations in a number of sequenced genes for which no standard mutagen induced knockout was known (HODGKIN, PLASTERK and WATERSTON 1995).

The previously mentioned features of *C. elegans* make it very attractive for the studies of genetics and of the genome. Currently, a project leading to a complete sequence of the *C. elegans* genome is underway. One can estimate that sequencing approaches will yield a great deal of information regarding the essential genetic requirements for function of a complete organism. Comparison of genomes from evolutionarily diverse species will reveal a great deal of previously unknown information regarding genome structure and organization. For example, this approach will uncover synteny between divergent genomes by comparison of gene order and the detection of ancient conserved regions. At the level of gene content, examination and comparison of gene types and numbers between organisms will allow predictions to be made regarding the minimum genetic complement required for a specific biological process, and will further illustrate increases in genome complexity associated with an ascension in physiological complexity. The recent completion of the bacterium *Mycoplasma*

genitalium genome sequence (FRASER *et al.* 1995) allows for a number of such determinations to made. At 580,070 base pairs, the genome of *M. genitalium* is the smallest known genome of any self-replicating organism, and contains 470 genes. Comparison of the genome sequence of *M. genitalium* with that of *Haemophilus influenzae*, a more complex gram-negative bacterium (FLEISCHMAN *et al.* 1995) allows the basic complement of genes conserved between the two species to be identified. The number of *M. genitalium* genes totals only 27 % of the number of genes found in *H. influenzae*, and the relative proportion of the genome encoding genes involved in replication, transcription, translation and many other cellular processes is similar between the two organisms. This indicates that the *H. influenza* genome contains more genes of each category relative to *M. genitaliam.* From this data one can estimate a minimum number of genes required for each function; 32 for DNA replication, 12 for transcription and 101 for translation. The increase in the number of genes required for each process in *H. influenzae* presumably reflects differences in physiology and metabolic capacity between these two organisms (FRASER *et al.* 1995).

The sequence of the yeast genome will provide data regarding the genetic complement required to specify a simple eukaryotic organism. And being a single cell organism, it is likely that most of the genes expressed in yeast will be represented within other eucaryotic cells and will identify genes involved in cell maintenance and physiology that are common to all eukaryotes. Having the complete sequence of the *Caenorhabditis elegans* genome will provide another benchmark with which to compare genome structure and content among organisms. *C. elegans* will be the first multicellular organism with a completely sequenced genome. The *C. elegans* genome sequence should identify genes whose products are required in metazoan processes such as embryogenesis, tissue differentiation, gametogenesis, myogenesis, neurogenesis and nervous system function.

However, the full potential of a sequenced genome will not be realized without the ability to relate sequence identified genes to their *in vivo* functions. This limitation is best

emphasized by the demonstration that approximately 47 % of the 9,700 *C. elegans* genes identified by the sequencing project at the time of this writing have unknown functions. Genetic data providing functional information on genes identified by mutation is invaluable in assigning roles to sequence identified genes. Therefore, one way to increase understanding of the gene function within a genome is through the assignment of mutationally identified genes to sequence identified coding elements. Currently, this task is most rapidly accomplished through correlation of the physical map with the existing genetic map. Union of these two media will undoubtedly aid in relating sequence with biological function.

The goal of this study is two-fold. First is the examination of the genetics of mutations in the *rol-3V* gene. The *rol-3* locus was initially identified by S. BRENNER (1974) and was represented by the recessive visible mutation *e754*. Worms homozygous for *e754* display aberrant movement typified by left handed rotation about their long axis during locomotion. This abnormal movement is also associated with morphological abnormalities which include left-handed twisted body wall musculature, gut and ventral nerve cord (HIGGINS and HIRSH 1978). *rol-3(e754)* is only one of nine other roller mutations, each of which define individual genetic loci. Previous dogma placed *rol-3(e754)* along with other roller mutants into the general class known as morphological mutants (COX *et al.* 1986). Other mutants defining this class include dumpy, long, squat and blister mutations. Such mutations are thought to exert their phenotypes through aberrations in the nematode cuticle; thus, loci defined by morphological mutations are thought to encode products whose normal roles in development are connected to the establishment and maintenance of a sound cuticle. An additional 12 recessive *rol-3* mutations, associated with developmental arrest during early- to mid-larval development, were previously identified in Dr. Baillie's laboratory (ROSENBLUTH *et al.* 1988, JOHNSEN and BAILLIE 1991). The existence of these alleles suggests that the *rol-3* gene product performs an essential function during larval development which is in stark

contrast to the adult specific non-vital alterations manifest in worms homozygous for *rol-3(e754)*. The first section of this thesis describes the genetic analysis of visible and essential *rol-3* mutations, as well as the identification and analysis of *rol-3* suppressor mutations *(srl-)*. This analysis has aided in interpreting the function of the *rol-3* gene product.

The second section of this thesis is concerned with the molecular localization of *rol-3(V)* and *srl-2(III)* through correlation of the genetic and physical maps. This is initially accomplished through the Polymerase Chain Reaction (PCR) based assay (BARSTEAD and WATERSTON 1991) aimed at aligning the extent of genetically characterized deficiencies within the *rol-3* region of LGV (ROSENBLUTH *et al.* 1988), and the *srl-2* region of LGIII (H. STEWART, *personal communication*) with the physical map. The results of this analysis led to the identification of cosmid clones making up a portion of the *C. elegans* physical map (COULSON *et al.* 1986, 1988) within the regions defined by these deficiencies. These clones were employed in the construction of transgenic *C. elegans* (MELLO 1991), and used in cosmid rescue experiments to rescue the phenotypes of mutations in essential genes placed onto the genetic map within the deficiency intervals. Cosmid rescue experiments of this type integrate the genetic and physical maps by positioning mutationally identified genes onto the physical map. These mutations are then accessible to cloning experiments which will aid in elucidating biological roles for sequence identified genes on LGV. Through the combined approaches described above, I have aligned a 2 Mb interval of the *C. elegans* physical map containing the *rol-3* locus to the LGV genetic map. Furthermore, I have demonstrated that the *srl-2* locus resides within a 9 kb interval of the *C. elegans* genome contained within the overlap region of the cosmid clones B0361 and F56C9.

SECTION I

THE GENETIC ANALYSIS OF THE CAENORHABDITIS ELEGANS ROL-3 GENE AND THE IDENTIFICATION AND CHARACTERIZATION OF SUPPRESSORS OF LETHAL ALLELES OF ROL-3.

INTRODUCTION

Caenorhabditis elegans has a typical nematode body plan consisting of two concentric tubes separated by a space: the pseudocoelom. The outer tube, composed of cuticle hypodermis, neurons and muscles, constitutes the nematode body wall. The inner tube is the intestine, and the adult gonad is contained within the pseudocoelomic space (LEE 1965, WOOD 1988). The epithelial cells and the cuticle they secrete establish the basic body form of *C. elegans* (PRIESS and HIRSH 1986). There are two distinct regions of the epithelium: the basolateral surface which is separated from the body muscle cells by basement membrane, and the apical side which contacts the cuticle (SULSTON *et al.* 1983). Many of the cells constituting the hypodermis are multinucleate arising from the fusion of a number of cells during development. The largest, *hyp-7*, is cylindrical in shape extending most of the body length and constitutes the majority of the main body hypodermal syncytium. A seam syncytium is present along each lateral line of the adult worm. These syncytia, formed by fusion of the lateral seam cells late in larval development, remain separated from the main body syncytium (SULSTON and HORVITZ 1977).

The hypodermis is responsible for secretion of a developmentally regulated proteinaceous extracellular structure which surrounds and encloses the animal. This structure, the nematode cuticle, has an intricate ultrastructure consisting of four major layers (COX, LAUFER, KUSCH and EDGAR 1980, COX, STAPRANS and EDGAR 1981, reviewed by KRAMER 1994, KRAMER 1994). These layers, from external to internal surface, are: the external cortical layer, the cortical layer, the fibrous layer and the basal layer. The fibrous layer consists of two separate layers composed of fibers which spiral around the animal in opposite directions at an angle of approximately 70 degrees from the longitudinal axis. The cortical layer and the fibrous layers are joined by means of columnar material termed struts, otherwise, a fluid filled space separates these layers.

Beneath the basolateral surface of the epithelium, separated by a layer of basement membrane, is the *C. elegans* body wall musculature. In the adult worm, this structure is

composed of 95 mononucleate, spindle shaped cells arranged into four strips, each composed of two parallel rows of muscle cells, which span the worm longitudinally along four quadrants: two sub-dorsal quadrants (left and right), and two sub-ventral quadrants (left and right) (SULSTON and HORVITZ 1977). Each body wall muscle cell has three components: a cell body containing the nucleus and cytoplasmic organelles, and an arm which extends to either the dorsal or ventral nerve cord and the region containing the myofilament lattice. The myofilament lattice makes up the contractile machinery of the muscle cell and is restricted to a 1-2 μm deep zone lying beneath the hypodermis. The contractile unit of *C. elegans* body wall muscle is analogous in structure to the sarcomere of vertebrate striated muscle. Each contractile unit is defined at either end by a Z line analog known as a dense body. Actin containing thin filaments extend from the dense body and overlap along a portion of their length with myosin containing thick filaments. The thick filaments associate at their center with an amorphous material analogous to the M line of vertebrate muscle (FRANCIS and WATERSTON 1985). Membrane associated attachment plaques to which the thin filaments are anchored are present at the termini of the myofilament lattice. The sarcomeric unit is anchored to the muscle cell membrane at the dense bodies and M line. These structures also project into the basement membrane which serves as the muscle cell/hypodermal cell interface (FRANCIS and WATERSTON 1985).

The hypodermal syncytium contains inner and outer membrane associated plaques resembling hemi-desmosomes from which filaments extend across the hypodermal cytoplasm (FRANCIS and WATERSTON 1991). These filaments, extending from those plaques lining the outer membrane, appear to extend into the cuticle. Although the fibrous organelles do not appear to be specifically concentrated under the dense bodies or M lines, their presence in regions of the hypodermis apposed to muscle cells strongly implicates them in the mechanical coupling of the myofilament lattice to the cuticle; and , they are therefore likely to transmit muscle generated tension to the cuticle. (FRANCIS and

WATERSTON 1991). Efforts taken toward generating monoclonal antibodies specific to components making up the sarcomeric unit or muscle cell attachment structures have enabled identification of constituents of each of the linking structures between myofilaments and the cuticle. This has defined a series of components beginning at the points of attachment of thick and thin filaments to dense bodies to M lines, continuing through the muscle cell membrane to the intercellular space, through the hypodermal cell to the overlaying cuticle (FRANCIS and WATERSON 1991).

C. elegans develops through four post embryonic larval stages (L1-L4) into an adult. The transitions from L1-L2, L2-L3, L3-L4, and finally L4-adult are marked by molts during which a new cuticle, physiologically and biochemically distinct from it's predecessor, is synthesized by the underlying hypodermis (COX, KUSCH and EDGAR 1981; COX, STAPRANS and EDGAR 1981; OUAZANA and HERBAGE 1981). The nematode cuticle is composed primarily of collagens, and is thought to play a role in the definition and maintenance of *C. elegans* body morphology (COX, KUSCH and EDGAR 1981; COX, STAPRANS and EDGAR 1981; COX and HIRSH 1985). Therefore, mutations in genes encoding cuticle components should affect gross morphology (KUSCH and EDGAR 1986).

Several morphology mutations have been isolated and analyzed genetically in *C. elegans*. These include Dumpy, Blister, Long and Squat mutants. Recent molecular analysis of some of these morphological mutants have confirmed their roles in cuticle formation. For example, *sqt-1* (KRAMER *et al.* 1988), *sqt-3* (KRAMER 1994), *dpy-2* (LEVY, YANG and KRAMER 1993), *dpy-7* (JOHNSTONE, SHAFI and BARRY 1992), *dpy-10* (LEVY, YANG and KRAMER 1993) and *dpy-13* (VON MENDE *et al.* 1988) have been demonstrated to encode cuticle collagens. Roller mutants display abnormally twisted cuticles and rotate about their long axis as they crawl. Previous theories suggest that roller mutants (Rol) are also the result of mutations affecting cuticle structure (HIGGINS and HIRSH 1977; COX *et al.* 1980; GREENWALD and MOERMAN 1989).

In support of this, the *rol-6* gene has been shown to encode a cuticle collagen (KRAMER *et al.* 1990). In addition, specific alleles of the known collagen genes *sqt-1*, *dpy-2*, *dpy-10* and *sqt-3* have Roller phenotypes (COX, LAUFER, KUSCH and EDGAR 1980).

I have undertaken a genetic analysis of mutations of a particular roller mutant: *rol-3*. The *rol-3* locus is on the left half of LGV (see Figure 1), and was initially defined by the recessive visible allele *e754* (BRENNER 1974). Worms homozygous for *rol-3(e754)* have abnormally left-hand twisted cuticles, body musculature, gut, ventral nerve cords, and display an aberrant left-handed rotation during locomotion. The *rol-3* phenotype appears after the final cuticle molt, which suggests that *rol-3* may act to establish adult cuticle (HIGGINS and HIRSH 1977; COX *et al.* 1980; KUSCH and EDGAR 1986). Here I report the characterization of 12 alleles of *rol-3* which are recessive for an early- to mid-larval lethal phenotype (ROSENBLUTH et al 1988, JOHNSEN and BAILLIE 1991). The existence of alleles causing arrest during larval development and those resulting in an adult specific phenotype suggest that *rol-3* may function at more than one developmental stage.

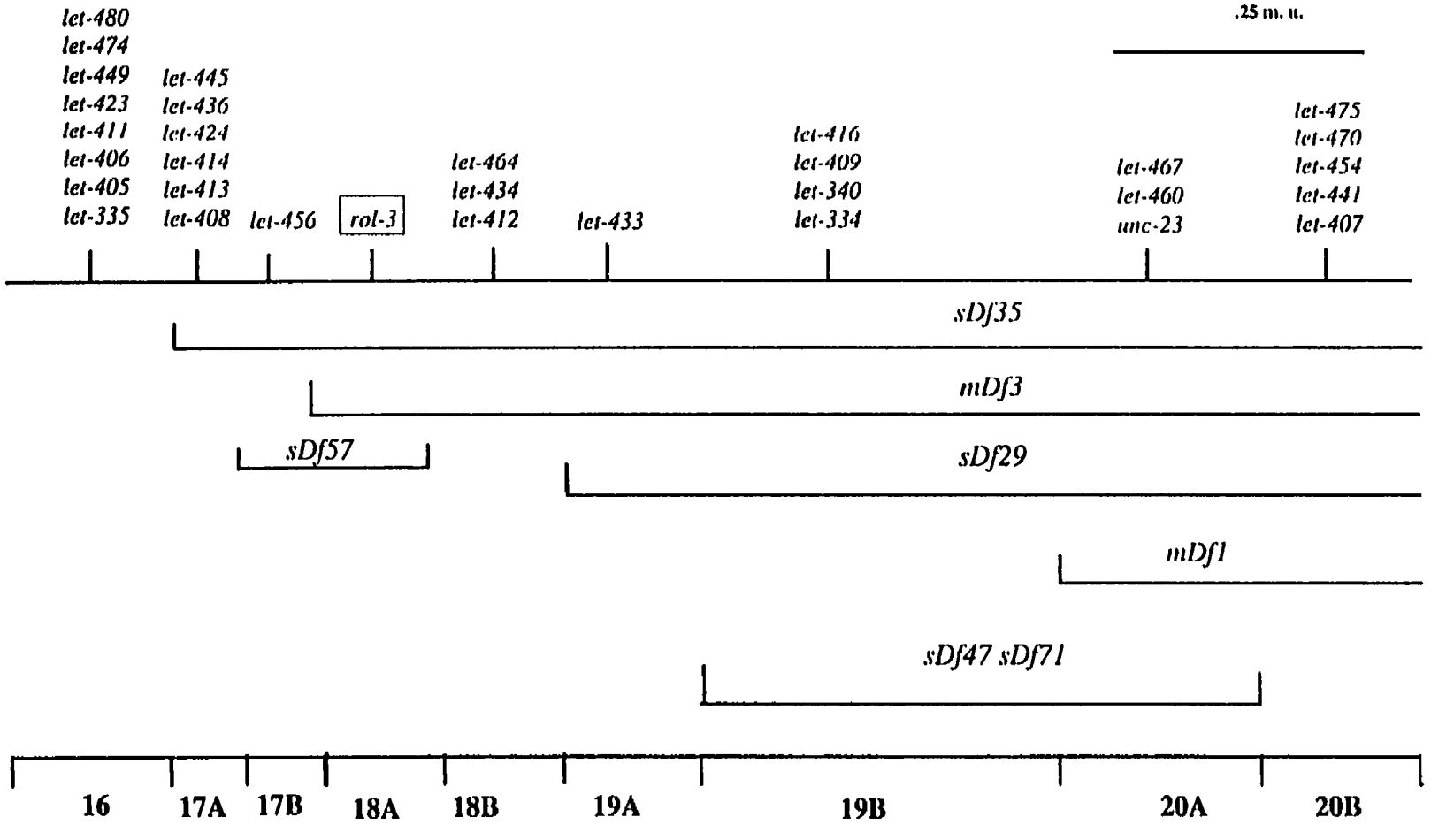
In an effort to examine the function of the *rol-3* gene product, I sought to identify mutations which would suppress mutations at *rol-3*. A suppressor mutation is a second mutation at a site distinct from the first, whose presence modifies the phenotypic expression of the original mutant such that it is reduced or corrected. There are two general classes of suppressor mutations: intragenic and intergenic suppressors. Intragenic suppression occurs as a result of a second mutation within the same locus as the original whereas intergenic suppression is caused by a second mutation at a site distinct from the first. Intergenic suppressors can be further categorized into two classes: informational suppressors and direct suppressors. Informational suppressors generally result from

Figure 1

Comprehensive Map Illustrating Zones 16 - 20 of the *eTI* Balanced Region of LGV.

rol-3 resides within zone 18A, as defined by the overlap of *sDf57* with *mDf3*. The gamma induced allele of *rol-3*, *s501*, was determined to be a deficiency based on its failure to complement *let-456*. *rol-3(s501) (this work)* has been subsequently renamed *sDf57*. The position of *let-433* and *let-434* were determined during the course of this work. This Figure was adapted from Johnsen and Baillie (1991).

Expansion of Zones 17 - 20 of the *eT1* Balanced Region of LG V left.



alterations of the cellular machinery involved in information processing and transfer. The best characterized examples of informational suppressors are mutations which affect tRNA molecules (MURGOLA 1985). Nonsense and missense suppressors both result from changes to particular tRNA molecules. Such mutations result in the association of a tRNA molecule with a codon that it would not normally recognize. In effect, mutations such as these serve to disturb the genetic code. Informational suppressors can also arise from mutations that effect RNA processing. The *C. elegans smg* suppressors (HODGKIN *et al.* 1988, PULAK and ANDERSON 1993) promote the accumulation to near wildtype levels of mRNAs containing frameshift or nonsense mutations which are otherwise unstable and rapidly degraded in a *smg+* background (PULAK and ANDERSON 1993). Correct processing of mRNAs carrying aberrant splice recognition sequences has been achieved in *vitro* with altered snRNA molecules (CONTO *et al.* 1987) The hallmark of informational suppressors is that they often affect (suppress) the phenotypes associated with specific alleles at a number of unrelated loci.

The second class of intergenic suppressors are known as direct suppressors. Such suppressor mutations are locus rather than allele specific. Early studies by JARVIK and BOTSTEIN (1973, 1975) demonstrated that intergenic second site suppressors can identify genes whose products interact with that of the first gene. Therefore suppressor analysis can be useful in identifying genes whose products are involved in a common process or pathway, and may also aid in understanding the function of that system. In *C. elegans*, this approach has been successful in identifying interacting genes involved in a number of processes, for example: genes involved in *glp-1* mediated control of cell fate

(MAINE and KIMBLE 1993), and *lin-12* mediated control of cell fate (SUNDARAM and GREENWALD 1993) have been identified through suppressor screens. *sli-1* (JONGEWARD, CLANDININ and STERNBERG 1995) and *sur-2* (SINGH and HAN 1995), members of the vulval induction pathway of *C. elegans*, were isolated as suppressors of *let*-60 ras and *let-23 EGF* respectively; and, suppressor analysis has identified a number of genes thought to effect excitation contraction coupling in muscle (LEVIN and HORVITZ 1993, GREENWALD and HORVITZ 1980,1982,1986). Likewise, I expected that identification of suppressors of *rol-3* would likely identify genes whose products interact with that of *rol-3* during development; and, may aid in the determination and comprehension of the *C. elegans* development process which requires the *rol-3* gene product.

Toward this end, I generated and characterized suppressors of the temperature sensitive mid-larval lethal phenotype of *rol-3(s1040ts)*. These suppressor mutations have been named *srl-* for suppressor of roller lethal, and define two novel loci, *srl-1(II)* and *srl-2*(III). I provide evidence suggesting that the *srl* gene products are involved in some aspect of *C. elegans* posterior pattern formation, and that this includes, but may not be restricted to, the male sex-specific lineages. Although these suppressor mutations have been induced in a *rol-3(s1040ts)* background, alleles of *srl-2* are able to suppress other *rol-3* lethal alleles suggesting that it is not an allele specific suppressor. The results from experiments designed to determine *srl-2*'s ability to suppress *rol-3* lethal alleles, as well as studies directed at determining the cuticle requirements for expression of the roller phenotype, have led us to argue that *rol-3* influences morphology in a much more global

manner than would be expected for a simple cuticle collagen. In fact, I suspect that *rol-3* functions either directly or indirectly in the developmental establishment of *C. elegans* posterior morphology. Finally, based on our genetic data, I present a model in which the wildtype *srl-1* and *srl-2* gene products normally function as down-regulators of *rol-3* gene activity.

MATERIALS AND METHODS

General:

The nomenclature follows the uniform system adopted for *C. elegans* (HORVITZ *et al.* 1979). The nematodes were cultured in petri dishes on a simple agar nematode growth medium streaked with *Escherichia coli* strain OP50. For details of maintenance, as well as procedures for observing and handling of the nematodes, refer to BRENNER (1974). All manipulations were performed at 20 ^{o}C except where otherwise noted.

Mutations:

The wildtype *C. elegans* (var. Bristol) N2 strain and strains carrying the following mutations were obtained from the MRC, Cambridge, England, from the *Caenorhabditis* Genetics Center at the University of Missouri, Columbia Missouri, or as cited:

LGI *dpy-5(e61).*

LGII *dpy-10(e128), lin-29(n1440)* (AMBROS and HORVITZ 1984) received from A.M. ROSE; *mab-9(e1243)* (CHISHOLM and HODGKIN 1989).

LGIII *dpy-1(e1), dpy17(e164), sma-2(e502), dpy-18(e364); pal-1(e2091)* (WARING and KENYON 1991) received from C. KENYON; *mab-5(e2088)* (KENYON 1986) received from C. KENYON; *smg-6(r896)* (HODGKIN *et al.* 1989); *mab-10(e1248)* (HODGKIN 1983).

LGIV *dpy-4(e1166), dpy-13(e184).*

LGV *dpy-11(e224), unc-46(e177), rol-3(e754), rol-3(s126, s422, s502, s742, s833, s1030)* (ROSENBLUTH, *et al.* 1988); *rol-3(s1040*ts, *s1408, s1409, s1494, s1519)* (JOHNSEN and BAILLIE 1991); *unc-42(e270,), let-456(s1479) (JOHNSEN and BAILLIE 1991), let-433(s950)*(H. STEWART *unpublished results*), *let-434(s1904)* (H. STEWART *unpublished results*).

LGX *lin-14(n179*ts) (AMBROS and HORVITZ 1984, 1987) received from A. M. ROSE. The LGV deficiency *mDf3* (BROWN 1984) was received from D. L. RIDDLE; and the reciprocal translocation *eT1 (III; V)* was characterized previously by ROSENBLUTH and

BAILLIE (1981). All mutations denoted with the *s* prefix arose in this laboratory. All mutations and genes have been named according to the conventions of HORVITZ *et al.* (1982).

Characteristics of *eT1(III;V)*:

eT1(III;V) is a reciprocal translocation that recombinationally balances the right half of LGIII and the left half of LGV. A total of about 43 map units (m.u.) (14 % of the genome) is balanced (ROSENBLUTH and BAILLIE 1981). The balanced regions of each chromosome are approximately the same size recombinationally. On LGV, recombination appears to be completely suppressed from the left end to a region between *dpy-11* and *unc-42* near the center of the chromosome. Ten sixteenths of the progeny of *+/eT1(III); +/eT1(V)* hermaphrodites stop maturing early in development, this is considered to be the result of those animals having aneuploid genomes. Because of the aneuploidy and no crossing over between markers and *eT1* breakpoints, markers on LGIII(right) and LGV(left) are pseudolinked. The breakpoint of *eT1* on LG(III) had been mapped close to and may be within *unc-36*. Worms homozygous for *eT1* are viable and exhibit an Unc-36 phenotype.

Developmental blocking stage:

Developmental blocking stages of the *rol-3* lethal alleles were previously determined (JOHNSEN and BAILLIE 1991). Briefly, this determination was based on terminal length measurements made with an ocular micrometer. Homozygous *dpy-18(e364)* ; *unc-46(e177)* animals reach a mature length of 0.8 - 0.9 mm. Worms shorter than 0.2 mm were considered to have not developed past hatching. Worms 0.2 - 0.3 mm were classified as early larval blockers, 0.3 -0.5 mm mid-larval blockers, 0.5 - 0.6 mm late larval blockers and greater than 0.6 mm with no internal fertilized eggs were called sterile

adults. Rough estimates based on length were further confirmed by examining the extent of hermaphrodite gonad development (KIMBLE and HIRSH 1979) in *rol-3* homozygotes.

***rol-3 inter se* complementation:**

Complementation tests between *rol-3* alleles were conducted by one of two methods.

1. lethal *rol-3* allele vs lethal *rol-3* allele:

Strains were either of the genotype *dpy-18(e364)/eT1(III) ; unc-46(e177) rol-3(sx)/eT1(V)* where sx represents *s501, s422, s742, s1030, s1040, s1408, s1409, s1494 s1519,* or *dpy-18(e364)/eT1(III) ; dpy-11(e224) rol-3(sx) unc-42(e270)/eT1(V)* where sx represents *s126* or *s833*. In the case of *s501, s422, s742, s1030, s1040, s1408, s1409, s1494* or *s1519*, WT males of one *rol-3* bearing strain were mated to WT hermaphrodites of another. If two alleles of *rol-3* complement, I expected DpyUnc : Wildtype : Unc-36 F1 progeny with a frequency of 1 : 4 : 1. Failure to observe DpyUnc F1 progeny indicates failure to complement. In the case of *rol-3(s126)* vs *s833*, complementation was indicated by the presence of DpyDpyUnc : Wildtype : Unc-36 progeny at a frequency of 1 : 4 :1. Failure to observe DpyDpyUnc progeny indicated failure to complement. Complementation between *rol-3(s126)* or *s833* vs *s501, s422, s742, s1030, s1040, s1408, s1409, s1494* or *s1519* was indicated by the observation of 1 Dpy : 4 Wildtype : 1 Unc-36 in the F1 generation. Failure to observe Dpy F1 progeny indicates failure to complement.

2. *rol-3* lethal alleles vs *rol-3(e754)*:

Males bearing lethal *rol-3* alleles, of the genotype *dpy-18(e364)/eT1(III) ; unc-46(e177) rol-3(sx)/eT1(V),* were mated to BC3008 *dpy-18(e364) ; rol-3(e754)* hermaphrodites. F1 progeny were scored for the presence or absence of DpyRol animals. If *rol-3(e754)* complements a given *rol-3* lethal allele, Dpy,

but no DpyRol progeny are expected. If *rol-3(e754)* fails to complement a given *rol-3* lethal allele, DpyRoller and Wildtype F1 progeny should be equally represented.

Construction of *rol-3(e754)* homozygotes in mutant backgrounds effecting cuticle molting:

lin-29(n1440) and *lin-14(n179*ts) demonstrate reciprocal effects on the timing of expression of the adult cuticle. *lin-29* loss of function alleles fail to make the L4 to adult cuticle switch, and reiterate the L4 stage cuticle (AMBROS and HORVITZ 1984). This is the only known effect of *lin-29* mutations. *The lin-14(n179*ts) allele results in the precocious expression of the adult cuticle at 25 °C. Homozygous *lin-14(n179*ts) produce an adult cuticle rather than L4 cuticle during the third larval molt. The effect of either *lin-14* or *lin-29* on the expression of the adult specific roller phenotype associated with *rol-3(e754)* was assayed in the presence of the linked mutation *dpy-11(e224).*

1. Construction of worm homozygous for *rol-3(e754)* and *lin-29(n1440)*:

Adult Hermaphrodite worms homozygous for *lin-29(n1440)* display a vulval "blip" which is recognizable under a dissection microscope Worms homozygous for both *rol-3(e754)* and *lin-29(n1440)* were constructed as follows: Hermaphrodites of the genotype *lin-29(n1440)/mnC1* were outcrossed to N2 males. F1 males segregated from the previous mating were mated to *dpy-11(e224) rol-3(e754)* hermaphrodites. Outcrossed wildtype hermaphrodites were isolated and allowed to self fertilize. Dpy F2s which displayed the vulval "blip" associated with homozygous *lin-29(n1440)* were examined for evidence of the Rol-3 phenotype. The presence of *lin-29* was confirmed further by examining DpyRol Vulvaless animals under Nomarski optics. All DpyRol F2 animals examined had fully formed gonads indicating that the worm had attained adult stage, however, the adult specific cuticular alae were missing which suggested that the adult cuticle was absent.

2. Construction of worms homozygous for *rol-3(e754)* and *lin-14(n179*ts):

At restrictive temperature, adult hermaphrodite worms homozygous for *lin-14(n179*ts) exhibit an egg laying defect. This defect is manifest as a failure to expel eggs which, therefore, develop within the mother. Worms doubly homozygous for *rol-3*(e754) and *lin-14(n179ts)* were constructed as follows. *dpy-11(e224) rol-3(e754)* double homozygous hermaphrodites were outcrossed to *lin-14(n179*ts) ; *him-5(e1490)* males which had been picked from the self fertilization progeny of *lin-14(n179*ts) ; *him-5(e1490)* hermaphrodites at permissive temperature (15 °C). Wild type F1s from the aforementioned cross were placed on plates individually, allowed to self fertilize at restrictive temperature (25 °C) and dpy animals were picked. These were assayed for presence of the Rol-3 phenotype and the *lin-14(n179*ts) egg laying defect. Progeny from self fertilized *dpy-11(e224) rol-3(e754) ; lin-14(n179*ts) worms were allowed to develop at 25 °C and observed under Nomarski optics. All animals observed expressing the Rol-3 phenotype also attained adulthood based on their extent of gonad development.

Test for *sup-7(st5)* mediated suppression of essential *rol-3* alleles:

sup-7(st5) is a *C. elegans* nonsense suppressor (WATERSTON 1981, WILLS *et al.* 1983, BOLTEN *et al.* 1984) which suppresses the phenotypes associated with specific mutations of a number of unrelated genes (WATERSTON 1981). The *dpy-18(e364)* mutation is suppressed by *sup-7(st5)* (WATERSTON 1981). Typically, *RW2070 dpy-18(e364) III* ; *sup-7(st5) X* animals have terminal lengths intermediate to that of *dpy-18(e364)* homozygous animals and N2 animals. Heterozygous *rol-3* lethal allele bearing males of the genotype *dpy-18(e364)/eT1(III)* ; *unc-46(e177) rol-3* (sx)*/eT1*(V) were mated to *RW2070 dpy-18(e364) III ; sup-7(st5) X* hermaphrodites. All F1 progeny were expected to carry one *sup-7(st5)* marked X chromosome. One half of the F1 progeny received a paternally contributed *dpy-18* marked LGIII and *unc-46 rol-3* marked LGV,

the remaining F1 progeny were recipients of *eT1* marked LGs III and V. F1 progeny heterozygous for *rol-3* were also homozygous for *dpy-18(e364)*. Since all F1s carry a *sup-7(st5)* marked LGX, those carrying *rol-3* display a *sup-7(st5)* mediated suppressed Dpy-18 phenotype. Therefore, these F1s are slightly short and readily distinguishable from those than those not carrying *rol-3*. Short F1 hermaphrodites of the genotype *dpy-18(e364)/dpy-18(e364) ; unc-46(e177) rol-3(sx)/+ + ; sup-7(st5)/+* were picked and individually placed on plates to lay eggs. The adults were transferred to fresh plates at 24 hour intervals and the F2 progeny were allowed to develop and were scored for the presence of Unc-46 animals. If the *rol-3* lethal allele being tested was suppressed by *sup-7(st5)*, Unc-46 animals were expected to represent 3/16 of the F2 progeny.

<u>*rol-3(s1040*ts) A recessive temperature sensitive lethal allele of *rol-3*</u>:

Worms homozygous for *rol-3(s1040)* arrest during mid-larval development at 20 - 25 ^{o}C. However, at 15 ^{o}C, homozygous *rol-3(s1040*ts) animals develop to adulthood, are fertile, and display a weak left-handed roller phenotype. BC1941 *dpy-18(e364)/eT1(III); unc-46(e177)rol-3(s1040*ts) hermaphrodites were placed at 15 ^{o}C and individual F1 Dumpy-Uncs were collected and maintained at 15 ^{o}C. A single line was retained, and named BC3129. All progeny of adult BC3129 worms shifted to 20 ^{o}C -25 ^{o}C display terminal lengths of approximately 0.35 mm, indicative of mid-larval arrest. The BC3129 strain [*dpy-18(e364)/eT1(III);unc-46(e117)rol-3(s1040*ts)*/eT1(V)*] was used as the background for the generation of suppressors of Rol-3 lethality.

<u>Temperature sensitive period of *rol-3(s1040*ts)</u>:

Several plates, each with ten gravid BC3129 hermaphrodites were placed at permissive temperature (15 ^{o}C) for one hour. After one hour at 15 ^{o}C. (this point is time zero, t_o). the hermaphrodites were removed and the plates containing eggs were transferred to 25 ^{o}C (restrictive temperature) at recorded times. The plates were kept at

25 °C for a minimum of four days after which five worms were picked at random, and their terminal lengths were measured. A second set of plates were placed at 25 °C immediately following removal of the adult hermaphrodites. These plates were treated in an identical manner to those above, though they were shifted down to 15 °C. at recorded times. All data were normalized to 15 °C.

Mutagenesis:

Two suppressor screens were performed, one using gamma irradiation and one using EMS. In both cases 100,000 haploid genomes were treated (see Figure 2).

EMS mutagenesis:

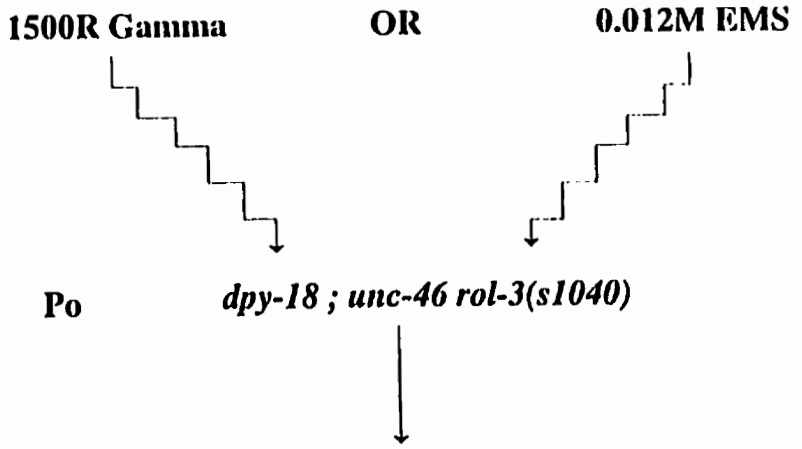
Mutagenesis of BC3129 was carried out according to BRENNER (1974) except that the dose was decreased to 0.012 M EMS to minimize the frequency of second hits (ROSENBLUTH, CUDDEFORD and BAILLIE 1983). Hermaphrodites were treated for four hours at room temperature. After treatment a total of 10 plates were set up with 50 worms per 10 cm petri plate, and incubated at 15 °C for 7-10 days (two generations). The plates were then shifted to 20 °C and monitored for the presence of developing and fertile Dpy Uncs. Such individuals were maintained (one per mutagenesis plate) and the nature of the suppression was analyzed.

Figure 2

Mutagenesis Screens

This figure depicts the gamma-ray or ethylmethylsulfonate (EMS) mediated induction of *srl* mutations. 100,000 haploid genomes were screened in each experiment for induction of suppressors of the temperature sensitive mid-larval lethality associated with homozygous *rol-3(s1040*ts).

Mutagenesis experiment for generation of suppressors of *rol-3(s1040*ts)



Set up 50 Po per plate for a total of 10 plates (10cm)
Each Po will produce approx. 100 F1s
Allow worms to go through two generations at 15 ℃
Shift to restrictive temperature and screen for fertile DpyUncs.

Gamma irradiation mutagenesis:

The mechanics of the gamma irradiation screen were the same as the EMS screen described above except BC3129 Po hermaphrodites were treated with 1500 R of Gamma radiation emitted from a 60 Co radiation unit (Gamma Cell 200, Atomic Energy of Canada). The suppressor mutations were named *srl-* for Suppressor of Roller Lethal.

Mapping suppressor mutations to linkage groups:

Testing for linkage to LGs III, V or X: (see Figure 3 A and B). All recombination experiments were carried out at 20 ^{o}C (ROSE and BAILLIE 1979). Advantage was taken of the fact that *dpy-18 III, unc-46 V* lie in the regions recombinationally balanced by *eT1 (III;V)* and therefore appear pseudolinked in the progeny of *eT1* heterozygotes. Hermaphrodites from each suppressor (*srl*) mutant strain of the genotype *srl-(sx)...dpy-18(e364)/eT1 (III); unc-46(e177) rol-3(s1040ts)/eT1(V)* were mated to BC4015 *dpy-18(e364)/eT1 (III); unc-46(e177) rol-3*(s1040ts)*/eT1 (V)* males. The F1s were scored for the presence of Dpy Unc-46 males. Absence of such males indicated that the suppressor is recessive and that it is not on LGX. Wild-type F1 hermaphrodites were picked and the F2s were scored. Due to aneuploidy effects, only 6/16 of an *eT1* heterozygote's progeny are normally viable (ROSENBLUTH and BAILLIE 1981). If the *srl* mutation was within the *eT1* balanced region of LGIII or LGV, and thus pseudolinked to *dpy-18 III, unc-46 V* and *rol-3 V*, I expected to see Dpy Unc-46 Rol : wildtype : Unc-36 progeny in a 1:4:1 ratio. If the *srl* assorted independently of LGIII and LGV, then I expected the ratio to be 1:16:4.

Distinguishing between linkage to LGIII or LGV:

Homozygous *srl-(sx) ...dpy-18 III; unc-46 rol-3(s1040ts) V* hermaphrodites were mated to N2 males and the F2 progeny scored. The Dpy Unc-46 *Rol-3*/Unc-46 Rol-3ratio

was used to distinguish between the *srl*'s linkage to LGIII or to LGV. Taking into account the map distance of approximately 3.6 map units between *unc-46* and *rol-3* (M. L. EDGLEY and D. L. RIDDLE, personal communication), tight linkage of the *srl* mutation to *dpy-18 III* would give a ratio of 4.2:1; while in the case of tight linkage to *unc-46 V*, the ratio would be 0.33:1.

Testing for linkage to markers 'm' on LGI, II, III, or IV:

Hermaphrodites, homozygous for *srl-(sx)*, marker '*m*', *unc-46* and *rol-3(s1040*ts*)*, were mated to N2 males and the F2 progeny were scored. The ratio M Unc-46 : Unc-46 was used to determine whether the *srl* mutation was linked to the marker. Tight linkage to the marker would give a ratio of 4.2:1. Linkage to neither the marker nor *unc-46* would give a ratio of 0.31 : 1 (see Figure 3).

2-factor mapping *srl-2* relative to LGIII markers:

srl-2(s2507) was 2-factor mapped relative to *dpy-1 (III)*, *dpy-17 (III)* and *sma-2 (III)*. Hermaphrodite strains of the genotype *M srl-2(s2507) ; unc-46 rol-3(s1040*ts*)* (where M=*dpy-1*, *dpy-17* or *sma-2*) were mated to N2 males. Individual wildtype F1 hermaphrodites were allowed to self-fertilize at the standardized mapping temperature of 20°C (ROSE and BAILLIE 1979), and the F2s scored for the presence of recombinant Unc progeny. Given that *rol-3* is separated from *unc-46* by 3.6 map units (M. L. EDGLEY and D. L. RIDDLE personal communication), the number of Unc progeny expected due to recombination between *unc-46* and *rol-3* rather than due to recombination between M and *srl-2(s2507)* was determined from the formula U=T[1-(1-P)2]/3 where U is the number of Uncs, T is the total number of worms, and P is the frequency of recombination between *unc-46 - rol-3*. Once determined, the value U was subtracted from the total number of Unc-46 recombinants scored, and the corrected value for Unc-46 recombinant progeny was used to calculate the recombination distance

between the marker and *srl-2*. The formula used to determine the recombination distance between M and *srl-2* was p= 1-[1-(13R/T)]1/2, where p is the recombination frequency between M and *srl-2*, R is the corrected number of Unc recombinants and T represents the total number of worms. 95 % confidence limits are based on the limits of Unc-46 recombinants. These limits were taken from Table 1 of CROW and GARDENER (1959).

Figure 3A

Mapping Scheme for Suppressors of *rol-3(s1040*ts) Lethality

Hermaphrodite worms heterozygous for *srl-2* and *rol-3(s1040*ts) in the presence of the reciprocal translocation *eT1* were constructed and self-crossed. Pseudo linkage between LG(III) and LG(V) imposed by the presence of *eT1* was used to assay whether or not the *srl-* mutation was linked to either breakpoint of *eT1*, and therefore linked to either LG(III) or LG(V), as well as to determine whether *srl-* is dominant or recessive.

Mapping Scheme for Suppressors of *rol-3(s1040)*ts Lethality

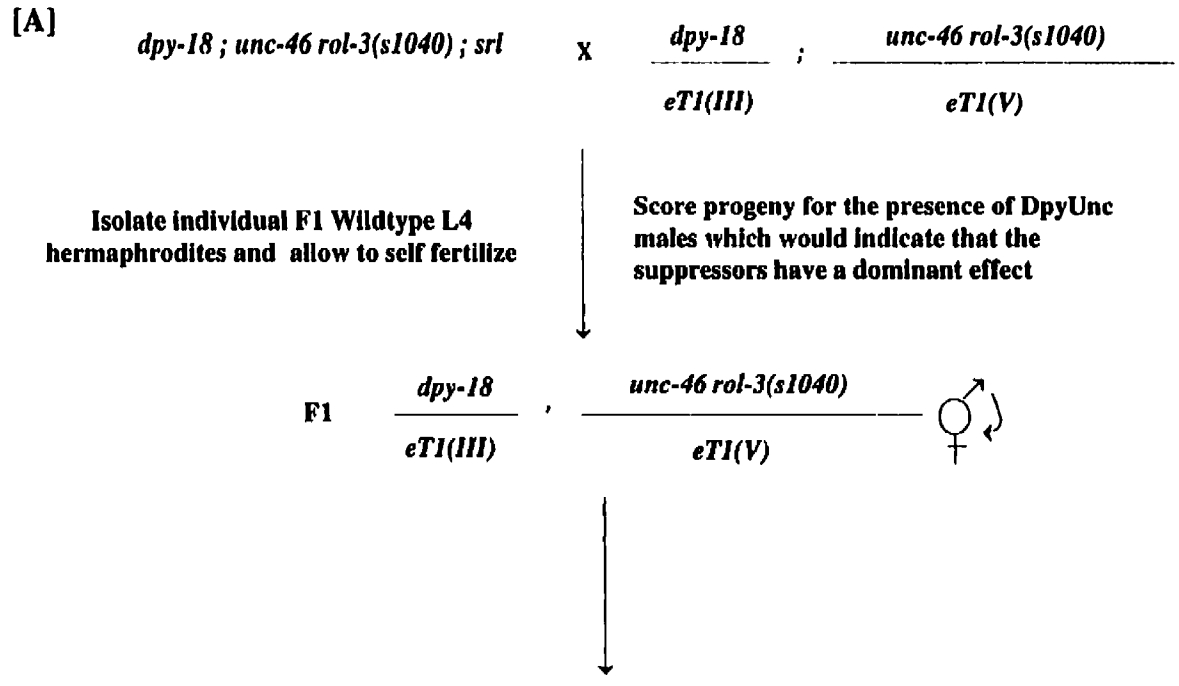


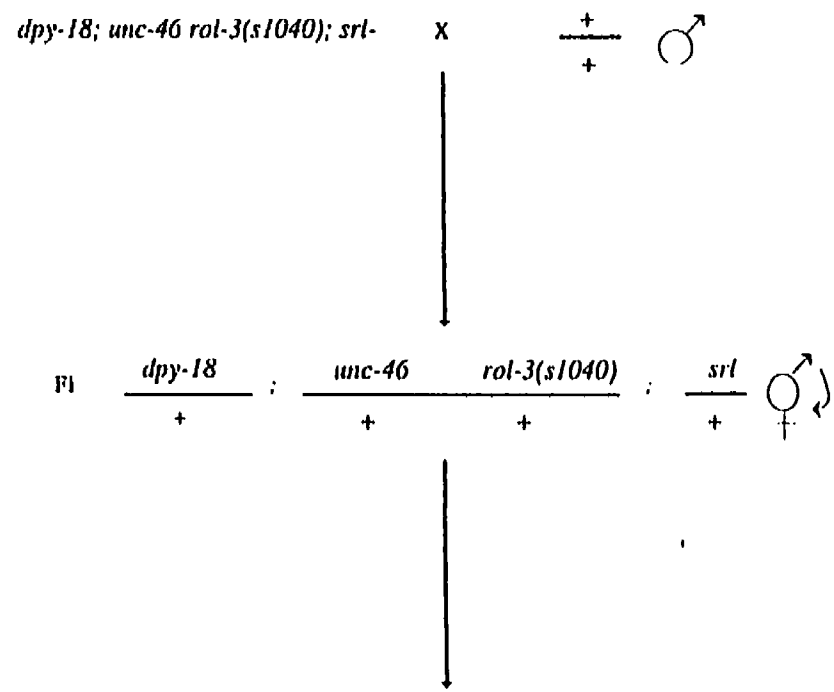
Figure 3B

Mapping Scheme for Suppressors of *rol-3(s1040*ts) Lethality

Outcrossing hermaphrodites of the genotype *srl; dpy-18(e364); unc-46(e177) rol-3(s1040*ts) to N2 males and scoring the F2 progeny was used to confirm *srl* linkage results determined for A. In the case of linkage, the number of Dpy or Unc progeny generated serves as 2 factor mapping data for *srl* to *unc-46* or *dpy-18* respectively.

Mapping Scheme for Suppressors of *rol-3(s1040*ts) Lethality

[B]



Score F2s. If srl is unlinked to either of dpy-18 or unc-46 then expect 1 DpyUnc:3 Unc46: 12 Dpy: 36 WT.
If srl- does not reside on LG III or V, linkage to dpy-5 *(I)* *dpy-4 (IV)*, *dpy-13 (IV)* and *dpy-10 (II)* was tested.

***srl- inter se* complementation:**

All complementation experiments were conducted at 20 °C. Hermaphrodites carrying one *srl* allele to be tested (designated srl-A in Figure 4) were crossed to *dpy-18/eT1(III); unc-46 rol-3(s1040ts)/eT1(V)* males and the resulting F1 males were picked. These males were mated to a second hermaphrodite carrying a different *srl-* allele. The presence of Dpy Unc males indicated a failure to complement. If the *srl-* alleles tested were linked to either LGIII or LGV then failure to complement would result in a ratio of Dpy Unc to wildtype males approximately equal to 1:1. Conversely, if the *srl-* mutants are not linked to LGIII or LGV, then a ratio of Dpy Unc to wildtype males of 1:2 would occur. In some cases *srl-; unc-46 rol-3(s1040ts)* was used rather than *srl-; dpy-18; unc-46 rol-3(s1040)ts.* In these instances the ratios discussed above are applicable although the frequency of Unc to wildtype males was scored rather than Dpy Unc to wildtype males.

Test of *srl-2*'s ability to suppress *rol-3* lethal alleles other than *s1040ts*:

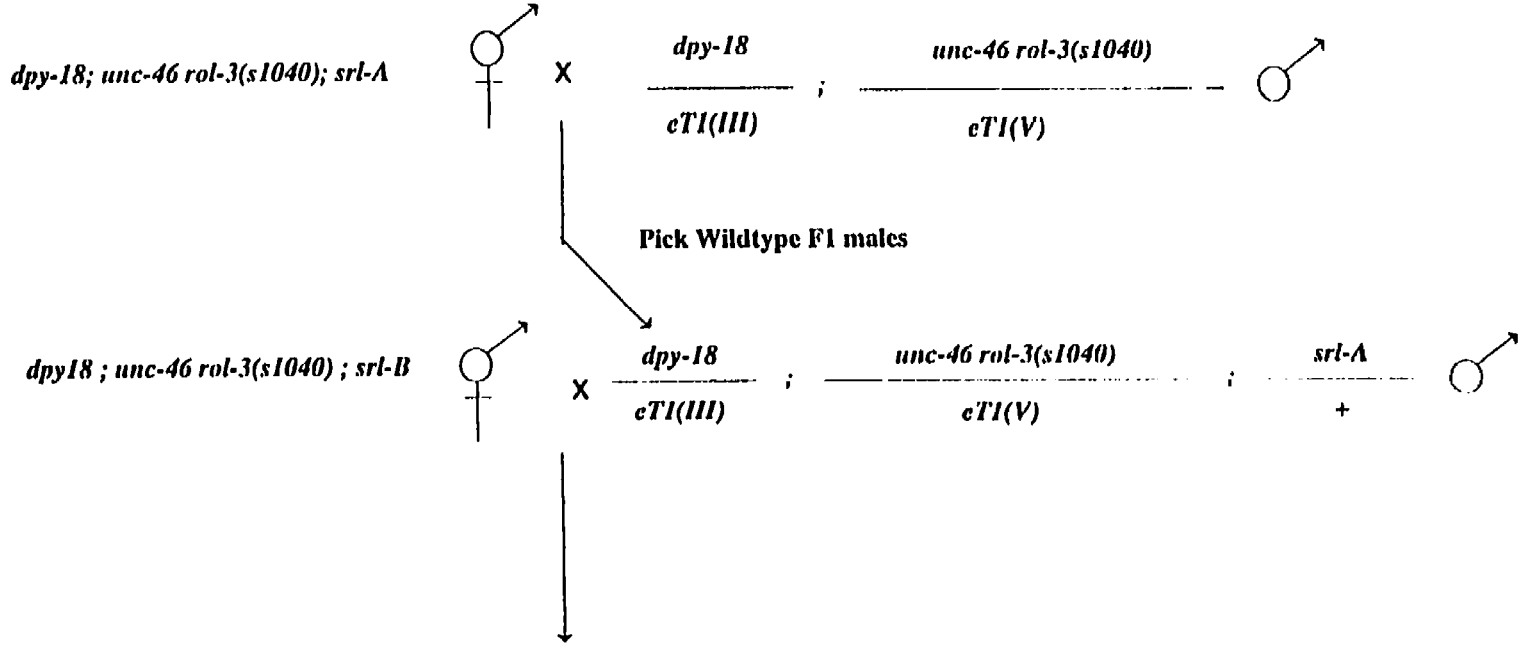
srl-2 dpy-18 /++ males were mated to *eT1* balanced *rol-3* lethal allele bearing strains of the type *dpy-18/eT1(III) ; unc-46 rol-3/eT1(V)*, or *dpy-18/eT1(III) ; dpy-11 rol-3/eT1(V)* (in the case of *rol-3(s833)*, or *dpy-18/eT1(III) ; dpy-11 rol-3 unc-42/eT1(V)* (in the case of *rol-3(s126)*. F1 Dpy hermaphrodites were picked, and allowed to self-cross. F2 Dpy Unc-46's were scored and picked up (or Double Dpys in the case of *s833*, or Double Dpy-Unc-46s in the case of *s126*). For suppression I expected a Dpy Unc F2 frequency of approximately 1/16. Furthermore, I expected Dpy Unc F2s at a frequency of 2/100 due to recombination between *unc-46* and the *rol-3* allele being tested. Therefore, progeny of the putative *srl-2 dpy-18; unc-46 rol-3* were mated to N2 males and F1 hermaphrodites were picked. The F2's were observed for the presence of arresting larvae confirming that the *rol-3* lethal was present.

Figure 4

Determination of *srl* Allelism

Crosses used in the determination of *srl* allelism. The presence of F2 Dpy Unc males indicates that the heteroallelic combination of *srl-A* and *srl-B* are able to suppress *rol-3(s1040*ts), indicating failure to complement. Complementation data is presented in Figure 9.

Complementation Scheme for Determination of *srl* Allelism



Presence of F2 DpyUnc males indicates failure to complement.

If *srl-A* is unlinked to the breakpoints of *eT1*:
Expect 2 : 1 WT males to DpyUnc males.

If *srl-A* is linked to either breakpoint of *eT1*:
Expect WT males and DpyUnc males with equal frequency.

Microscopy:

General morphology, cuticular alae and posterior and male specific alterations were observed and photographed using differential interference contrast (Nomarski) optics (SULSTON and HORVITZ 1977). *C. elegans* musculature was observed under polarized light. Worms were mounted on 5% agar pads containing 2 µl S Buffer (BRENNER 1974). Photographs were taken with Kodak Technical Pan film, a.s.a 64 - 100. To avoid movement during long exposure times worms were anaesthetized with sodium azide (final concentration of 5 mM). A cover slip was then added, and photographs were taken with as little time delay as possible to avoid desiccation. All Nomarski microscopy was performed on either an Olympus AHBS3 Research photomicrographic microscope system equipped with dual Olympus C35AD-4 35 mm cameras, or a Zeiss axiophot. All polarized light microscopy was carried out on an Olympus AHBS3 Research photomicrographic microscope.

RESULTS

Phenotype of the *rol-3* recessive visible allele *e754.*

rol-3(e754) is a member of the original set of *C. elegans* visible mutants isolated by Sydney Brenner in 1974. The wild type or N2 worm crawls on the agar media in a sinusoidal wave motion. Worms homozygous for *rol-3(e754)* move aberrantly. Specifically, *e754* homozygotes rotate about their longitudinal axis as they crawl failing to move in the graceful sinusoidal wave motion associated with N2. Furthermore, they usually crawl along a circular or semi-circular path. Early morphological studies on *C. elegans* roller mutants by HIGGINS and HIRSH (1977) established that *e754*'s adult specific left hand rolling phenotype is coincident with left-hand helically twisted body wall musculature, ventral nerve cord, gut and cuticle. COX (1980), further determined that the degree of body wall muscle helicity correlates with the degree of twisting observed in the cuticle as evidenced by the cuticular alae (COX *et al.* 1980).

The alae are cuticle specializations produced by the hypodermal seam cells, and only present on L1, dauer and adult cuticles (SINGH and SULSTON 1978). Their function is not well understood. The seam cells, which run along each lateral line, divide in a stem cell-like pattern during the first three larval molts. During the L4 molt, these cells terminally differentiate and fuse together to produce the lateral seam syncytia which remain separate from the main body syncytia (SULSTON and HORVITZ 1977). COX *et al.* (1980) found that the alae of *rol-3(e754)* homozygotes demonstrate a helicity of 1/4 turn over the length of the animal. This degree of helicity was considered low in comparison to other roller mutants (most displayed a helicity of 1/2 - 1), but is thought to reflect the relatively subtle phenotype of *e754* with respect to other roller mutations such as *rol-6*.

As mentioned, the body wall musculature of *rol-3(e754)* mutants is also helically twisted. The body wall muscles are distributed in four longitudinal rows along the length of the animal. The myofibrilar lattice of the body wall muscle cells is obliquely striated

and lies just beneath the cell surface facing the hypodermis (FRANCIS and WATERSTON 1985). The structure of body wall muscle is rapidly assessed by polarized light microscopy (WATERSTON, THOMPSON and BRENNER 1980). Under polarized light, the wild type body musculature is visible as bifringent rows which run along the length of the animal (WATERSTON, THOMPSON and BRENNER 1980). HIGGINS and HIRSH (1978) demonstrated that the body wall musculature in *rol-3(e754)* is twisted in a left hand fashion relative to the long axis of the worm, and makes 0.72 +/- 0.05 helical rotations along the length of the worm. The twisted body wall muscle phenotype of *rol-3(e754)* described by HIGGINS AND HIRSH (1978) is evident in the polarized light micrograph of a homozygous *rol-3(e754)* adult Roller hermaphrodite presented in Figure 5a. Figure 5b displays the body wall musculature of an *unc-46(e177)* homozygous adult hermaphrodite. The bands of body wall muscle visible in this micrograph run anterior to posterior in a straight line. Viewed with polarized light microscopy, adult *unc-46(e177)* homozygous worms display body wall muscle bands which are essentially indistinguishable from that of wildtype worms (HIGGINS and HIRSH 1977, WATERSTON, THOMPSON and BRENNER 1980, COX *et al.* 1980). In contrast, the body wall musculature of *rol-3(e754)* homozygous animals is obviously twisted about the long axis of the worm.

Disruption of Adult Cuticle:

To further illuminate the association of *rol-3* function with the adult cuticle I studied the interaction of *rol-3(e754)* with mutations of the heterochronic genes *lin-14(X)* and *lin-29(III)*. *lin-29(n1440)* and *lin-14(n179*ts) demonstrate reciprocal effects on the timing of expression of the adult cuticle. *lin-29* encodes a zinc-finger protein (ROUGVIE and AMBROS 1995). Animals homozygous for *lin-29(n1440)* fail to execute the larval / adult switch which results in the failure of seam cells to terminally differentiate and the failure to synthesize an adult cuticle. Rather, *lin-29* mutant worms reiterate a larval cuticle during the adult molt (AMBROS and HORVITZ 1984). It is thought that *lin-29*

Figure 5

Examination of Adult Body Wall Musculature by Polarized Light Microscopy

Polarized light microscopy of [A] *rol-2(e754)* and [B] *unc-46(e177)*. Note the twisted body wall musculature evident in the *rol-3(e754)* hermaphrodite. Homozygous *unc-46(e177)* appears to have no twisting effect on body wall musculature. This is consistent with N2 (HIGGINS and HIRSH 1977, COX et al. 1980). Arrows indicate body wall muscle bands.

A
50μm
B
50μm

regulates gene expression of adult cuticle specific components such as collagens (BETTINGER, LEE and ROUGVIE 1996, PAPP, ROUGVIE and AMBROS 1991, AMBROS 1989). Therefore, I expected that if the rolling phenotype associated with *rol-3(e754)* was dependent on the presence of an adult cuticle, rolling should be suppressed in worms doubly mutant for *rol-3(e754)* and loss of function mutations in *lin-29*. Double mutants for *rol-3(e754)* and *lin-29(n1440)* mutations were constructed and scored for the presence of adult rollers. All *lin-29(n1440) ; rol-3(e754)* double mutants roll as adults. This result suggests that the expression of *rol-3(e754)* is temporally constrained to the adult stage, but does not require the presence of an adult cuticle. In contrast to *lin-29(n1440)*, the *lin-14(n179*ts) allele results in advancement of cuticle expression such that an adult cuticle is formed after the third molt rather than the fourth at the restrictive temperature of 25 ^{0}C (AMBROS and HORVITZ 1984; 1987). If the expression of the rolling phenotype requires an adult cuticle, then, *rol-3(e754); lin-14(nt179)* doubles are expected to roll one molt earlier than wildtype. All such double mutants constructed do roll, but only as gravid adults, demonstrating that the precocious expression of an adult cuticle does not alter the expression of the *rol-3(e754)*'s visible phenotype.

Generation and identification of lethal alleles of *rol-3 V*:

Our laboratory has undertaken an extensive mutagenic dissection of a 23 m.u. portion of LGV(left) balanced by the reciprocal translocation *eT1* in an attempt to saturate this region for essential genes (JOHNSEN and BAILLIE 1991). Toward this end, gamma irradiation, EMS (JOHNSEN, ROSENBLUTH and BAILLIE 1986; ROSENBLUTH *et al.* 1988; JOHNSEN 1990; JOHNSEN and BAILLIE 1991), formaldehyde (JOHNSEN and BAILLIE 1988), Tc1 transposon (CLARK *et al.* 1990), and UV (STEWART, ROSENBLUTH and BAILLIE 1991) mutagenesis screens have been instrumental in generating a total of 242 mutations defining 101 essential genes to date within this region. These mutations were complementation tested against known deficiencies, and

subsequently tested against known genes in the appropriate regions. These analyses resulted in the identification of 12 alleles of *rol-3*, all of which have recessive lethal phenotypes (JOHNSEN and BAILLIE 1991). Two of these alleles (*s501* and *s742*) had been induced with gamma irradiation. I subsequently demonstrated that *s501* is associated with a disruption in a neighboring essential gene *let-456;* and, based on this observation, have determined s501 to be a deficiency. *rol-3(s501)* has been renamed *sDf57* (see Figure 1). The remaining ten alleles had all been isolated following EMS treatment.

Characterization of *rol-3* lethal alleles:

The stages of developmental arrest for each of the *rol-3* lethal alleles were determined previously (JOHNSEN and BAILLIE 1990) and are presented in Table 1. It is evident that all but four of the eleven lethal *rol-3* alleles are recessive for an early larval lethal phenotype. *s422*, *s742* and *s833* are recessive for a mid-larval phenotype, while *s1040* is a conditional lethal: at 20 °C worms homozygous for *rol-3(s1040)* display a mid-larval lethal phenotype, but at 15 °C develop as viable weak adult rollers. Nomarski optics were used to examine the extent of gonad development in dying hermaphrodite larvae homozygous for *rol-3* lethal alleles (see Figure 6). By this criteria I determined that those alleles designated as early blockers by JOHNSEN and BAILLIE have L1 stage gonads while those designated as mid larval blockers have early to mid L2 stage gonads (KIMBLE and HIRSH 1979).

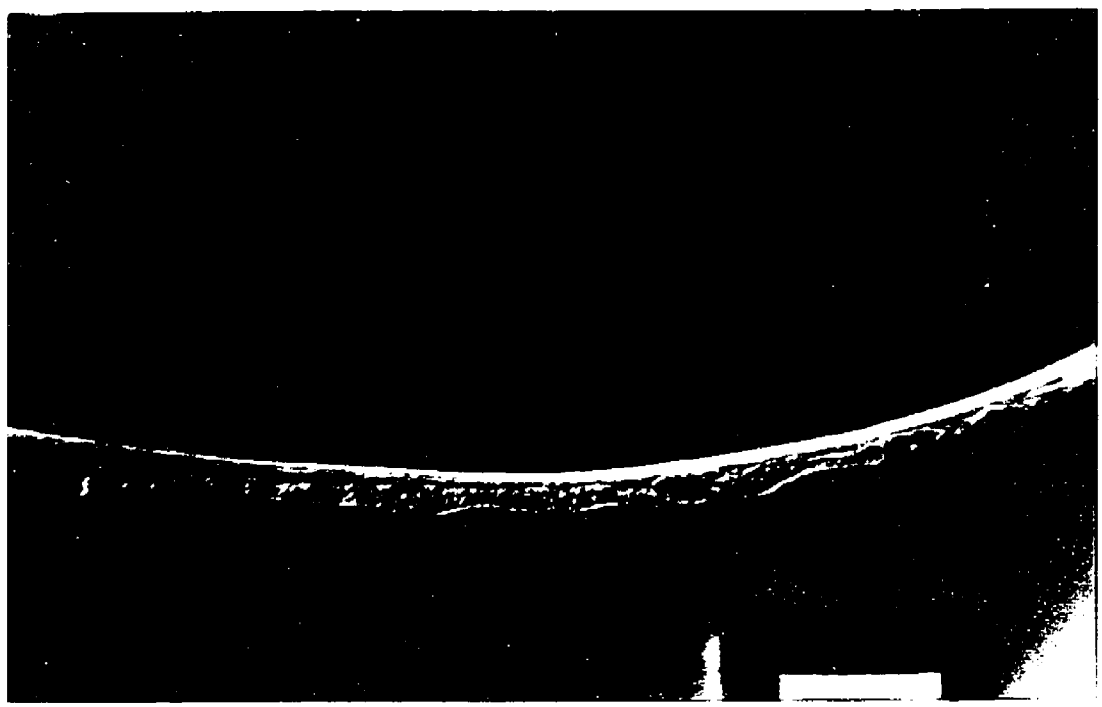
Figure 6A

Differential interference contrast (DIC) micrographs of Dpy-18 Unc-46 and Dpy-18 Unc-46 Roller *C. elegans* larvae.

[A]: *dpy-18(e364) ; unc-46(e177)* larva, judged to be L1 based on the extent of gonadogenesis (KIMBLE and HIRSH 1979)

[B]: *dpy-18(e364) ; unc-46(e177)rol-3(s1040ts)* arrested larva. The extent of gonad formation suggests that these Dpy Unc Rollers arrest at in L2. Note that there is no obvious morphological difference between the Dpy Unc worm in A, and the Dpy Unc Roller worm in B. The DpyUnc Roller depicted was raised at restrictive temperature (20 °C).

A



B

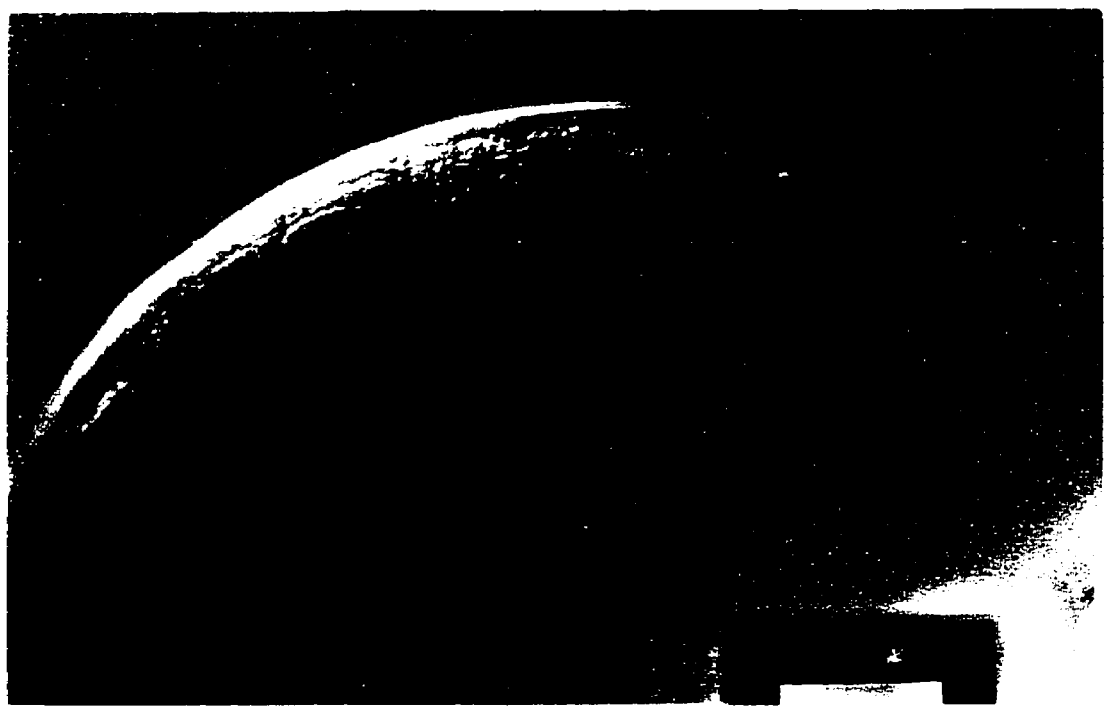


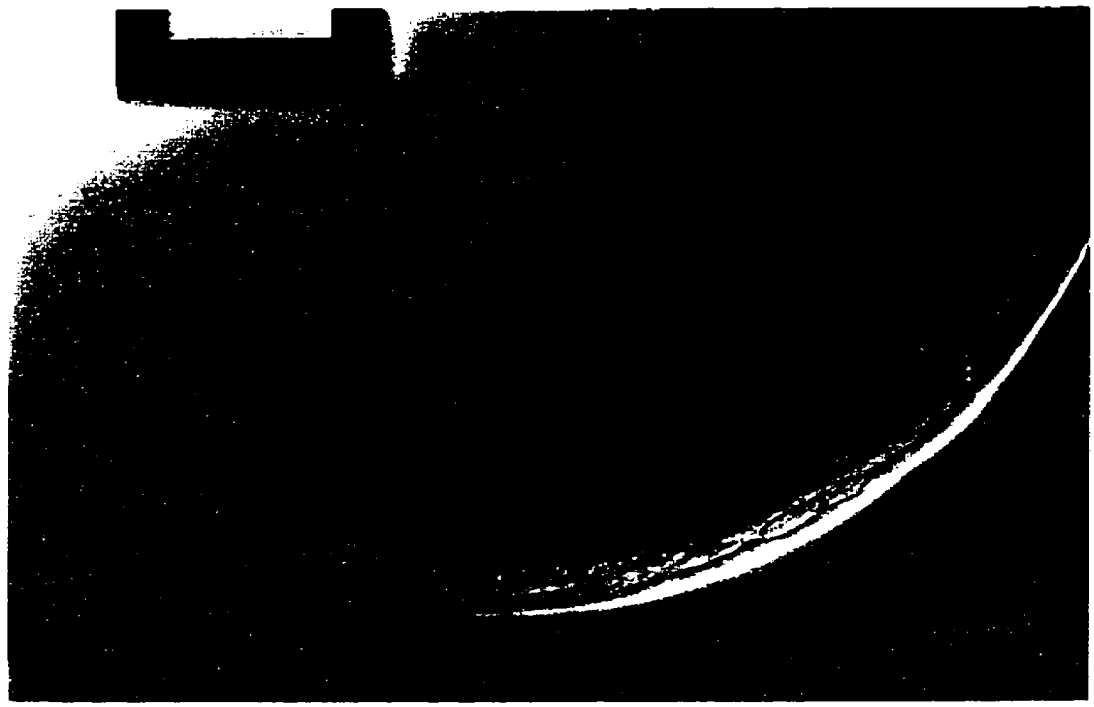
Figure 6B

Differential interference contrast (DIC) micrographs of Dpy-18 Unc-46 and Dpy-18 Unc-46 Roller *C. elegans* larvae.

[C]: Same individual as in Figure 3A a, but focal plane has been altered such that the lateral surface is now visible.

[D]*dpy-18(e364) ; unc-46(e177) rol-3(s1519)* arrested larva. This larva has arrested as an L1 based on the extent of gonad development (KIMBLE and HIRSH 1979). Note the absence of any obvious morphological defect associated with the developmental arrest phenotype of homozygous *rol-3(s1519).*

C



D

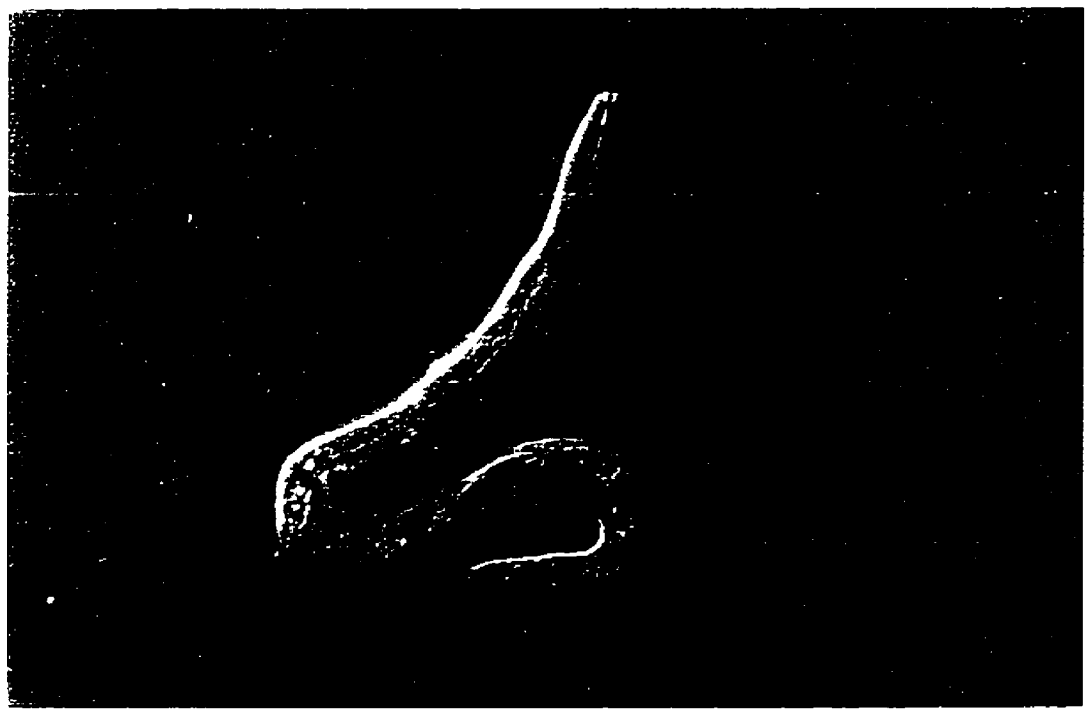


TABLE 1

Alleles of *rol-3* and their Phenotypes.

Arrest at early larval stage	Arrest at mid-larval stage	Fertile left hand-rollers
s126	*s422*	*e202*
s1408	*s742*	*e754*
s1409	*s833*	
s1494	*s1040*ts[a]	
s1519		

[a] Arrest during mid-larval development at 20-25 °C

Fertile, weak left-hand roller at 15 °C.

Data from JOHNSEN and BAILLIE (1991)

JOHNSEN (1990) calculated the average EMS hit frequency for essential genes within the *eT1* balanced region to be 1.25 mutations per essential gene. EMS induced recessive lethal mutations of the *rol-3* loci occur at a frequency six times that of the average essential gene. This suggests two possibilities: First, it is possible that the high EMS mutability associated with *rol-3* reflects the presence of a physically large coding region. Indeed, *unc-22* (MOERMAN, BENIAN and WATERSTON 1986) and *unc-54* (MACLEOD, KARN and BRENNER 1981) are two highly mutable loci which also constitute large physical targets. Alternatively, *rol-3* may contain one or more hot spots for EMS induced mutation. That *rol-3* may code for a large gene product, as well as the fact that *rol-3* lethal alleles display differential blocking stages suggests that the *rol-3* product may be organized into discrete functional domains, and that these may play separate roles during *C. elegans* development. Independently functioning domains can also be

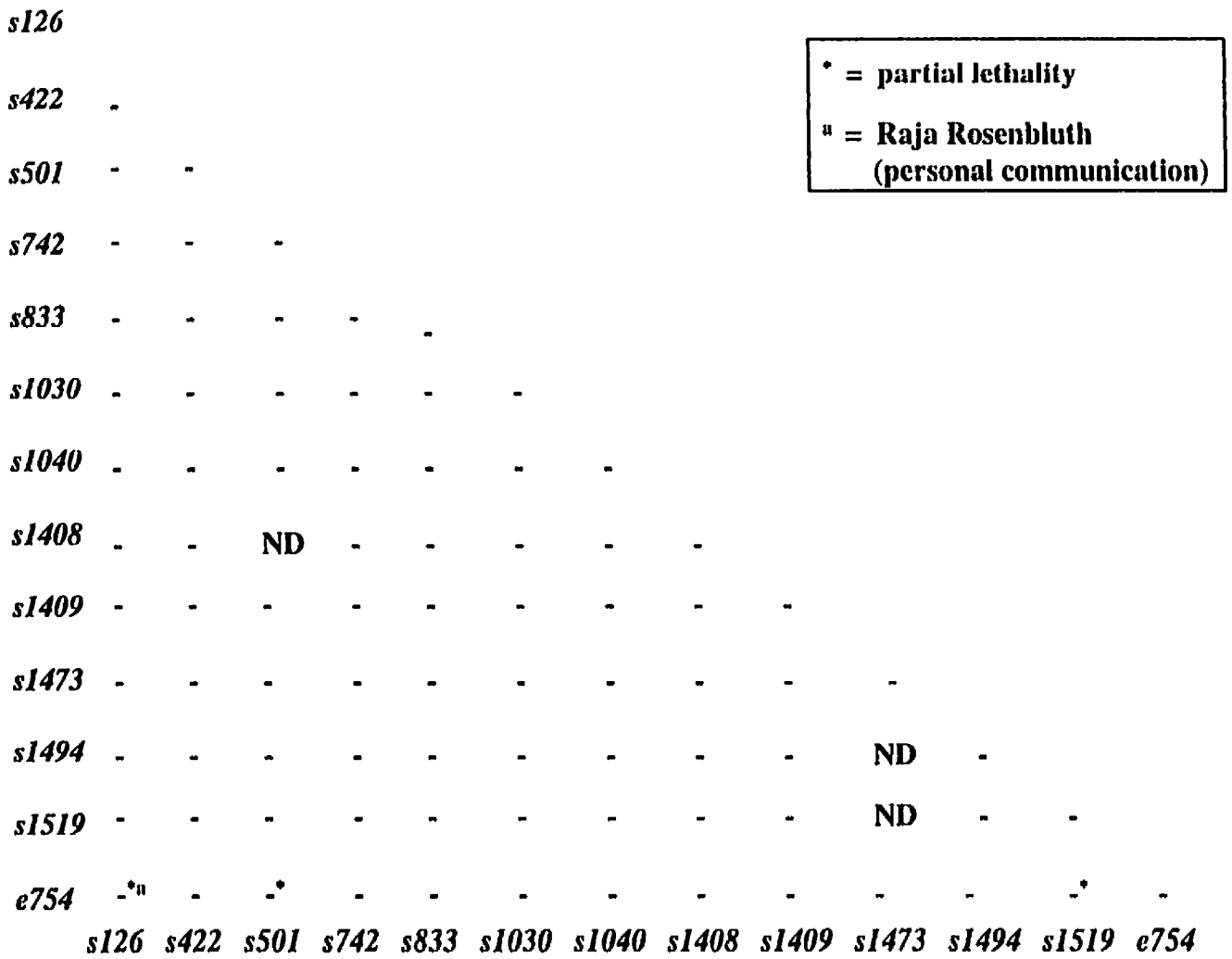
considered independently mutable, (RAND 1989) and as such, may elicit intragenic mutations which complement in *trans*. To test the possibility that *rol-3* might represent a complex locus of this type I performed *rol-3 inter se* complementation tests. I observed no complementation between *rol-3* alleles suggesting that *rol-3* defines a genetically simple locus, and that the *rol-3* protein likely does not function as a homo-multimer. Worms carrying the *rol-3(e754)* mutation in trans to the deficiency *mDf3* exhibit partial lethality. When worms homozygous for *rol-3(e754)* are mated to *mDf3* heterozygous animals, roller F1s are produced, but at a frequency greatly reduced from 1 : 1. Furthermore, many of the rolling F1 animals observed exhibit retarded growth which may reflect poor fitness. The semi lethal effects of worms hemizygous for *rol-3(e754)* are paralleled in *rol-3(e754)/sDf57*, *rol-3(e754)/rol-3(s126)* (*R. Rosenbluth unpublished results*) and *rol-3(e754)/rol-3(s1519)* heteroallelelic combinations. The presence of a temperature sensitive lethal allele which rolls at permissive temperature, and a genetically simple complementation pattern implies that weak alleles cause rolling, whereas stronger alleles affect viability. This is further supported by the observed behavior of *rol-3(e754)* over *mDf-3*. The reduced viability observed for *rol-3(e754)/Df* in comparison to *rol-3(e754)/rol-3(e754)* suggests that *e754* is a loss of function allele (hypomorph) as opposed to a null mutation. Moreover, the phenotypic severity of *rol-3* heteroallelic combinations follows the trend *rol-3(e754)/rol-3(s1519) = rol-3(e754)/rol-3(s126) = rol-3(e754)/Df > rol-3(e754)/rol-3(sx)* (where sx represents any *rol-3* lethal allele exclusive of *s126* or *s1519*) which in turn suggests that *s126* and *s1519* represent null mutations of *rol-3*. The remaining *rol-3* lethal alleles are likely hypomorphs. The results of *rol-3 inter se* complementation tests are presented in Figure 7.

Figure 7

Intra-allelic Complementation Results for Mutations at the *rol-3* Locus

All complementation tests were performed at 20 °C. *rol-3* lethal alleles versus one another were scored as non-complementing based on an absence of DpyUnc males (see materials and methods in this section). Lethal alleles of *rol-3* versus the recessive visible allele *e754* were scored as non-complementing based on the presence of Roller-3 males.

rol-3 vs *rol-3* Complementation Matrix



A sub-set of the lethal alleles of *rol-3* were tested for their suppression by the informational suppressor mutation, *sup-7(st5)* (Table 2). The *sup-7(st5)* mutation, which suppresses specific alleles of many unrelated genes (WATERSTON 1981), has been demonstrated to allow read through of mRNA molecules harboring amber terminators (WILLS *et al.* 1983). Cloning of the *sup-7* locus by BOLTEN *et al.* (1984) established that the *sup-7* gene encodes a tryptophan tRNA molecule which normally recognizes the codon UGG. In the case of *sup-7(st5)*, the anti-codon of the $tRNA^{Trp}$, which normally would be CCA has been altered to read as CTA. This alteration results in the recognition of the UAG codon by the *sup-7(st5)* encoded form of $tRNA^{Trp}$.

TABLE 2

Suppression of *rol-3* Recessive Lethal Alleles by *sup-7(st5)*

rol-3 allele tested	normal blocking stage	suppressible by *sup-7()* +/-
s126	early larval	-
s422	mid larval	-
s742	mid-larval	-
s1040	mid-larval @ 20-25 °C	-
s1519	early-larval	+

This codon, which is normally recognized by release factors which elicit the cessation of protein synthesis, when read by the *sup-7(st5)* $tRNA^{Trp}$, does not result in chain termination. Worms homozygous for *rol-3(s1519)* are viable in a *sup-7(st5)* mutant background. This suggests that the nature of the *rol-3(s1519)* defect is a pre-maturely truncated polypeptide product which is, for all intents and purposes, non-functional. Many amber mutations result in severely truncated peptides which are incapable of

performing their wildtype functions, as such, many null or nearly null mutations in a number of genes have been associated with amber mutations (HODGKIN, KONDO and WATERSTON 1989). The fact that *rol-3(s1519)* exhibits genetic criteria supportive of being a null allele and that worms homozygous for *s1519* display a very severe *rol-3* associated phenotype suggests that non-functional or null alleles of *rol-3* result in early larval arrest, weaker hypomorphic alleles result arrest later during development and viable rolling alleles are likely weak hypomorphs.

Analysis of *s1040*ts, a temperature sensitive lethal allele of *rol-3*:

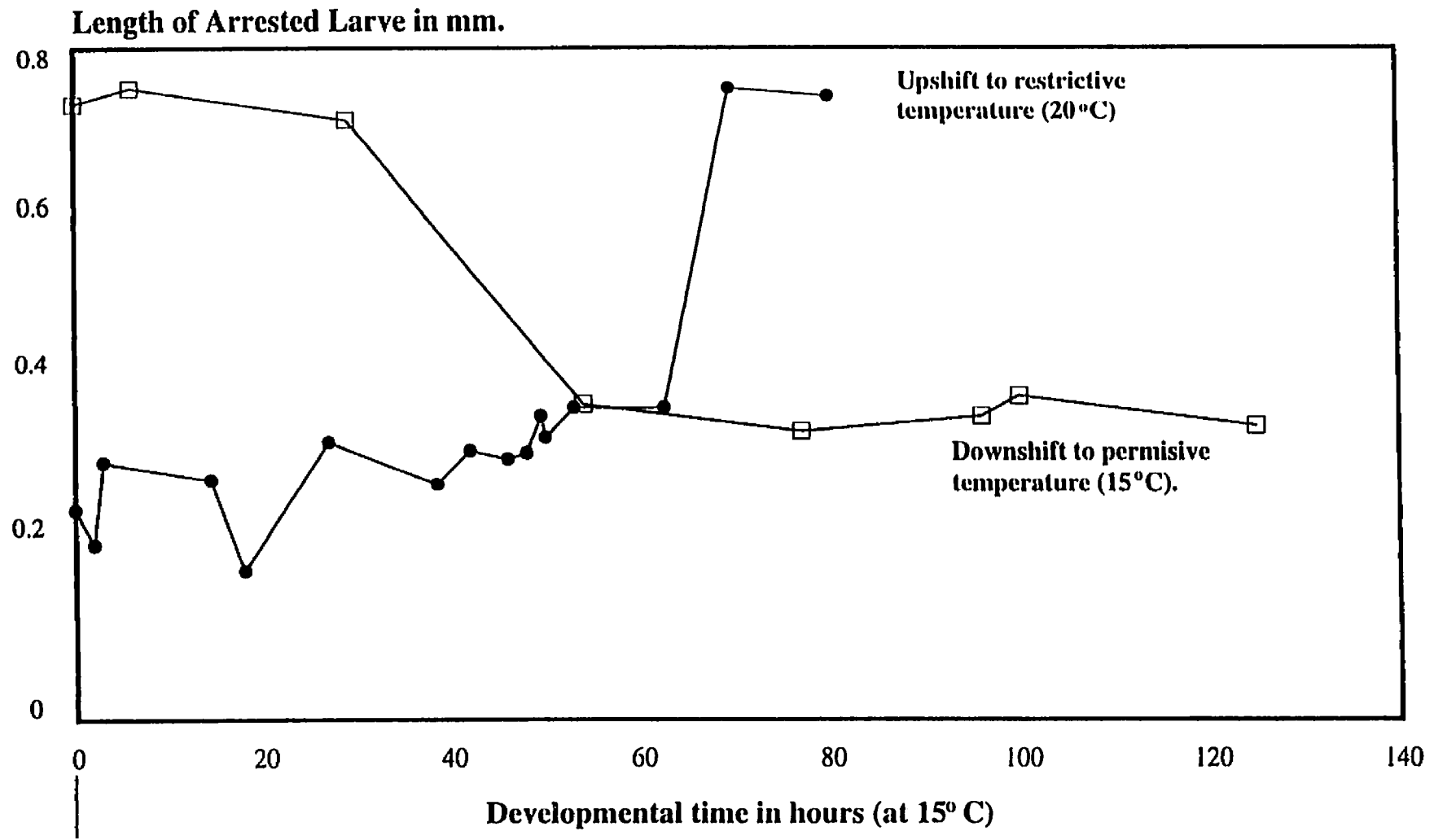
As previously mentioned, *s1040* is a recessive lethal allele of *rol-3* which displays a temperature dependent phenotype. When subject to a temperature of 20-25 ^{o}C , worms homozygous for *rol-3(s1040)* arrest development at a mid-larval stage. However, when grown at 15 ^{o}C, homozygous *s1040* worms develop to adulthood, are fertile, and display a weak left-handed roller phenotype. I studied the temperature sensitive period (TSP) for *rol-3(s1040*ts*)* in an effort to determine when in development functionally active *rol-3* gene product is required. As evidenced by the TSP for *s1040* (see Figure 8) I propose that a functional *rol-3* gene product is first required at approximately 30 hours after egg lay at 15 ^{o}C, and this requirement ceases at 70 hours. With respect to *C. elegans* growth at 25 ^{o}C the *rol-3* gene product is required from 15 to 35 hours after egg lay. Correlation of this time with the *C. elegans* developmental profile suggests that *rol-3(+)* is required mid L1 to mid L3 stage (WOOD *et al.* 1980). It should be noted that this TSP suggests the stage at which active *rol-3* gene product is required, but does not yield any information regarding the stage at which gene activation or protein synthesis occurs (HIRSH and VANDERSLICE 1976).

Figure 8

Temperature Sensitivity Period for *rol-3(s1040*ts)

Worms homozygous for *rol-3(s1040*ts) were upshifted (•) to restrictive temperature (25 °C), or downshifted (□) to permissive temperature (15 °C) at the times indicated along the X axis. These times were correlated with the terminal length of the worms shifted. Adults were expected to reach ~ 0.8 mm in length while those which arrested development at mid-larval stage were only expected to reach 0.35 mm. All time points were normalized to 15 °C. This experiment suggests that the requirement for *rol-3*'s essential function may occur as early as 30 hours after egg lay at 15 °C (15 hours at 25 °C) and may continue until 70 hours after egg lay at 15 °C (35 hours at 25 °C).

*rol-3(s1040*ts) Temperature Shift Experiment.
Determination of Temperature Sensitive Period.



All previous analyses concerning *rol-3* have been focused upon *e754*, the original recessive visible allele first isolated by BRENNER (1974). COX *et al.* (1980) determined that the visible roller phenotype associated with *rol-3(e754)* is not manifest until after the L4 molt, subsequent to adult cuticle formation. However, the TSP for *s1040* demonstrated that the *rol-3* gene performs an essential function at a stage much earlier than this. The observation that *rol-3* not only plays an essential role during early development but also disrupts adult cuticle morphology, suggests that the developmentally essential function may be separable from a second function which is required to establish or maintain the integrity of the adult cuticle.

Generation of suppressors of *rol-3(s1040)*: In an effort to elucidate the essential developmental role performed by the *rol-3* gene product I attempted to generate suppressors of the recessive temperature sensitive mid-larval lethal phenotype of *rol-3(s1040)*. Both gamma irradiation and chemical (EMS) mutagenesis were performed and a total of 100,000 treated F1 chromosomes were examined for each mutagen. I recovered two gamma irradiation induced suppressors (*s2500* and *s2501*) and eight EMS induced suppressors (*s2502-s2509*). Difficulties associated with outcrossing *s2505* and *s2509* prevented their further analysis. The eight suppressors analyzed function as recessive suppressors of *s1040*ts's temperature sensitive lethal phenotype, yet do not suppress the rolling phenotype. I have named these suppressors *srl* for suppressor of roller lethal.

Linkage mapping *srl* mutations: Linkage of the *srl* mutations was first tested with respect to LGs III, V and X (MATERIALS and METHODS). All *srl* mutations were found to be recessive and none were on LGX. Table 3 presents the progenies from *srl-(sx)/+ ...dpy-18/eT1; unc-46 rol-3(s1040*ts*)/eT1(V)* hermaphrodites. I interpret the data to show that the mutations fall into two classes 1 and 2. Class 1 (*s2500-s2503*) comprises those mutations that are linked to neither LGIII nor LGV; while class 2

mutations (*s2504-s2508*) are linked to either LGIII or LGV, but outside the region balanced by *eT1(III;V)*.

The mutations of class 2 were tested to determine whether they are linked to LGIII or LGV (MATERIALS and METHODS). The segregation patterns obtained in the absence of *eT1* (see Table 4) demonstrate that all four mutations are linked to LGIII rather than to LGV. Further support for class 2 mutation linkage to LGIII was obtained when *s2507* was tested with other LGIII markers: *dpy-1(e61)*, *dpy-17(e164)* and *sma-2(e502)* (Table 4). Our data suggest that the *s2507* is tightly linked to the *sma-2 - dpy-17* interval of LGIII.

Two of the class 1 mutations (*s2501* and *s2503*) were tested for linkage to *dpy* markers on LGs I, II, III, IV (MATERIALS and METHODS). The Dpy Unc-46/Unc-46 ratios (see Table 5) suggest that both *s2501* and *s2503* are loosely linked to *dpy-10* on LGIII (see Table 6).The average brood size, and lengths (based on five individuals) of the *dpy-18 ; unc-46 rol-3(s1040ts)* strains containing each of the eight suppressor mutations are displayed in Table 7. Worms triple homozygous for *dpy-18*, *unc-46* and *rol-3(s1040ts)* are inviable at restrictive temperature, but have a brood size of 100 at permissive temperature. This is a 50% decrease in brood size relative to worms doubly homozygous for *dpy-18 ; unc-46* alone. The presence of a homozygous suppressor mutation renders worms homozygous for *rol-3(s1040ts)* viable at restrictive temperature and also increases brood sizes relative to the *rol-3(s1040ts)* animals grown at permissive temperature. However, all Srl Dpy Unc Roller strains display brood sizes which are lower that those seen in *dpy-18 ; unc-46* mutant strains. The average length of a Srl Dpy Unc-46 Roller individual remains slightly lower than either a *dpy-18 ; unc-46* or a *dpy-18 ; unc-46 rol-3(s1040ts)* homozygous strain.

Table 3

Testing for linkage of *srl-(sx)* to either LGIII or LGV

Progeny from *srl-(sx)/+...dpy-18/eT1(III) ; unc-46 rol-3(s1040ts)/eT1(V)*					
***srl-* mutation**	**Viable phenotypes**			**Ratio[b]**	**Class**
	DpyUnc-46	**Wild-type**	**Unc-36[a]**	**DpyUnc-46 : WT : Unc-36**	
s2500	4	221	33	1 : 55 : 8	1
s2501	3	225	25	1 : 75 : 8	1
s2502	2	87	14	1 : 43 : 7	1
s2503	9	272	49	1 : 30 : 5	1
s2504	11	183	26	1 : 9 : 2	2
s2506	11	178	27	1 : 16 : 2	2
s2507	33	596	99	1 : 18 : 3	2
s2508	9	92	17	1 : 10 : 2	2

a Phenotype of homozygous *eT1*.

b If *srl-(sx)* is on LGIII or LGV, within the eT1 balanced region, expected ratio = 1 : 4 : 1.
If *srl-(sx)* is linked to neither LGIII nor LGV, expected ratio = 1 : 16 : 4.

Table 4

Testing class 2 *srl-* mutations for linkage to LGIII or LGV

Progeny from *srl-(sx)/+...dpy-18(III)/+ ; unc-46 rol-3(s1040ts) (V)/+ +*						
	Viable phenotypes				Ratio[a]	
srl- mutation	Dpy Unc-46	Unc-46	wild-type	Dpy	Dpy Unc-46 : Unc-46	Location
s2504	32	29	215	704	1 : 1	LGIII
s2506	27	12	140	439	2.2 : 1	LGIII
s2507	24	14	244	739	1.7 : 1	LGIII
s2508	22	10	120	433	2.2 : 1	LGIII

a If *srl-(sx)* is tightly linked to *dpy-18*, expected ratio = 4.2 : 1

If *srl-(sx)* is tightly linked to *unc-46*, expected ratio = 0.33 : 14

Table 5

2-Factor mapping of class 2 *srl* mutations to LGIII markers.

Progeny from *M srl-2(s2507)/+ + ; unc-46 rol-3(s1040*ts)/+ +

	Viable phenotypes					
LGIII Marker	**Dpy Unc-46 or Sma Unc-46**	**Unc-46**	**Dpy**	**wild-type**	**Adjusted Unc-46 total [a]**	**Distance [b] m.u. (95 % confidence)**
dpy-1	111	36	388	1360	15	6.59 - 20.87
dpy-17	55	7	200	671	0	0 - 2.6
sma-2	59	35	212	520	0	0 - 2.6

a Number of Unc-46 worms expected due to loss of *rol-3(s1040)*ts) was determined by the formula U=T[1-(1-P)2]/3, where U is the number of Unc-46, T is the total number of worms, and P is the *unc-46 - rol-3* distance in mu. divided by 100. This value was subtracted from the observed number of Unc-46 animals to yield the adjusted total.

b Recombination distance between the *srl-* mutation and the LGIII marker in question was determined by the formula p=1-[1-13R/T]1/2 where p is the recombination frequency between M and *srl-2*, R is the adjusted Unc-46 total and T is the total number of worms.
95% confidence limits are based on the limits of Unc-46 recombinants.
These limits are from Table 1 of CROW and GARDNER (1959).

Table 6

Testing class 1 *srl* mutations for linkage to Dpy markers on LGs I, II, III and IV.

Progeny from *srl-(sx)/+...dpy/+ ; unc-46 rol-3(s1040ts)/+ +*

		Viable phenotypes				Ratio[a]	
***srl* mutation**	**Dpy marker**	**Dpy Unc-46**	**Unc-46**	**Dpy**	**wild-type**	**DpyUnc-46 : Unc-46**	**Linkage**
s2501	*dpy-5 (I)*	16	58	302	764	0.3 : 1	Unlinked
	dpy-10 (II)	51	29	352	1140	1.8 : 1	Loose linkage
	dpy-18 (III)	3	7	40	126	0.4 : 1	Unlinked
	dpy-13 (IV)	32	45	258	549	0.7 : 1	Unlinked
s2503	*dpy-5 (I)*	8	30	160	385	0.3 : 1	Unlinked
	dpy-10 (II)	55	31	337	1039	1.8 : 1	Loose linkage
	dpy-18 (III)	3	6	49	220	0.5 : 1	Unlinked
	dpy-4 (IV)	30	55	337	628	0.5 : 1	Unlinked
	dpy-13 (IV)	21	28	251	459	0.7 : 1	Unlinked

a If *srl-(sx)* is tightly linked to dpy marker, expected ratio = 4.2 : 1
If *srl-(sx)* is linked to niether the marker nor unc-46, expected ratio = 0.3 : 1

Table 7

srl-mutations: Mutagen, Linkage group, Fecundity, and Terminal length.

GENE	MUTATION	MUTAGEN	LINKAGE GROUP	BROOD SIZE	LENGTH
srl-1 [1]	*s2500*	GAMMA	II	187	0.59 mm
	s2501	GAMMA	II	121	0.57 mm
	s2502	EMS	II	203	0.63 mm
	s2503	EMS	II	110	0.60 mm
srl-2 [1]	*s2504*	EMS	III	181	0.61 mm
	s2506	EMS	III	121	0.61 mm
	s2507	EMS	III	137	0.70 mm
	s2508	EMS	III	155	0.63 mm
CONTROLS					
dpy-18 ; unc-46				212	0.78 mm
dpy-18 ; unc-46 rol-3(s1040ts) [2]				100	0.72 mm

1 *dpy-18 ; unc-46 rol-3(s1040ts)* background

2 data taken at permissive temperature

The *srl* mutations fall into two complementation groups, *srl-1 II* and *srl-2 III*:

Figure 9 displays the complementation results observed between the various *srl* mutations. It is immediately obvious that the eight mutations fall into only two complementation groups, designated *srl-1* and *srl-2*. Furthermore, the complementation results are consistent with the linkage data. All mutations that were found to be unlinked to either LGIII, LGV or LGX (class 1) failed to complement one another and are the alleles of *srl-1*. Since two of these mutations (*s2501* and *s2503*) were shown to be on LGII, *srl-1* is located on LGII. All mutations that were found to be linked to LGIII outside the *eT1* balanced region (class 2) failed to complement each other and are the alleles of *srl-2*. Thus *srl-2* is located on the left half of LGIII, tightly linked to the *sma-2* - *dpy-17* interval. While the combined linkage and complementation data show that the mutations are in two distinct loci, the complementation pattern displayed by *srl-2(s2506)* III was unexpected. All alleles of both *srl-1* and *srl-2* failed to complement *s2506* indicating the existence of a complex genetic interaction between the *srl* loci.

Although the frequency at which these *srl* alleles were generated suggests that they probably represent loss of function alleles, (approx. 1 in 12,000 for the 0.012M EMS induced mutations) there are small differences within the complementation pattern which suggests that some of these alleles are not null alleles. For instance, *srl-1(s2502)* by *srl-2(s2507)* clearly complements. Although I do observe some exceptional males, they only represent 20% of the number expected if these alleles were to fail to complement. Evidently, the doubly heterozygous combination of these alleles result in weak suppression. This interaction occurs for these alleles regardless of which parent contributes which allele.

Another unusual feature of the data in Figure 9 concerns the complementation results of *srl-1(s2500* and *s2501)* hermaphrodites by *srl-2(s2506)* males. As previously mentioned, *srl-2(s2506)* fails to complement all analyzed *srl* mutations; however, in these two doubly heterozygous combinations only one half of the expected exceptional males

Figure 9

inter se Complementation Results for the *srl* Mutations

srl Complementation Data

		srl-1(II)				*srl-2(III)*			
		s2500	s2501	s2502	s2503	s2504	s2506	s2507	s2508
srl-1(II)	s2500	-	-	-	-	+	-[1]	+	+
	s2501	-	-	-	-	+	-[1]	+	+
	s2502	-	-	-	-[1]	+	-	+[2]	+[2]
	s2503	-	-	-	-	+	-	+	+
srl-2(III)	s2504	+	+	+	+	-	-	-	ND
	s2506	-	ND	-	-	-	-	-	-
	s2507	+	+	+[2]	+	-[1]	-	-	ND
	s2508	+	+	ND	+	-[1]	-	ND	-

1 Progeny class indicating failure to complement is present but at only one half of the expected frequency.

2 Observe some heteroallelic escapees, approximately one-fifth of the frequency expected for failure to complement.

that indicate failure to complement are observed. Furthermore, these are clearly maternal effects since *srl-2(s2506)* hermaphrodite vs. *srl-1(s2500)* male generates the expected number of exceptional males.

The suppression of lethality produced by *srl-2* is not restricted to *rol-3(s1040*ts):

The alleles of *srl-1* and *srl-2* were recovered as suppressors of *rol-3(s1040*ts). To determine whether or not these suppressors act in an allele general fashion with respect to *rol-3*, seven *rol-3* alleles were tested with three *srl-2* alleles (MATERIALS and METHODS), and the results are displayed in Table 8.

Table 8

rol-3* Alleles Tested for Suppression by *srl-2

	***srl-2* allele**		
***rol-3* lethal allele**	*s2506*	*s2507*	*s2508*
s126	-	-	-
s422	+	**ND**	+
s742	+	+[a]	+
s833	+	+	**ND**
s1040	+	+	+
s1409	+	**ND**	+
s1494	-	-	-
s1519	-	-	-

I have been unsuccessful in suppressing the lethal phenotype of either *s126* or *s1519*, both of which cause arrest at an early larval stage. However, I have demonstrated suppression of *rol-3(s422, s742, s833,* and *s1409)* by some *srl-2* alleles. Of particular interest is the

suppression of *rol-3(s742)* by *srl-2(s2506, s2507* and *s2508)*. When either *srl-2(s2506)* or *srl-2(s2508)* is crossed into a strain carrying *rol-3(s742)* yielding Dpy hermaphrodites and these are subsequently self-crossed, some Dpy Unc F2s are produced. These are morphologically similar to our *dpy-18 ; unc-46* reference strain (see Figure 10A). However, the F3s produced by these Dpy Unc F2s are not morphologically characteristic of the Dpy-18 Unc-46 reference strain. These worms exhibit varying degrees of posterior deformation and some internal disorganization, but develop to adulthood and are viable (see Figure 10C). If, however, *srl-2(s2507)* is crossed into a *rol-3(s742)* background Dpy Unc F2's again resemble our *dpy-18 ; unc-46* reference strain. However, *srl-2(s2507) dpy-18 ; unc-46 rol-3(s1040*ts) F3s have much different morphological characteristics. These F3s have severely disrupted posterior morphology, an abnormal cuticle surface, disrupted internal structures and are inviable (see Figure 10D and E). It is evident that, although these are inviable, they are morphologically distinct from the arrested *dpy-18(III) ; unc-46 rol-3(s742)* homozygotes (see Figure 10B). Therefore, on the basis that viability of the *srl-2(s2507)* ; *rol-3(s742)* combination is restricted to first generation while second generation individuals are inviable (clearly a maternal interaction), and that *srl-2(s2508)* ; *rol-*3 individuals display no maternal effect on viability; with respect to *rol-3(s742)*, I classify *srl-2(s2507)* as a weak suppressor and *srl-2(s2508)* as a strong suppressor. These results not only confirm that suppression by *srl-2* is not restricted to *rol-3(s1040*ts), but indicate that *srl-2* exhibits allele specific variation in the degree of suppression.

Phenotypes associated with *srl-1* and *srl-2*:

Hermaphrodites homozygous for any of the suppressor mutations have no readily discernible phenotype in the absence of *rol-3(s1040*ts). However, males homozygous for *srl-1* or *srl-*2 display aberrant male tail morphology. The morphology of the *C. elegans* male tail has been well defined (SULSTON, ALBERTSON and THOMSON 1980;

BAIRD *et al.* 1991; EMMONS 1992) and is highly specialized for copulation. This specialization entails both the execution of male-specific post-embryonic cell lineages, and the morphogenesis of adult body shape (SULSTON and HORVITZ 1977; SULSTON, ALBERTSON, and THOMSON 1980). Genes required for male tail development have been identified and studied by Hodgkin (1983), and some such as *mab-3* (SHEN and HODGKIN 1988), *mab-5* (KENYON 1986; COSTA *et al.* 1988 WARING and KENYON 1991; SLASER and KENYON 1992) and *mab-9* (CHISHOLM and HODGKIN 1989) effect cell lineage thus implying regulatory functions. The *C. elegans* male tail is composed of nine bilateral pairs of sensory rays, each comprising the dendritic ending of two neurons and one structural cell (SULSTON and HORVITZ 1977; SULSTON, ALBERTSON and THOMSON 1980). These three processes are contained within a tube-like extension of hypodermis forming a ray, which is surrounded by a tube of inner layer cuticle. Wild-type rays project radially from the tail and are embedded in the fan composed of outer-layer cuticle (BAIRD and EMMONS 1990; EMMONS 1992). The effects of *srl-1* and *srl-2* on male tail morphology are shown in Figure11. Figure 11A shows a ventral view of a wildtype *C. elegans* male tail illustrating an orderly ray pattern. Males homozygous for *unc-46 rol-3(s1040*ts*)* display a similar structure suggesting that neither *rol-3* or *unc-46* influence ray or fan morphology (see Figure11B). However, the presence of either *srl-1* (see Figure 11C) or *srl-2* (see Figure 11D, E) result in a general reduction of the rays and fan. Furthermore, the presence of *srl* mutations results in the extrusion of the copulatory spicules. This may reflect a role for the *srl* gene products within the structures responsible for the extension/retraction of the copulatory spicules. It is unclear whether or not the spicules in srl mutants are lengthened relative to those in wildtype.

dpy-18 homozygotes display disorganized tail morphology concomitant with a thickening and shortening of the rays (see Figure 11F). BAIRD and EMMONS (1990) identified and characterized five genes comprising a class of mutations known as *ram*

Figure 10

Differential Interference contrast (DIC) Micrographs of *srl-2 dpy-18(e364) III ; unc-46(e177) rol-3(s742) V* hermaphrodites

[A] *dpy-18(e364) III; unc-46(e177).* [B] *dpy-18(e364); unc-46(e177) rol-3(s742)* arrested during mid-larval development. [C] *srl-2(s2508) dpy-18; unc-46(e177) rol-3(s742).* The animals in C exhibit a posterior rounding followed by an obvious cuticle taper. Despite the internal disorganization, these animals develop into fertile adults. [D], and [E] *srl-2(s2507) dpy-18(e364); unc-46(e177) rol-3(s742).* Posterior development appears incomplete lending to a croissant-like ap3pearance to animals of this genotype. These animals exhibit aberrant cuticle surfaces and severe internal disorganization. Note that immediate anterior development appears normal as evidenced by a morphologically wild-type pharynx, although hyper-contraction of the body has imposed a kink between the pharyngeal bulbs. Both of the animals depicted in [C] and [D] are six days old.

Bar in [C], [D], and [E] = 20 μm.


E

Figure 11

Differential Interference contrast (DIC) Micrographs illustrating adult male posterior morphology.

[A] Wild-type (*N2*); [B] *unc-46 rol-3(s1040*ts*)* at 15 °C. Worms homozygous for *rol-3(s1040*ts*)* at 20-25°C are inviable. Based on the comparison between [A] and [B], there is no apparent male tail defect associated with either *rol-3* or *unc-46*. [C] *srl-1(s2501) ; unc-46 rol-3(s1040*ts*)*, [D] *srl-2(s2507) ; unc-46 rol-3(s1040*ts*)*. Note a general reduction in tail structures in worms homozygous for *srl* mutations, as well as fully irreversibly extended spicules. [E] *srl-2(s2507) ; unc-46 rol-3(s1040*ts*)*, the abnormally extended spicules are conspicuous in this lateral view of a male homozygous for *srl-2(s2507)*, [F] *dpy-18 ; unc-46* male. Tail defects present are associated solely with *dpy-18* since *unc-46* does not influence male tail morphology. [G] *srl-2(s2506) dpy-18* male, [H] *srl-2(s2507) dpy-18* male. In both [G] and [H] the defects observed are likely the additive effect of *dpy-18*'s male tail defect and those attributed to *srl-2*. [I] *srl-1(s2501) ; dpy-18 ; unc-46 rol-3(s1040*ts*)*. Note that the *srl-1-dpy-18* combination results in a severe disorganization of the male posterior and lack of recognizable male copulatory apparatus.

Bar in [G], [H] and I = 20 μm.

A
I

mutants for their effect on ray morphology (1990). They stated that *dpy-18*'s Ram phenotype is presumably due to cuticle effects on ray morphology. Figure 11G and 11H illustrates the effects of *srl-2(s2506)* and *srl-2*(s2507) respectively in a *dpy-18(e364)* background. It is evident that *srl-2* has an additional effect on the morphology of the male tail as these worms display increased tail disorganization including a further shortening and/or absence of rays, as well as an obvious decrease in fan size. The effect of *srl-1* on male tail development is even more pronounced. Figure 11I shows a lateral view of a *srl-1(s2501); dpy-18(e364); unc-46(e177) rol-3(s1040*ts*)* adult male. In this case the male posterior structures are so deformed that there no recognizable male tail structures present. I believe that with respect to overall male tail morphology, the mutations within the *srl-1* locus behave synergistically with mutations within the *dpy-18 (III)* locus.

<u>*srl-2* modifies the body wall muscle phenotype associated with *rol-3*</u>:

Homozygous *rol-3(s1040*ts*)* adult worms maintained at permissive temperature were assayed under polarized light for helically twisted body wall muscle. The muscle band morphology defects associated with *rol-3(s1040*ts*)* were assayed in the presence, of either *unc-46(e177)* (see Figure 12A) or *dpy-18(e354)* ; *unc-46(e177)* (see Figure 13B) homozygous backgrounds. In both cases, the presence of *rol-3(s1040*ts*)* results in body wall musculature which is twisted relative to control animals (see Figure 5B *unc-46(e177);* Figure 13A *dpy-18(e364); unc-46(e177)* respectively). Therefore, as observed in animals homozygous for *rol-3(e754)*, the *rol-3(s1040*ts*)* mutation also confers a helical twist to the *C. elegans* body wall muscle. No attempt to quantify differences in severity with respect to the degree of body wall muscle twisting observed in *e754* and *s1040*ts homozygotes was made. The ability of *srl-2* to suppress the *rol-3* associated twisting of the body wall musculature is apparent in Figure 12B and 13C.

Figure 12

The effect of the *srl-2(s2507)* mutation on the twisted body wall muscle apparent in *unc-46(e177) rol-3(s1040ts)* adult hermaphrodites.

[A]: *unc-46(e177) rol-3(s1040ts)* [B]: *srl-2(s2507) ; unc-46(e177)rol-3(s1040ts)*

The *unc-46(e177)* mutation has no apparent effect on the gross morphology of *C. elegans* body wall musculature, as demonstrated in Figure 5B.

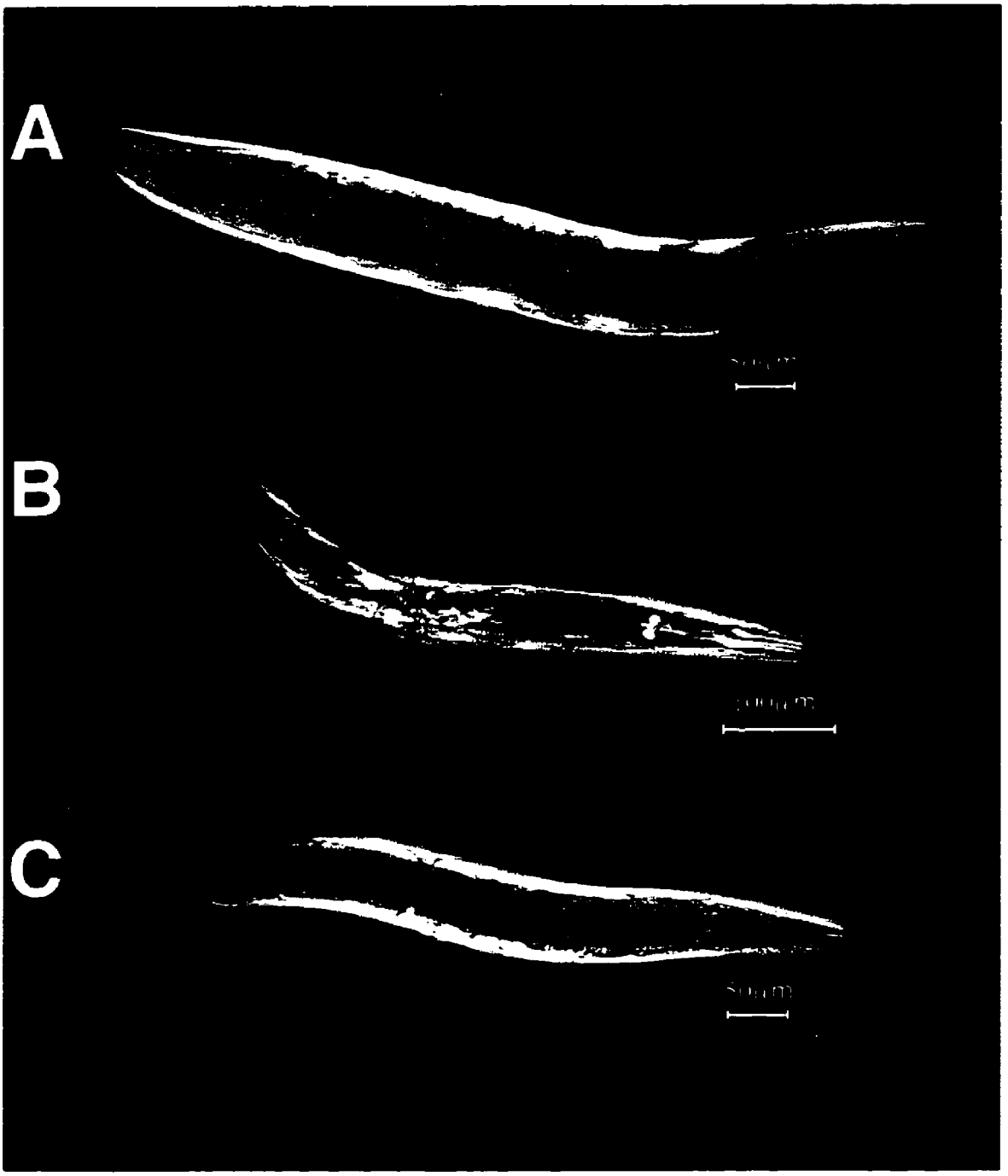
A
B
50μm

Figure 13

The effects of the *srl-2(s2507)* mutation on the gross morphology of body wall musculature of *dpy-18(e364)* ; *unc-46(e177)rol-3(s1040*ts) adult hermaphrodites.

[A]: *dpy-18(e364) ; unc-46(e177)* [B]: *dpy-18(e364) ; unc-46(e177)rol-3(s1040*ts*)*

[C]: *srl-2(s2507)dpy-18(e364) ; unc-46(e177)rol-3(s1040*ts*).*


A
B
C

Introduction of *srl-2(s2507)* into an *unc-46(e177) rol-3(s1040*ts) background appears to correct body wall musculature helicity such that, when assayed for twisting by polarized light microscopy, the body wall muscle bands of *srl-2(s2507)* ; *unc-46(e177) rol-3(s1040*ts) animals are indistingiushable from those of *unc-46(e177)* animals (see Figure 12B). The presence of srl-2(s2507) also decreases twisting of the body wall muscle bands within *dpy-18(e177)* ; *unc-46(e177) rol-3(s1040*ts) animals (see Figure 13C).

The body wall muscles and the nematode cuticle are indirectly linked by a series of structural components which act to interface the two structures. The existence of these components which likely act in the transmission of muscle tension across the basement membrane separating the body wall muscle cells from the hypodermis, through the hypodermis to the cuticle, has been demonstrated via immunocytochemisty (FRANCIS and WATERSON 1991). Therefore, if *srl-2* acts to straighten the body musculature in Roller-3 animals, perhaps the cuticle is affected in a similar fashion. In order to assay twisting in the cuticle I observed the alae under Nomarski optics. Adult control animals of the genotype *unc-46(e177)* or *dpy-18(e364)* ; *unc-46(e177)* double mutants have lateral alae which run straight along the animal cuticle. The alae of these animals resembles that of wildtype (N2) animals (data not shown). The alae of adult *srl-2(s2507)* ; *unc-46(e177) rol-3(s1040*ts) and that of *srl-2(s2507) dpy-18(e364)* ; *unc-46 rol-3(s1040*ts) are demonstrated in (see Figure 14A and B respectively). The lateral alae of *srl-2(S2507)* ; unc-46(e177) *rol-3(s1040)* hermaphrodites lie straight along the animal with no apparent deviation in direction (see Figure 15A). However, the alae of adult srl-2(s2507) dpy-18(e364) ; unc-46(e177) *rol-3(s1040*ts) worms remain slightly twisted compared to wildtype.

Since, srl-2(s2507) can restore normal body wall muscle band pattern in homozygous *rol-3(s1040*ts) Roller animals, I sought to determine whether *srl-2(s2507)*

Figure 14

Differential interference contrast (DIC) micrographs taken in the focal plane of the cuticle

This Figure depicts (DIC) Micrographs of the cuticle and illustrates the adult cuticle alae of *srl-(s2507) rol-3(s1040*ts) hermaphrodite worms in either an [A]: *unc-46(e177)* genetic background, or a [B]: *dpy-18(e364) ; unc-46(e177)* background. Only the posterior one-fifth of the Srl Unc Roller-3 is shown.

Figure 15

The effect of *srl-2(s2507)* on the body wall musculature of *rol-3(e754)* adult hermaphrodites.

[A]: *dpy-18(e364) ; rol-3(e754)* [B]: *srl-2(s2507)dpy-18(e364) ; rol-3(e754)*

The presence of *srl-2(s2507)* appears to have some corrective effect on the twisted body wall musculature characteristic of the *rol-3(e754)* mutation; however, *srl-2(s2507)* appears to have no corrective influence on Rolling during locomotion.


A


B
60

would have an effect on the unconditional visible allele e754. *srl-2(s2507) dpy-18(e364); rol-3(e754)* worms Roll indicating that *srl-2* does not correct the Rolling phenotype associated with locomotion. Examination of the body wall muscle bands in Srl-2 Dpy Roller-3 animals suggests thar *srl-2(s2507)* can partially correct twisting of the body wall musculature resulting from the *rol-3(e7564)* mutation.

It is interesting to note that Srl-2 Unc Roller-3 animals display no evidence of rolling, whereas, Srl-2 DpyUnc Roller-3 animals remain left-handed rollers. This supports the suggestion that the rolling phenotype observed during locomotion is a result of cuticle structure (HIGGINS and HIRSH 1977; COX et al. 1980), and that twisted cuticle, even in the presence of straight body musculature, is sufficient to effect rolling. It is possible that *dpy-18* may interact with *srl-2, rol-3* or both, with respect to influencing cuticle morphology. The observation that *srl-2(s2507)* is unable to repair locomotion and alae defects associated with mutations in *rol-3(s1040*ts) when *dpy-18* remains in the genetic background, but these defects are absent in *srl-2(s2507) dpy-18*$^{+}$; *unc-46(e177)rol-3(s1040*ts) animals suggests that mutations in dpy-18 may enhance cuticle defects associated with mutations in *rol-3.*

. I tested the *srl-1* and *srl-2* mutations to determine if they represent alleles of previously identified genes which map in the same vicinity and exhibit male tail defects. I suspected that *srl-1* mutations might represent alleles of *mab-9 II*,(CHISHOLM and HODGKIN 1989) and *srl-2* mutations might represent alleles of *mab-10 III* (HODGKIN 1983), *mab-5 III* (KENYON 1986) or *pal-1 III*.(WARING and KENYON 1991). However, loss of function alleles of the above genes both fail to suppress *rol-3(s1040*ts), and complement the tail phenotypes of the *srl-1* mutations tested against. *smg-6* is a recessive suppressor of mutations within a variety of different genes, and exhibits a male abnormal phenotype (HODGKIN *et al.* 1989). The *smg-6* allele *(r896)* also fails to suppress *rol-3(s1040*ts), complements the tail phenotype of *srl-2* alleles, and; alleles of *srl-2* do not suppress the *smg* suppressible *dpy-10* allele *e61.*

DISCUSSION

Analysis of lethal alleles of *rol-3*

In this section I describe the genetic characterization of *rol-3* and the isolation and analysis of its intergenic suppressors. *rol-3* has at least two distinct developmental functions. First, *rol-3* has an essential function during early to mid larval development. Second, *rol-3*'s adult-specific visible phenotype is rotation about the nematode long axis called rolling. Roller mutants have long been considered to be a subset of the morphological mutants (KUSCH and EDGAR 1986). Roller mutations, grouped together with Squat, Long, Blister, and Dumpy mutants, are thought to exert their effects on overall nematode morphology by disrupting functions or components which normally act to establish and/or maintain cuticle integrity. Indeed, many of these morphological mutants are now known to result from mutations in genes encoding collagens or other cuticle components (KRAMER *et al.* 1988; VONMENDE *et al.* 1988, KRAMER *et al.* 1990). Worms homozygous for *rol-3(e754)* not only display aberrant adult cuticles, but also display epistatic interactions with other cuticle mutants (HIGGINS and HIRSH 1977; COX *et al.* 1980; KUSCH and EDGAR 1986). Until this report it was assumed that the *rol-3* gene would simply encode a cuticle component.

If an altered adult cuticle component is responsible for *rol-3(e754)*'s rolling phenotype, I would expect that heterochronic mutations effecting cuticle development would also effect the expression of the rolling phenotype. I have shown that this is not the case. The heterochronic mutation *lin-14(n179ts)* allows the early expression of an adult cuticle (AMBROS and HORVITZ 1984, 1987) to occur at restrictive temperature (25^{o}C). *rol-3(e754); lin-14(n179ts)* roll as gravid adults at the restrictive temperature, suggesting that formation of an adult cuticle one molt earlier then in wildtype does not elicit a parallel effect on the onset of rolling. In addition, *lin-29(n1440)* causes reiteration of larval cuticles in adult worms such that an adult cuticle is not produced (AMBROS and HORVITZ 1984). From the fact that all *rol-3(e754); lin-29(n1440)* worms roll as adults I

argue that the expression of the rolling phenotype is not dependent on the presence of an adult cuticle. Therefore, *rol-3* itself is unlikely to encode an adult cuticle specific component.

There are an estimated 40 to 150 collagen genes in the *C. elegans* genome (COX, KRAMER and HIRSH 1984; KINGSTON 1991) suggesting redundancy within members of this gene family. As such, one would predict that mutations in many collagen genes would be non-lethal. *rol-6* and *sqt-1* are good examples of this, both of these genes encode collagens, and both display genetic behavior expected from redundant gene products, including wildtype null alleles (PARK and HORVITZ 1986; KRAMER *et al.* 1988; 1990). *rol-3*, on the other-hand, has eleven recessive lethal alleles, and only two viable alleles. In contrast to *rol-6* and *sqt-1*, I suspect that *rol-3* null alleles affect viability.

I have four pieces of evidence from which I argue that *rol-3* lethal alleles constitute severe loss of function alleles. First, from the presence of a temperature sensitive allele which rolls at permissive temperature it can be argued that the more severe alleles result in lethality. Second, weaker *rol-3* lethal alleles display more severe phenotypes as heterozygotes over a deficiency then as homozygotes, while the strong alleles exhibit no such effect (data not shown). Third, the phenotypic severity of *rol-3* heteroallelic combinations are related as follows: *rol*-3(e754)/rol-3(s1519 or s126) = rol-3(e754)/Df > rol-3(e754)/rol-3(sx) (where sx represents any *rol-3* lethal allele exclusive of *s126* or *s1519*). The observation that *rol-3(e754)* in trans to the early larval lethal alleles s126 and s1519 behaves the same as when trans to a deficiency suggests that *s126* and *s1519* are likely null alleles. Fourth, *rol-3(s1519)* is suppressible by the amber mutation *sup-7(st5)*. The identification of an amber allele of a given gene, whether by the criterion of being suppressed by an amber suppressor or some other means, indicates that this gene produces a protein, and also suggests that this gene may be null or nearly null for that gene activity (HODGKIN, KONDO and WATERSTON 1989). These results suggest that those *rol-3* alleles which cause early developmental arrest are likely null

mutations, and the weaker lethal alleles are hypomorphs. I suspect that the relatively rare visible alleles constitute weak hypomorphs, or represent minor alterations of the *rol-3* gene product.

What function does *rol-3* perform during larval development, and how does this relate to the adult phenotype? It is possible that the *rol-3* gene product is multifunctional and performs an essential developmental role, as well as an unrelated role during adulthood. Alternatively, the rolling phenotype may be a consequential manifestation of altered essential gene function during early larval development, and thus only detected in the weakest of alleles. If this is the case then it's possible that *rol-3* may play a role within *C. elegans* basement membrane or basal lamina. Since basement membranes are believed to play roles in a variety of important biological functions, including tissue morphogenesis, maintenance of tissue structure, cell attachment and may also serve as a substrate upon which cells migrate (GUO and KRAMER 1989; GUO, JOHNSON and KRAMER 1991; MACDONALD 1989; HUGHES and BLAU 1990; DISPERSIO, JACKSON and ZARET 1991), mutations effecting such genes would be expected to have repercussions in overall morphology, as well as viability (GUO and KRAMER 1989, . For example, *emb-9* encodes the *C. elegans* alpha 1(IV) collagen chain and mutations of this locus cause temperature sensitive lethality during late embryogenesis (Guo *et al.* 1991). Furthermore, unc-52 encodes the basement membrane component perlican, and some alleles of unc-52 also cause arrest during embrogenesis (ROGALSKI 1993).

Generation and analysis of suppressors of rol-3 lethal alleles:

Early studies by JARVIK and BOTSTEIN (1973; 1975) showed that intergenic second-site suppressors can identify genes whose products interact with the defective product of the first gene, thus identifying additional genes involved in a particular process, and perhaps providing information regarding the function of that system. This

approach has also proven effective in *C. elegans* (RIDDLE and BRENNER 1978; GREENWALD and HORVITZ 1980; 1982; 1986; MOERMAN *et al.* 1982, WATERSTON *et al* 1982; HODGKIN, KONDO and WATERSTON 1987; SCHNABEL, BAUER and SCHNABEL 1991; LEVIN and HORVITZ 1993; MAINE and KIMBLE 1993; SUNDARAM and GREENWALD 1993; JONGEWARD, CLANDININ and STERNBERG 1995; SINGH and HAN 1995). The main requirement underlying a successful suppressor screen is the ability to apply very sensitive selection criteria to a large number of individuals. One powerful screening method for the identification of suppressors involves the use of a temperature sensitive mutant background. Successful applications of this approach have resulted in the identification of extragenic suppressors of temperature sensitive alleles of the *Saccharomyces cervisiae* RAD52 gene (KAYTOR and LIVINGSTON 1996) and of temperature sensitive alleles of glp-1 in *C. elegans* (MAINE and KIMBLE 1993). The temperature sensitive lethal allele *rol-3(s1040*ts) provided a sensitive background for the rapid assay of second site suppressors. At 15°C, worms homozygous for the recessive, temperature sensitive lethal mutation *rol-3(s1040*ts) develop to adulthood and are fertile. At 20-25°C, homozygous *rol-3(s1040*ts) worms arrest development at a mid larval stage. Therefore, large numbers of homozygous *rol-3(s1040)* animals can be mutagenized and allowed to self fertilize at permissive temperature. The induction of a suppressor mutation can then be rapidly assayed by transfer of the worms to restrictive temperature.

Using this method, I was successful in generating recessive mutations within two genes which suppress the mid-larval lethal phenotype of *rol-3(s1040*ts): *srl-1(II)* and *srl-2(III)*. The identification of two loci which in turn affect the lethal phenotype of *rol-3(s1040*ts) suggests that *srl-1* and *srl-2* may act in a common biochemical or regulatory pathway with the *rol-3* gene product. The complementation data for alleles of *srl-1* and *srl-2* indicate that there exists a complex, allele specific, genetic interaction between these two loci. Specifically, in a *rol-3(s1040*ts) background at restrictive temperature, worms

heterozygous for *srl-2(s2506)* and any of the four *srl-1* alleles develop to fertile adulthood. Therefore, *srl-2(s2506)* fails to complement all *srl-1* alleles analyzed to date, despite their residence on different chromosomes. Intergenic noncomplementation between recessive mutations is indicative of functional interactions between the products of the genes involved (KUSCH and EDGAR 1986; TRICOIRE 1988; DANBLY-CHAUDIERE *et al.* 1988; HOMYK and EMERSON 1988; BAIRD and EMMONS 1990). Our observed intergenic noncomplementation suggests that *srl-1* and *srl-2* participate in a common developmental pathway. *srl-1, srl-2* double mutants have been constructed, and are viable, but the effect on mutations in *rol-3* has not been assayed.

Based on the relatively high forward mutation frequency (1 in 10,000) at which *srl* mutations are generated, I suspect that the majority of our mutations are gene loss of function mutations. Although the true nature of these mutations is not known, the complementation results of Figure 9 suggest that some alleles may retain partial function. In the crosses of *srl-1(s2502)* hermaphrodite by either *srl-2(s2507)* or *srl-2(s2508)* male, and *srl-2(s2507)* hermaphrodite by *srl-1(s2502)* male, I observed a small number of escapees doubly heterozygous for mutations in *srl-1* and *srl-2* indicative of a very weak dominant effect. It is possible that *srl-2(s2507)* and *s2508* each harbor a similar defect to that seen in *s2506* since all three mutations exhibit dominant effects. However, in the case of *srl-2(s2507)* and *s2508*, this dominance is only exhibited in the presence of *srl-1(s2502)*, while *srl-2(s2506)* displays interalleleic noncomplementation with all *srl-1* alleles.

Our observations that males homozygous for either *srl-1* or *srl-2* display aberrant tail structures suggests a possible role for the *srl* gene products. I suspect that these genes normally function in some aspect of *C. elegans* posterior patterning. Mutations in many such genes are conspicuous in the male due to the disruption of the well defined and prominent male tail. However, most such genes affecting the male tail also show less obvious hermaphrodite effects (HODGKIN 1983). Genetic evidence suggests that many

of the genes affecting male tail morphology encode products which function as regulators of downstream gene expression. In the case of *mab-5* (COSTA *et al.*) and *pal-1* (WARING and KENYON 1991) this has been corroborated with molecular data. Both of these genes encode homeo-domains which are thought to have DNA binding capabilities. It is possible that the *srl* gene products also act to regulate gene expression. Based on our genetic evidence I propose that *srl-1(+)* and *srl-2(+)* are co-regulators of the *rol-3* gene. However, since no detectable tail morphological defect has been associated with *rol-3*, the tail phenotype associated with mutations in the *srl-* loci suggests that they may also have roles outside of their association with the *rol-3* gene.

I observe suppression of a number of *rol-3* lethal alleles by mutations at the *srl-2* loci. In no case is suppression of the presumptive *rol-3* null alleles *(s126 and s1519)* observed; however, suppression is observed for a number of less severe mid larval alleles of *rol-3*. In general, suppressible mutations in *rol-3* constitute the weaker lethal alleles whereas strong alleles are not suppressible. The observation that strong alleles of *rol-3* are not suppressible suggests that mutations in *srl-2* do not bypass a reguirement for *rol-3* gene function. Instead, it's likely that *srl-2* allows a disabled rol-3 product to act more efficiently.

The question remains as to the nature of the relationship between *rol-3* and the *srl* gene products. One model supporting the observation that null alleles of *rol-3* are not suppressible by *srl-2* proposes that the *srl-1*(+) and *srl-2*(+) gene products participate in regulation of the *rol-3*(+) gene product. Specifically, the wildtype *srl* gene products may act in a concerted fashion as negative regulators of *rol-3* expression. In light of this, I expect that subjecting hypomorphic *rol-3* alleles to down-regulation by wildtype *srl-* gene products would result in a substantial decrease of functional *rol-3* gene product relative to the levels in wildtype worms. However, in the absence of down regulation, a hypomorphic allele of *rol-3* may produce enough functional *rol-3* product to meet the essential function requirements, thus avoiding developmental arrest. The left-handed

rolling phenotype is not suppressed presumably because such a phenotype is the result of a weakly defective *rol-3* product which impairs a specific aspect of *rol-3* function. Up-regulating the expression of this mutant product would not be expected to suppress the rolling phenotype.

The suppression of *rol-3(s742)* by *srl-2* gives further insight into the developmental roles played by the *rol-3* and *srl* gene products. As discussed previously, hermaphrodite worms which are homozygous for *rol-3(s742)* and *srl-2(s2507),* and some which are homozygous for *srl-2(s2506* or *s2508)* and *rol-3(s742)* display abnormal cuticle and internal morphology as well as disrupted posterior morphology. As illustrated in Figure 5C, D, and E the posteriors are rounded and blunt, while the heads and pharynx appear normal.

In general, I observe *srl-2* mediated suppression of roller lethality in only the weaker of the *rol-3* alleles. The observation that neither the most severe lethal *rol-3* alleles, nor the rolling defect are suppressible is consistent with the *srl* gene products acting as negative regulators of functional *rol-3* expression. However, the male tail defects associated with *srl-1* and *srl-2* suggest that these gene products are pleiotropic, and may regulate the expression of genes other than *rol-3.*

srl-2(s2507) appears to have a corrective effect on the twisted musculature associated with mutations in *rol-3.* Worms of the genotype *unc-46(e177) rol-3(s1040ts)* or *dpy-18(e364) ; unc-46(e177) rol-3(s1040ts)* roll as adults when reared at permissive temperature. Coincident with rolling, their cuticle and musculature is twisted. In the presence of *srl-2(s2707),* the body wall muscle bands run straight along the length of the worm. However, whereas the cuticle twists appear to have been repaired in *srl-2(s2507)* ; *unc-46(e177) rol-3(s1040ts)* worms, the cuticles of *srl-2(s2507)dpy-18(e364)* ; *unc-46(e177) rol-3(s1040ts)* animals remain slightly twisted. Furthermore, these animals roll,

whereas Srl-2 Unc Rol-3 animals do not. This suggests the possibility that cuticle morphology may be the primary influence on the Roller phenotype.

I can not exclude the chance that mutations in *dpy-18* enhance mutations in *rol-3*; however, *dpy-18(e364) ; rol-3(e754)* animals are no more severe Rollers than worms solely homozygous for *rol-3(e754)*. It is also possible that the Roller phenotype is indicative of an interaction between mutations in *srl-2* and *dpy-18*, indeed, I have observed *srl* and *dpy-18* mutations have a synergistic effect on male tail morphology (see results, section I) . It is unlikely that mutations in *dpy-18* alone are responsible for the Roller phenotype. While BAIRD and Emmons (1992) suggest that the *dpy-18* gene may produce a cuticle component, possibly a collagen, and mutations in some collagen genes lead to a Roller (KRAMER 1994), I have yet to observe homozygous *dpy-18* animals exhibit a Roller phenotype.

SECTION II

ALIGNMENT OF THE PHYSICAL AND GENETIC MAPS AT THE *ROL-3* LOCUS OF LGV AND THE *SRL-2* LOCUS OF LGIII

SECTION IIA

CORRELATION OF THE PHYSICAL AND GENETIC MAPS WITHIN THE *ETI(V)* BALANCED REGION DEFINED BY DEFICIENCY MAP ZONES 16 - 20

INTRODUCTION

Traditionally, the method used to examine the contents of the genome has revolved around the systematic disruption of coding elements, followed by a phenotypic examination which may assign a role, at least in a gross sense, to the wildtype gene product which has been disrupted. Such mutagenic dissections are instrumental in identifying genes based solely on the principle of association by necessity. In other words, if treatment with a mutagen results in a heritable change, or phenotype, which can be associated with the segregation of a discrete portion of a single linkage group, then it is thought that the new phenotype is a result of a gene mutation. This type of analysis can be very directed; aimed at identifying all or most of the genes involved in a specific biological process. BRENNER (1974) isolated a number of mutations which affect movement in *C. elegans*. Further characterization demonstrated that a number of these mutants disrupt muscle structure (WATERSTON, THOMSON and BRENNER 1980). This type of analysis is further illustrated in the identification of genes which influence coat color in mice (JACKSON 1994; BARSH 1996 and references therein). Although all coding elements in the genome are subject to the mutagen, disruptions in genes coding for muscle components are easily recovered since mutagenized worms harboring such mutations often generate paralyzed or motion compromised progeny. In *C. elegans*, screens for mutants with identifiable disruptions of specific structures or processes have been instrumental in the genetic analysis of a number of systems such as muscle formation (BRENNER 1974), vulval induction (reviewed in STERNBERG and HORVITZ 1993) and cuticle structure (COX *et al.* 1996).

Alternatively, the mutagenesis can be more generally applied, aimed at identifying all genes within a defined region of the genome which can be mutated to confer a lethal phenotype. This method has been applied to many regions of the *Drosophila melanogaster* genome including the *rosy* region on chromosome III (HILLIKER, CLARK and CHOVNICK 1980), and the *dorsal Bicaudal-D* region on the left end of chromosome II (STEWARD and NUSSLEIN-VOLHARD 1986). Mutagenesis of this sort has also

been extensively applied to *C. elegans* in attempts at saturating defined portions of the genome for mutations in essential genes. These include the *dpy-5* region (ROSE and BAILLIE, 1890, HOWELL *et al.* 1987, HOWELL 1989) and the *dpy-14* region (McKIM *et al.*, 1992) of LGI; the region balanced by the recombination suppressor *mnC1* of LGII (SIGURDSON *et al.*, 1984); the region near the *unc-22* gene on LGIV (ROGALSKI *et al.*, 1982; ROGALSKI and BAILLIE, 1985; CLARK *et al.*, 1988; CLARK, 1990; CLARK and BAILLIE, 1992; MARRA 1994); the region around the ama-1 gene of LGIV (ROGALSKI and RIDDLE, 1988); the region of LGV balanced by the reciprocal translocation *eT1* (ROSENBLUTH, CUDDEFORD and BAILLIE, 1983; 1985; ROSENBLUTH *et al.*, 1988; JOHNSEN, 1990; JOHNSEN and BAILLIE, 1991); portions of the X chromosome (MENEELY and HERMAN, 1979;1981); and recently the *dpy-17 - unc-36* region of LGIII (H. STEWART *unpublished results).*

Perusal of the most recent release of ACeDB (**A C. elegans Data Base**, R. DURBIN and J. THIERRY-MIEG *unpublished*) indicates that there are over 1,400 mutationally identified genes representing 150 gene categories. By far the largest class of identified genes is that of the essential genes (*let-* for lethal) whose members number 367. However, a number of genes from other classes which were identified by virtue of alleles with other phenotypic characteristics also have lethal alleles. Taking these into account, approximately 47% of all mutationally identified genes have lethal alleles suggesting that their gene products perform essential functions during the *C. elegans* life cycle.

Another, more recent, approach to the identification of genes in *C. elegans* is through the determination of the complete nucleotide sequence of the genome. The *Caenorhabditis elegans* genome project is directed by the *C. elegans* sequencing consortium led by Dr. J. Sulston at the Sanger center in Cambridge England; and Dr. R. Waterston at Washington University in St. Louis. The *C. elegans* genome is represented by the physical map which is largely composed of overlapping cosmid and YAC clones. Currently, over 52 Mb of the 100 Mb of sequence making up the *C. elegans* genome has

been completed to date, and the remainder is expected to be finished by 1998 (WILSON 1994; WATERSTON AND SULSTON 1995).

Both avenues of genome analysis will provide insight into the numbers, types and organization of genes within the *C. elegans* genome; however, on their own, each method has its limitations. The key to understanding the synthesis of a complex organism like *C. elegans* lies in determining the role of each individual gene product both at the molecular level, and within the context of *C. elegans* development. Perhaps the most direct route towards this understanding is through the correlation of sequence identified protein coding elements to known and well characterized genetic loci. The coupling of this independently obtained information will greatly increase our understanding of gene function within *Caenorhabditis elegans*, and ultimately, in all organisms.

Presently, at the current size of the public domain protein databases, approximately 47 % of all sequence identified coding elements from *C. elegans* code for protein products which display a significant match with previously identified proteins (WATERSTON AND SULSTON 1995 M. MARRA and S. JONES *personal communication*). Unfortunately, the function of the remaining 53 % cannot be inferred from sequence data alone. In these cases, it is desirable to correlate sequence identified coding elements to genes previously identified mutationally. Such correlations will provide information as to the *in vivo* consequences of disrupting a specific coding element. Conversely, a number of mutationally identified genes in *C. elegans* are known to be essential for development based on their knock-out phenotypes. However, stringent examination of phenotype leading to the identity of a specific cellular, developmental or physiological defect associated with many of the known essential genes is not a trivial task, and thus has not been forthcoming. Correlation of genetically identified loci with sequence identified genes will yield valuable information into the role, in *vivo*, of identified gene products, and may aid in understanding of their biological relevance. Furthermore, intimate knowledge of a gene sequence will facilitate more in depth molecular studies of gene function. Once the

sequence is known, molecular manipulations involving the construction and utilization of gene specific molecular probes, PCR based techniques and anti-sense techniques will become routine.

Correlation of the *C. elegans* genetic and physical maps has been accomplished by a variety of techniques. The most notable of these involve Southern blotting or PCR based technologies to map deficiency breakpoints to the physical map, transposable element insertion, and germline transformation experiments in which clones containing a small portions of the *C. elegans* physical map are used to repair the mutant phenotype associated with a genetically mapped mutation.

Southern blot based mapping technology, as applied to the correlation of the physical and genetic maps of *C. elegans*, involves probing genomic DNA isolated from both wildtype control animals and animals heterozygous for a deficiency, or other chromosomal rearrangement, with labeled portions of the *C. elegans* physical map (most often cosmids). In the case of a deficiency, blots prepared with quantitative amounts of wildtype control and deficiency heterozygote genomic DNA will exhibit identifiable differences in the intensity of signal generated for the target DNA if the labeled probe falls within the portion of the genome affected by the deficiency. If a set of cosmid clones lies outside the region spanned by a deficiency, then the signal intensity corresponding generated by equivalent amounts of wildtype control and deficiency heterozygote DNA will be equal. If the cosmids used as probes carry genomic DNA which falls within the limits of the deficiency, the intensity of signal generated at the deficiency heterozygote target will be approximately 1/2 that generated at the wildtype control target. This technique has been used recently determining the physical map position of *bli-4* (THAKER *et al.* 1995, PETERS 1992) and *lag-2* (TAX, YEARGERS and THOMAS 1994).

The Polymerase Chain Reaction (PCR) is another tool used to align the physical and genetic maps of *C. elegans*. Primer pairs derived from cosmids or YACs are used to

amplify DNA from deficiency homozygous nematodes as per the protocols described by BARSTEAD and WATERSTON (1991), and WILLIAMS *et al.* (1992). The presence of a PCR product indicates that the deficiency does not delete the DNA corresponding to the primer pair. If the deficiency in question deletes the DNA from which one or both of the primers are designed then no PCR product is detected. This method, like the southern blot based strategy discussed previously, allows the portions of the physical map falling within the breakpoints of a genetically defined deficiency to be determined. This technique was instrumental in the cloning of *unc-60* (McKIM *et al.* 1993); and in placing 9 anchor points between the LGV physical map and the genetic map within the *eT1* balanced region of LGV (WAKERCHUCK 1992).

A third method of anchoring the *C. elegans* genetic and physical maps involves the use of transposable genetic elements. Five transposable elements have been identified in *C. elegans*. These are Tc1 (EMMONS *et al.* 1983; LIAO, ROSENZWEIG, and HIRSH 1983; MOERMAN and WATERSTON 1984, MORI *et al.* 1988), Tc2 (LEVITT and EMMONS 1989), Tc3 (COLLINS *et al.* 1987), Tc4 (YAUN et al. 1991) and Tc5 (COLLINS and ANDERSON 1994). Transposable elements can disrupt coding regions thus providing a molecular tag which will anchor the physical and genetic maps at this locus. The element most often used for mutagenesis is Tc1 which is structurally reminiscent of the P elements in *Drosophila*, the Ac-Ds elements of maize and the bacterial IS elements (ROSENZWEIG *et al.* 1983, reviewed by HERMAN and SHAW 1987). Tc1 insertion has been used to aid in cloning *unc-22(IV)* (MOERMAN, BENIAN and WATERSTON 1986), *lin-12(III)* (GREENWALD 1985), and *bli-4(I)* (PETERS, McDOWALL and ROSE 1991). Alternatively, transposable elements can be used to generate Restriction Fragment Length Polymorphisms (RFLP) which can be recombinationaly mapped relative to genetically defined loci (BAILLIE, BECKENBACH and ROSE 1985).

Germline transformation is the process of generating heritable change through the introduction of exogenous DNA into an organism's germline. This technique has been applied to many organisms such as the zebrafish, *Brachydanio rerio* (STUART *et al.* 1990), mouse (PETERSON *et al.* 1993), and *C. elegans* (STINCHCOMB 1985; FIRE 1986, MELLO *et al.* 1991) Formation of *C. elegans* which express transgenes in a stable and heritable fashion was initially performed by injecting *C. elegans* DNA clones directly into oocytes (FIRE 1986). This technique has been used to elicit transgene based rescue of mutations in a number of *C. elegans* genes (CLARK and BAILLIE 1992; SPENCE, COULSON and HODGKIN 1990; FIRE and WATERSTON 1989; WAY and CHALFIE 1988) demonstrating that the injected clone carried a wildtype copy of the endogenous mutant gene and that this was regulated properly *in vivo*. Mello *et al.* (1990) revolutionized *C. elegans* germline transformation by developing a rapid and efficient injection technique as well as a dominant transformation marker. Currently, most microinjection is carried out by injecting DNA clones into the syncytial arm of the hermaphrodite gonad where they form large concatameric arrays, presumably through the process of homologous recombination (MELLO *et al. 1991*).

During oocyte maturation, these arrays are occasionally taken up into the oocyte nuclei. Upon fertilization, the array, now present in a diploid nucleus, is segregated into the daughter cells of each subsequent mitosis in an incomplete manner such that transgenic *C. elegans* are mosaic for the array (STICHCOMB 1985; WAY and CHALFIE 1988). If a transgenic *C. elegans* has an endogenous genetic mutation, and the array carried contains a wildtype copy of the mutant gene, then the phenotype associated with the genetic mutation may be "rescued" by the extrachromosomal array. In some cases, the progenitors of the germline carry arrays which are then segregated, in a non-Mendelian fashion, into the gametes. Transformed strains which segregate transformed progeny are said to be stable for the array in question. In such strains, the array is passed onto progeny in a fashion similar to that observed for free duplications (HERMAN 1984). Therefore, a

single array can be tested for rescue against any number of mutations using genetic manipulations no more complex or time consuming then those required to perform a complementation cross. As such, the injection process has only to be performed until a stable transgenic is produced rather than be repeated for each mutation. For this reason, transgenic arrays can be used to break up clusters of genes which map to a similar interval on the genetic map, but have not been right - left positioned. Analysis of this type not only refines the position of genes on the genetic map, but also provides an anchor to the physical map at each loci rescued. Recent use of transgenic arrays as mapping tools has resolved the position of seven essential genes in the *bli-4* region of LGI (McKAY 1993), 13 essential genes in the *dpy-5* - *unc-13* region of LGI, (McDOWELL 1996).and has further resolved the physical map in the *dpy-20* - *unc-22* region of LGIV (Schein 1994)

At the time this work was started the closest genetic map loci to *rol-3* that had been anchored to the physical map were *mec-1* and *her-1*. Both of these loci flank *rol-3*, with *mec-1* mapping approximately 1 mu. to the left, and *her-1* mapping approximately 1 mu. to the right. The physical distance between these two loci can be estimated using the assumptions and methods of BARNES *et al.* (1995), and is assessed at 2.1 Mb of DNA. Based on the data of the first 2.2 Mb of contiguous *C. elegans* genomic sequence from the central cluster of LGIII (WILSON *et al.* 1994) a region of this physical size could potentially contain upwards of 420 genes. It is obvious that a blind search for a single gene coding for a product of formally unknown function within a potentially gene dense region of this magnitude would likely be profitless.

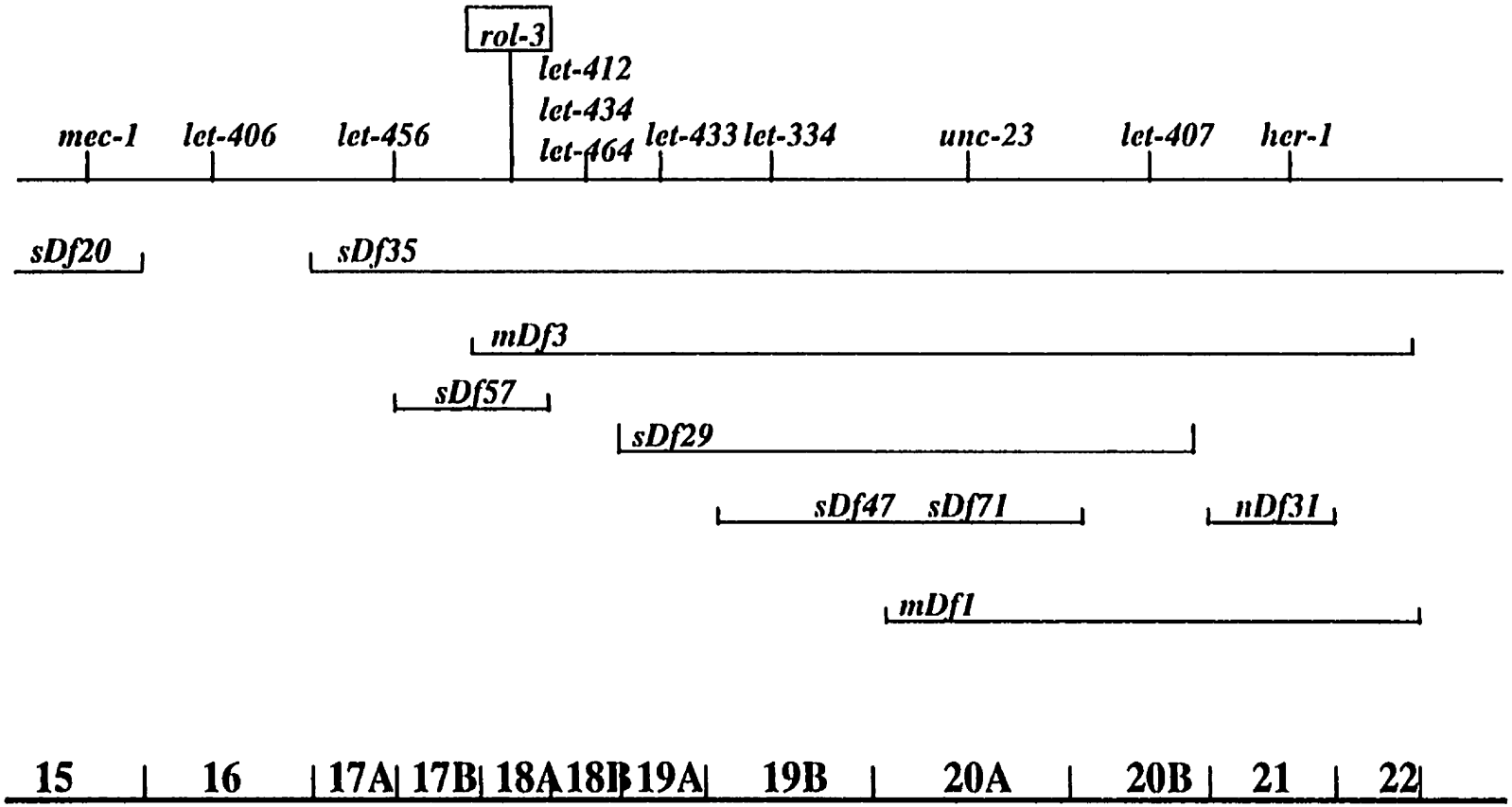
In this section I describe the molecular localization of *rol-3(V)* and *srl-2(III)* through correlation of the physical and genetic maps. As previously mentioned, the *eT1* balanced region of LGV has been the site of rigorous investigation in the attempt to saturate this region for essential mutations (ROSENBLUTH, CUDDEFORD and BAILLIE, 1983; 1985; ROSENBLUTH *et al.*, 1988; JOHNSEN, 1990; JOHNSEN and

Figure 16

Genetic Map of the *mec-1* to *her-1* Region of LGV

Partial genetic map of LGV illustrating the positions of deficiency breakpoints and the zones they define. A representative locus from each zone is displayed. For clarity, most of the essential genes mapping into these zones are not shown.

Genetic Map of *mec-1* to *her-1* Region of LGV (Zones 15 - 22)



BAILLIE, 1991). This work has produced a comprehensive genetic map of the region, which has been subdivided into 23 major zones on the basis of rearrangement breakpoints. The *rol-3* locus resides in zone 18A (Figure 16). To aid in locating *rol-3* on the *C. elegans* physical map, I attempted to correlate the physical and genetic maps about the *rol-3* locus. Initially, I used PCR in an attempt to place deficiency breakpoints defining the *rol-3* region to the physical map. I have aligned these two maps at eight distinctive sites which anchor zones 18, 19 and 20 to the physical map. Alignment of the physical and genetic maps by PCR enabled me to identify cosmid and YAC clones representing genomic DNA from the *rol-3* region. Using a transgenic approach I have attempted to rescue mutations in *rol-3*, as well as mutations in essential genes immediately surrounding the *rol-3* locus. This work has defined a limiting interval in which *rol-3* is expected to reside.

MATERIALS AND METHODS

Mutations:

The wildtype *C. elegans* (var. Bristol) N2 strain and strains carrying the following mutations were obtained from the MRC, Cambridge, England, from the *Caenorhabditis* Genetics Center at the University of Missouri, Columbia Missouri, or as cited:

LGIII *dpy-17(e502), dpy-18(e364), sma-3(e491), unc-32(e189), unc-36(e251)*

The LGIII deficiencies *sDf121, sDf125, sDf127,* and *sDf135* were provided by H. Stewart

LGIII duplications *sDp3 (III;f)* (ROSENBLUTH, CUDDEFORD and BAILLIE 1985) and *sDp8 (I;X)* (Stewart, Rosenbluth and Baillie 1991)

LGV *unc-46(e177), rol-3(s1040*ts, and *s1519)* (JOHNSEN and BAILLIE 1991); *unc-42(e270), let-433(s950)(H. STEWART unpublished result*s), *let-434(s1904) (H. STEWART unpublished result*s).

Mutations in the following LGV essential genes *(let-)* are described in JOHNSEN and BAILLIE (1991)*: let-335(s908), let-405(s388), let-406(s514), let-411(s1553), let-423(s1550), let-449(s1343), let-474(s1577), let-408(s195), let-413(s1451), let-414(s207), let-424(s384), let-436(s1403), let-445(s1419), let-456(s1479), rol-3(s422), let-412(s579), let-434(s1904), let-464(s1530), let-433(s950), let-340(s1622), let-409(s1480).*

LGV deficiencies *sDf29, sDf47, sDf71* (JOHNSEN and BAILLIE 1991)

mDf-1 and mDf3 (BROWN 1984) were received from D. RIDDLE

The reciprocal translocation *eT1 (III ;V)* (ROSENBLUTH and BAILLIE 1981) was described in section 1 (MATERIALS and METHODS).

Extrachromosomal arrays constructed from LGIII genomic clones*: sEx32, sEx67, sEx69, sEx73, sEx78, sEx93, sEx163,* and *sEx238* are from the Cosmid Transgenic Library

constructed in the laboratory of D. L. Baillie (D. JANKE *manuscript in preparation*). A *C. elegans* strain carrying the extrachromosomal array *upEx1* was obtained from E. Bucher All mutations and extrachromosomal arrays denoted with the s prefix arose in Dr. Baillie's laboratory.

Origin of Cosmid and YAC Clones:

Cosmid and YAC clones were obtained from A.R.Coulson and J. E. Sulston (COULSON *et al.* 1986) at the MRC, England. Purified cosmid DNA for the following clones was obtained from C. Mello and J. Priess: F58G4, F18B11, ZK427, F35E10, T24H5, ZK307. Purified cosmid DNA for the following clones was provided by Robert Waterston at the St. Louis Genome Sequencing Center: C08D8, F38E1, F52E1, K11D5. T23B12, ZK994, F45F2, F25G6.

Agarose gel electrophoresis:

DNA was electrophoresed through 0.8 - 1.0% (w:v) agarose gels containing 0.5 µg/ml ethidium bromide. Gels were prepared in 1 X TAE (Sambrook *et al.* 1989) using 1 X TAE as electrophoresis buffer. Gels were run at 1 - 5 V/cm as recommended by Sambrook *et al.* 1989.

Polymerase chain reaction (PCR) techniques:

Treatment of either whole worms or eggs for use as a source of template DNA was performed as described by BARSTEAD and WATERSTON (1991) with the

modification of WILLIAMS *et al.* 1992). Generally, 1-2 worms or 1-3 eggs were used per 25 µl reaction. Eggs to be used were treated with (20 mg/ml) chitenase solution (1-2 µl/egg) and were picked and placed into 500 ul Eppendorf tubes containing 2.5 µl of worm lysis buffer (WILLIAMS et al.1992), and frozen at -70 °C for 15 minutes. Tubes were then transferred to 65 °C for one hour followed by exposure to 95 °C for a further 15 minutes. Treatment of worms follows that described for eggs except that chitenase was omitted so that worms were picked directly into lysis buffer. Thermal cycling was accomplished on either an Idaho Technologies convection thermocycler model 1601 or a GTC-1 thermal cycler.

Experimental parameters for amplification using the Idaho technologies thermal cycler:

PCR was performed on the lysate following a whole worm (or egg) protocol of WILLIAMS *et al.* (1991). PCR amplifications were carried out in 25 µl volumes. The following reagents were added to each worm (or egg) lysate. 2.5 ul of 10X Idaho reaction buffer (50 mM Tris/HCL pH 8.3, 250 ug/ml BSA, 0.5% Ficoll, 1mM Tartrazine, 2 mM $MgCl_2$), 2.5 ul of deoxynucleoside triphosphates mixture (contains 25 µM each dNTP [Pharmacia]), 20 pMol (unless noted) of each member of a primer pair (usually two experimental primers, forward and reverse and two control primers forward and reverse), 0.625 units of *Taq* DNA polymerase (Promega) and H_20 to 25µl. Reactions were transferred to thin wall glass capillary tubes, sealed and placed directly into the thermal cycler. Unless noted otherwise, the following cycle conditions were used: reactions were

heated to 94 °C for 1 minute, followed by 30 cycles of [94 °C for 10 seconds, 20 seconds at the appropriate annealing temperature (see Table 9), 40 seconds at 72 °C] and followed by 2 minutes at 72 °C.

Experimental parameters for using the GTC-1 thermal cycler:

Each reaction volume was 25 µl. To the suspension of worms or eggs in lysis buffer the following reagents were added: 2.5 µl of 10X taq buffer (Promega) , 2.5 ul of 25 mM $MgCl_2$. 2.5 µl primer mixture (contains 25 µM each dNTP), 20 pMol (unless noted) of each primer used (usually two experimental primers, forward and reverse and two control primers forward and reverse), 0.625 units of Taq DNA polymerase (Promega) and H_20 to 25 µl. Unless noted otherwise, the reactions were heated to 94 °C for 1 minute, followed by 30 cycles of [94 °C for 1 minute, 1 minute at the appropriate annealing temperature, 1 minute at 72 °C] and followed by 2 minutes at 72 °C.

Preparation of cosmid DNA for Germline Transformation:

Cosmid DNA was prepared using the large scale alkaline lysis method as described by Sambrook *et al.* (1989). DNA was prepared from 500 ml LB liquid cultures containing 50 ug/ml ampicillin (for pJB8 cosmids clones) or 70 ug/ml kanamycin (for Lorist cosmid clones) incubated overnight at in a shaking incubator at 37 ^{0}C and 250 r.p.m. Cosmid DNA was purified on $CsCl_2$ gradients (SAMBROOK *et al.* 1989). The gradients were spun for 24 hours at 53,000 r.p.m using a Beckman L8-80 ultracentrifuge and Ti-70.1 rotor. Cosmid DNA bands were collected from centrifuge tubes and the ethidium bromide was removed by several extractions with water saturated butanol.

Table 9

Oligonucleotide primers used in this study

primer	primer sequence	product size	annealing temp (° C)	[primer] (pmol/rxn)	control primers used	target	ref
R5 R11	TGA TGA TGG ATT GGC TCG GC TAC TCG CAT CTT TAC CAT CG	380 bp	54-59	20	N\A	*hdl-1*	1
DC1 DC2	GCG GGG TTG CCT TAC TGG GTT ATG TGA CCG ATG AGC	618 bp	59	10	R5, R11	pJB8	2
Krp17 Krp18	CGT CCG GCG CAC AGA AGC GTG CTG AGC CCG GCC AAA	880 bp	59	20	R5, R11	Lorist 6	3
179 180	TGA TAC TTC CCT TTT TCG CAT TAC ACG GAG AAG ACG	380 bp	54	20	MAM2, DB19	cm7b4	-
B03f B03r	GCG GGC TCA CAA CCT GCT ATG GCG TCG CGC AGA AGA	699 bp	54	20	N/A	B0361	4
C07f C07r	GGG ATG TGT GCG GTT GAC ATA AGG CTG CGG CTG TGA	800 bp	57	20	N/A	C07G2	4
K08-3 K08-7	TCA AAA CGC ATC TAA ACT GG GCC ACA CTA AAA CAG GAT TC	900 bp	56-59	10	N/A	*unc-60*	6
T20f T20r	ATT CGG TGG GTG TGG TTG CTT TTG CTG CGT CGG TTA	353 bp	57	10	N/A	T20B12	4
yk49f yk49r	GCC AAT TCT TGA GAA TGT CAG CGG AGG CCA GAA CCC TTC TCC TCA CAA TGT ACC GG	284 bp	57	15	C07f & r	yk10a9.5	5
k100f yk100r	GGG AAA AGT GAA GAG GAA TAA CCG CG CCA ACA TGT CCT GTT GGC CCT CGA TC	220 bp	57	15	C07f & r	yk19h11.3	5

Table 9 continued

Oligonucleotide primers used in this study

yk647f yk647r	GCG ATT GGG CGA ACT GGT AAC CAC AG CCG TCG AGC CAA GCG ACA ACC ATC GA	216 bp	57	15	B03f & r	yk11d5.5	5
yk967f yk967r	GGA AGC CGA GGA GAA TTT CAG TGC CTT GTC CCT CGC TGA GCA TTG GTG G	200 bp	57	15	C07f & r	yk26a4.5	5
C08Df C08Dr	CCC ACT TCC TTC CAG ACC CGC GTT GCC TAC GAC ACA	350 bp	56[a]	20	K08f & r@5pMol/rxn	C08D8	-
W02f W02r	TGC GAA GGT TGC CGT TAC TTT TCG CTG CGC CTA TCA	500 bp	57	20	K08	W02D7	-
MAM1 DB19	CAT CCA AGT GCC AAA CCG GCT ATG ATG GAG GAA AGT G	550 bp	53	20	N/A	*nhe-1*	1
F21f F21r	ACG AAA GAA CGC AAG CAA CTC ACC CAC CCA CGC CGT	903 bp	57[b]	20	T20	F21F8	-

Reference 1 is MARRA, PRASAD and BAILLIE 1993. Reference 2 is D. JANKE personal communication, Reference 3 is McKAY 1993. Reference 4 is N. FRANZ, personal communication. Reference 5 is I. GREENWALD, personal communication. Reference 6 is K.S. McKim , personal communication.

CsCl was removed by precipitating the DNA in four volumes of water and eight volumes of 95% ethanol. Precipitated DNA was dissolved in 500 ul TE buffer (SAMBROOK *et al.* 1989). Quantification of the purified DNA was performed by measuring the absorbance of the DNA solution at 260 nm using an Ultraspec III UV/visible spectrophotometer (Pharmacia).

Germline Transformation:

The method of germline transformation was adapted from that of MELLO *et al.* (1991). This involves the injection of a DNA solution in to the large syncytium of one or both of the hermaphrodite gonad arms. The injection solution contained approximately 100 ng/ul of DNA in TE buffer. This concentration of DNA was considered optimal for the heritable formation of extrachromosomal arrays (MELLO *et al.* 1991). To facilitate identification of extrachromosomal array bearing animals, a plasmid carrying *rol-6(su1006)* (KRAMER *et al.* 1990), a dominant form of the *C. elegans rol-6 (IV)* gene, was included in the injection mix. The *rol-6* bearing plasmid was pCes1943, a derivative of pRF4 (KRAMER *et al.* 1990). Extrachromosomal arrays are composed of tandem copies of cosmid and *rol-6(su1006)* bearing plasmid, thought to have arisen by homologous recombination (MELLO *et al.* 1991). It is likely that homologous recombination is effected through the beta-lactamase genes contained within pJB8 cosmids (ISH-HOROWICZ and BURKE 1981) and pRF4 (KRAMER *et al.* 1990). To promote homologous recombination between pRF4 and kanamycin resistance gene containing Lorist vectors (CROSS and LITTLE 1986), I constructed pCes1943, which

contains a kanamycin cassette (Genblock™-Pharmacia) inserted next to *rol-6(su1006)* within the EcoR1 site of the Bluescribe polylinker of pRF4. In general, the relative concentration of the DNAs within the injection solution was 20 : 1 pCes1943 : cosmid DNA. Transgenic arrays constructed during the course of this study are listed in Table 10.

Determination of Extrachromosomal array fidelity using whole worm Polymerase Chain Reaction (PCR):

Incorporation of the Lorist and pJB8 cosmid clones into the extrachromosomal array in transgenic worms was verified by PCR analysis. Template from one or two Rol-6 worms from each transgenic strain was prepared and multiplex PCR reactions were carried out in an Idaho Technology Thermal-Cycler (as mentioned in Polymerase chain reaction (PCR) techniques). R5 and R11 were the control primers used for both the pJB8 and Lorist reactions (Table 9). The primers specific for the pJB8 vector are called DC1 and DC2 and amplify a 618 bp product, while those for the Lorist vectors are Krp17 and Krp18, and amplify a 880 bp product. Krp17 and Krp18 were a gift from A. M. Rose.

Table 10.

Construction of transgenic strains carrying extrachromosomal arrays.

Cosmid Clone Injected	[Cosmid] ng/ul	[pCes1943] ng/ul	Array Name
F58G4	5	93.5	*sEx60*
F18B11 ZK427 F38E10	5 5 5	80	*sEx40*
W03E11	10	100	*sEx104*
T24H5	2.3	100	*sEx103*
B0340	2.5	93.5	*sEx101*
ZK307	5	100	*sEx155*
W02D7	5	100	*sEx601*
C08D8	2.8	100	*sEx602*
C08D8 F38E1	2.5 2.5	100	*sEx603*
F38E1	5	100	*sEx604*
F38E1 F52E1	2.5 2.5	100	*sEx605*
F52E1 K11D5	2.5 2.5	100	*sEx606*
K11D5	5	100	*sEx607*
K11D5 T23B12	2.5	100	*sEx609*
T23B12	5	100	*sEx608*

T23B12 ZK994	2.5 2.5	100	*sEx610*
ZK994	5	100	*sEx611*
ZK994 F45F2	2.5 2.5	100	*sEx612*
F45F2	5	100	*sEx613*
F25G6	5	100	*sEx614*

Complementation testing of LGV lethal mutations with extrachromosomal arrays :

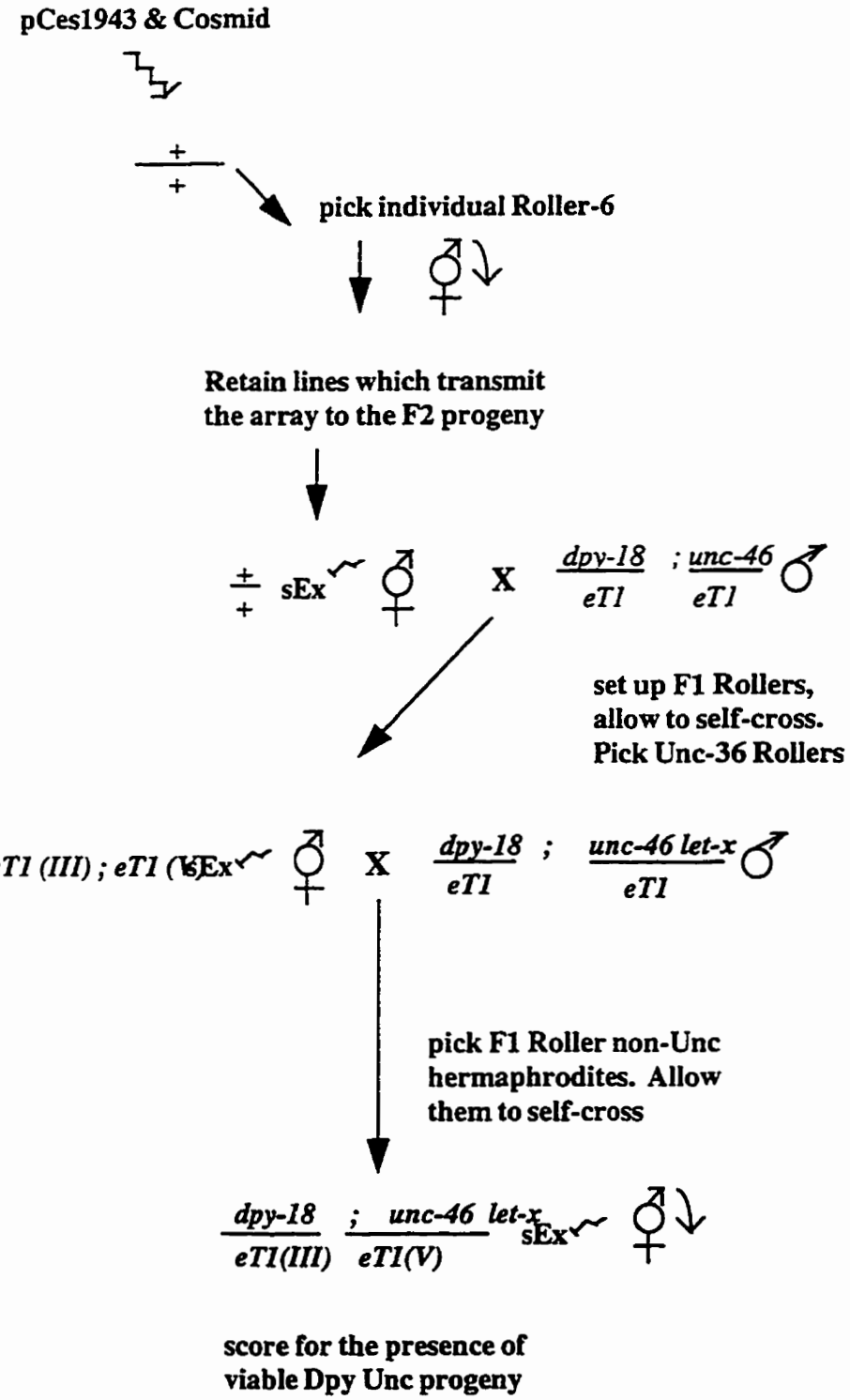
Strains carrying cosmid or YAC clones in stably inherited extrachromosomal arrays were used as duplication bearing strains in complementation tests with lethal mutations neighboring *rol-3* (V). Most arrays were produced via co-injection of clones and pCes1943 into N2 hermaphrodites. In order to facilitate complementation testing of LGV lethal mutations against cosmid bearing arrays, extrachromosomal arrays were moved into a homozygous *eT1* background. This was achieved by mating transgenic hermaphrodites to BC1265 *dpy-18(e364)III/eT1(V) ; unc-46(e177)V/eT1(III)* males. F1 Roller-6 hermaphrodite progeny were picked, set up individually on plates and allowed to self-cross. Those F1s which segregated wildtype, Dpy, Unc-46 and DpyUnc-46 F2s were discarded, while those which segregated wildtypes and *eT1* homozygotes (Unc-36) were retained. Unc-36 Roller F2s were isolated from these plates and used to establish stocks in which the extrachromosomal arrays are maintained in a homozygous *eT1* background (see Figure 17).

Figure 17

Protocol for Germline Transformation Rescue

Scheme outlining the protocol followed for the generation and genetic manipulation of extrachromasomal arrays. Any F1 Roller-6 progeny from the injected PO animal were isolated and allowed to self fertilize. An array was considered stable if Roller animals segregated in the F2 and subsequent generations. All arrays used were moved into a homozygous eT1 background prior to being assayed for ability to rescue LGV essential genes.

Scheme for Germline Transformation Rescue of LGV Essential Genes



These Unc-36 rollers, genotypically *eT1/eT1 sExn*, were then mated to lethal bearing males of the genotype *dpy-18(e364)/eT1(V) ; unc-46(e177) let-x/eT1(III)*. Typically, 5 F1 wildtype Roller-6 rollers were individually picked, set up on plates, and allowed to self-cross. The F1 adults were transferred to new plates every 24 hours. The F2 progeny were scored for the presence of fertile DpyUnc animals. Since *eT1* suppresses recombination in LGIII (right) and LGV (left), DpyUnc animals must be homozygous for the lethal mutation.

Arrays produced by the co-injection of the cosmids C08D8 + F38E1, or T23B12 + ZK994, or with W02D7 singly were formed by direct injection into hermaphrodites of the genotype *dpy-18(e364)/eT1(III) ; unc-46(e177) rol-3(s1519)/eT1(V)*. In these cases, F1 progeny of the P0s were scored for the presence of DpyUnc Roller-6 animals (indicative of rescue). Extrachromosomal array bearing strains carrying these arrays were established by maintaining lines derived from F1 Unc-36 Rollers.

Three-factor mapping of *srl-2*(s2507)

Three-factor mapping of *srl-2* was carried out by isolating animals in which recombination events between *srl-2* and flanking markers had occurred, and assaying for the presence of *srl-2* by virtue of it's suppression effect in a *rol-3* lethal background. Hermaphrodites of the genotype *srl-2*(s2507) ; *unc-46(e177) rol-3(s1040*ts) were mated to *dpy-18(e364) ; eT1(III) ; unc-46(e177) rol-3(s1040*ts*)/eT1(V)* males, and F1 males of the genotype *srl-2(s2507)/eT1(III)* ; *unc-46(e177) rol-3(s1040*ts*)/eT1*(V). These males were mated to hermaphrodite strains doubly homozygous for either *dpy-17(e502)and unc-*

36(e251) or *sma-3(e491) and unc-36(e251).* The progeny produced in this cross were either *unc-36(e251)/eT1(III) ; +/eT1(V),* which display an Unc-36 phenotype and discarded, or phenotypically wildtype worms of the genotype *srl-2(s2507)/M unc-36 ; unc46 rol-3(s1040*ts)/+ + where M is either *dpy-17* or *sma-3.* Approximately 50 such Wildtype hermaphrodites were set up individually and brooded twice at 24 hour periods. The F3 progeny were scored for the presence of Sma or Dpy recombinant animals depending on which marker (*dpy-17* or *sma-3)* was being tested. Recombinants picked up have one of two possible phenotypes. They are either Sma Unc-46 (Dpy Uncs if *dpy-17* is used as a marker) or Sma non-Uncs (Dpy non-Uncs if *dpy-17* used as a marker. Those recombinants which were non Unc-46 were set up, allowed to self cross and the progeny observed. If Sma Unc-46 (DpyUnc-46) animals were among the progeny then *srl-2*(s2507) was thought to be picked up by the recombinant chromosome. If no viable Sma Unc-46 animals were present in the progeny, but dying larvae were present, then *srl-2*(s2507) was thought to be excluded from the recombinant chromosome. If no dying larvae were present, then the recombinant failed to pick-up the *rol-3(s1040*ts) marked chromosome and the presence or absence of *srl-2*(s2507) could not be assayed. Since some recombination between *unc-46* and rol-3 is expected to occur, a small amount of both classes of LGIII recombinants (*sma-3 srl-2 +/sma-3 + unc-36* or *sma-3 + +/sma-3 + unc-36*) will segregate with LGV recombinant chromosomes of the genotype + *rol-3/+ +* or *unc-46 +/+ +*). With respect to these rare recombinant events, LGIII recombinants will receive either of the LGV recombinant chromosomes with equal frequencies. In the case of *unc-46 +/ + +* , SmaUnc-46s will be observed regardless of

whether *srl-2* was picked up in the recombination event. In the case of rol-3 + /+ +, no SmaUnc-46s will be observed, but dying larvae will be, so the assumption that *srl-2* was excluded during the recombination will be made. If p= the frequency of recombination between *sma-3* and *unc-36*, and q= the frequency of recombination between *unc-46* and *rol-3*, the number of Sma F1s which are heterozygous for a chromosome which has undergone recombination between *unc-46* and *rol-3* such that rol-3 is lost can be determined from the formula: $T\{[p-3/4(p)^2][(p/2 - p^2/4)]\}$ where T is the total number of worms scored. Recombinants of this type are falsely scored as *sma srl-2 +/sma + unc-36* Therefore, the number of observed Sma Srl-2 recombinants must be reduced by this amount. The number of Sma F1s which are heterozygous for a chromosome which has undergone recombination between *unc-46* and *rol-3* such that *unc-46* is lost can be determined by the formula: $T\{[p-3/4(p)^2][1/2(q-q^2)]\}$. where T is the total number of worms scored. Recombinants of this type are falsely scored as *sma + +/sma + unc-36.* Therefore, the observed number of Sma non Srl-2 recombinants must be reduced by the value calculated.

Mapping *srl-2* against the duplication *sDp8 (III ; I).*

sDp8 (III ;I) (STEWART, ROSENBLUTH and BAILLIE 1991) is a duplication of a portion of the central cluster of LGIII which is linked to LGI. The minimum region reiterated in *sDp8* spans from *sma-3* to *dpy-19*. The strain BC4272 *sDp8(III;I) eT1(III) ; eT1(V)* was used to determine if *srl-2* is covered by *sDp8*. Since Unc-36 is covered by sDp8, BC4272 has a wildtype phenotype. BC4272 males were mated to hermaphrodites

of the genotype srl-2(s2507) ; unc-46(e177) *rol-3(s1040*ts). F1 hermaphrodites of the genotype sDp8(III ; I) ; *srl-2*(s2507)/*eT1*(III) ; unc-46(e177) *rol-3(s1040*ts)/*eT1*(V) were set up individually and allowed to self-cross. If *sDp8* does not cover the *srl-2* locus, Unc-36 and Unc-46 Roller progeny are produced with equal frequencies. If *sDp8* covers the *srl-2* locus, Unc-46 Rol-3 progeny are present at one half the frequency of Unc-36 progeny.

Deficiency mapping of *srl-2*(s2507)

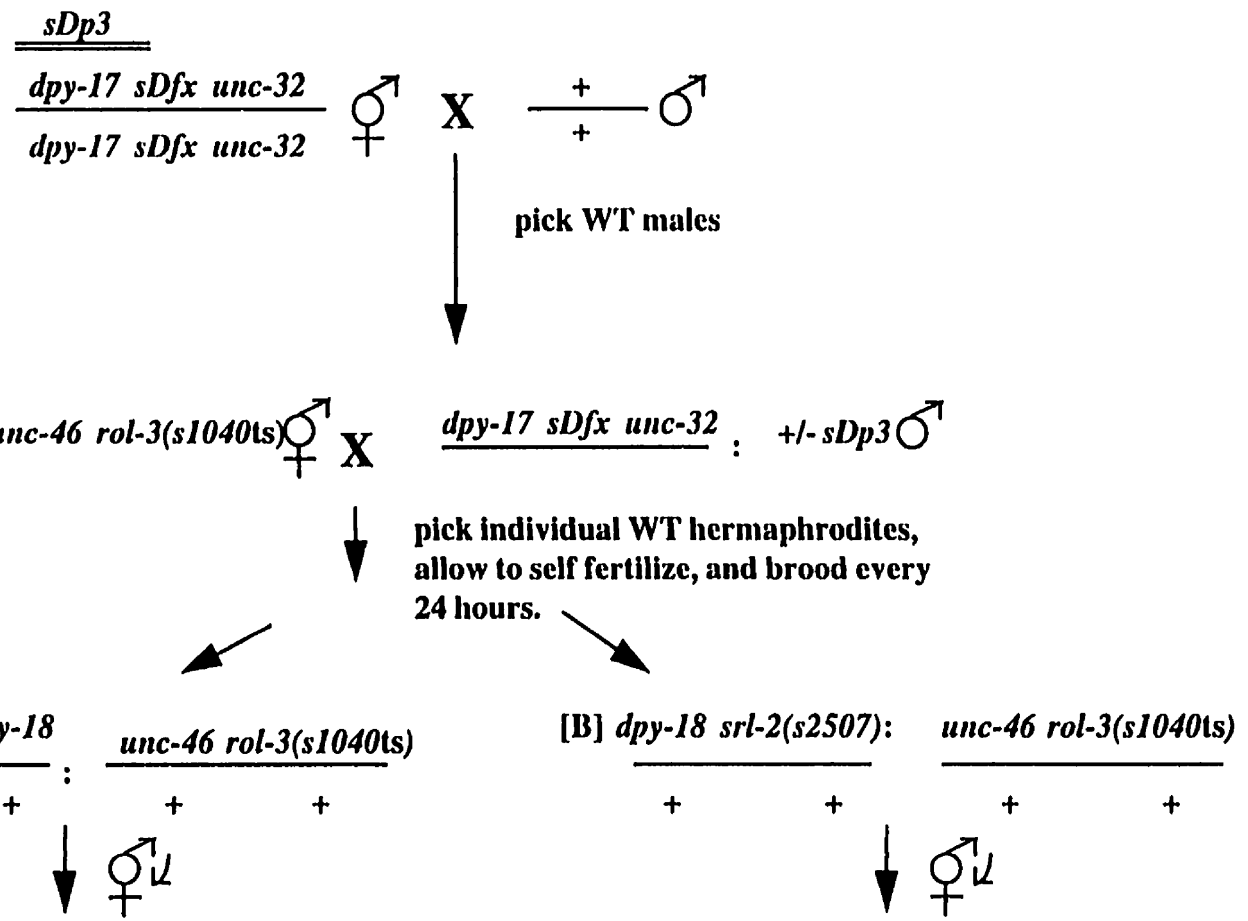
The Chromosome III deficiencies used to map *srl-2* were induced by exposing worms to ultraviolet radiation (UV) by H. STEWART (*unpublished data*). Homozygous deficiency strains are maintained in the presence of the large free duplication *sDp3 (III; f)* (ROSENBLUTH, CUDDEFORD and BAILLIE 1985). The deficiency strains used were of the genotype: *sDp3*(III;f) ; *dpy-17(e164) sDfx unc-36(e251) (III).* Strains carrying *sDf121, sDf125, sDf127* and *sDf135* were tested. *sDp3* does not cover the *unc-32* locus so each of the *Df* strains used exhibit Unc-32 phenotypes. Success of the deficiency mapping relies on the assumption that most of the mutations at *srl-2* represent gene knock-out events. Therefore, *srl-2(s2507) / Df* should confer viability to an animal homozygous for *rol-3(s1040*ts). The protocols used for the deficiency mapping of *srl-2* are diagrammed in Figure 18. Deficiency mapping of *srl-2* was initiated by outcrossing hermaphrodites of the genotype *sDp3(III;f) dpy-17(e164) sDfx unc-36(e251) III* to wildtype males. F1 males were isolated and mated to *srl-2*(s2507) dpy-18(e364) ; unc-46(e177) *rol-3(s1040)* animals. Approximately 20 wildtype F2 hermaphrodites were set up individually, allowed to self cross and transferred to fresh plates at 24 hour intervals.

Figure 18

Scheme for Deficiency Mapping srl-2(s2507).

Homozygous deficiency bearing strains were outcrossed in order to generate males which were subsequently mated to srl-2(s2507) dpy-18 ; unc-46 rol-3(s1040ts) hermaphrodites. Phenotypically wildtype F1 hermaphrodites are brooded at 24 hour intervals, and the self-cross progeny scored for the presence of Srl Unc-46 Rol-3 animals. A high frequency of occurrence (1 : 6) was indicative of failure to complement between srl-2 and the sDf being tested.

Mapping of *srl-2(s2507)* Relative to *sDf121, sDf125, sDf127* and *sDf135*



The F2 hermaphrodites were either of the genotype *dpy-18 srl-2/dpy-17 sDfx unc-32 ; unc-46 rol-3(s1040ts) /+ + or dpy-18 srl-2/+ + ; unc--46 rol-3(s1040ts)/+ +*, and each of these animals may or may not be carrying *sDp3* as well. Any F2 individual which segregates Unc32s among it's F3 progeny was assumed to carry *sDp3*, and discarded. The F3 progeny of the remaining F2 hermaphrodites were scored for Unc-46 individuals. If the F2 set up was of the genotype *srl-2 dpy-18/+ +, unc-46 rol-3(s1040ts)/+ +* the F3 animals were expected to display Wildtype, Dpy and DpyUnc-46 phenotypes in 36:18:1 proportions. Unc-46 non-Dpy individuals would occur rarely as a result of recombination between *unc-46* and *rol-3*. If the F3 animal set up was of the genotype *srl-2(s2507) dpy-18/dpy-17 sDfx unc-32 ; unc-46 rol-3(s1040ts)/+ +*, and the *srl-2* locus was deleted by *sDfx* then I expected to see Wildtype:Dpy:DpyUnc-46:Unc-46 non-Dpy animals at a frequency of 6:3:2:1. In this case, the number of Unc-46 non-Dpy animals is substantial: approximately 1/6 of the total animals should represent this class. If the deficiency fails to delete *srl-2* locus, only rare Unc-46 non-Dpy F3s will be observed. Wildtype, Dpy and DpyUnc-46 animals are represented in a 6:3:1 ratio respectively. Since the aforementioned Wildtype F2s set up are present in equal frequencies, approximately one half of all the F2s set up and progeny tested should carry the deficiency being tested.

Complementation testing of *srl-2(s2507)* with extrachromosomal arrays.

Transformation rescue of *srl-2* was accomplished using previously constructed transgenic arrays (D. Janke and J. Schein, *personal communication*) which were present

extrachromosomaly in otherwise genotypically wildtype strains. These strains are members of the *C. elegans* LGIII transgenic cosmid library and carry cosmid clones representing portions of the LGIII physical map. The arrays and the clones with which they were constructed are listed in Table 15 (see results, this section). The transgenic library strains used were constructed in an N2 hermaphrodite backgrounds and thus were of the genotype *+/+ ; sEx*. BC1265 *dpy-18(e364)/eT1(III) ; unc-46(e177)/eT1(V)* males were mated to hermaphrodites of each of the transgenic library strains used. F1 Roller-6 hermaphrodite progeny were picked, set up individually on plates and allowed to self fertilize. Those F1s which segregated Wildtype, Dpy, Unc-46 and Dpy Unc-46 F2s were discarded while those which segregated Wildtypes and *eT1* homozygotes (Unc-36) were retained. Unc-36Rol-6 F2s (genotypically *eT1*(III) ; *eT1*(V) ; sEx) were isolated from these plates and used to establish stocks which were subsequently used to introduce the arrays into an *srl-2 rol-3(s1040*ts) background. Hermaphrodites of the genotype *srl-2(s2507) ; unc-46 rol-3(s1040*ts) were mated to BC4015 *dpy-18(e364)/eT1(III) ; unc-46 rol-3(s1040ts)/eT1(V)* males and F1 wildtype males of the genotype *srl-2(s2507)/eT1(III) ; unc-46 rol-3(s1040ts)/eT1(V)* were isolated. These males were subsequently mated to the Unc-36 Rol-6 hermaphrodites constructed as detailed above. Individual F2 Roller-6 hermaphrodites were isolated and allowed to self-cross. The F3 progeny was scored for the presence or absence of Unc-46 Rol-6 progeny. If the cosmid carried in the transgenic array does not carry a wildtype *srl-2* gene then Unc-36 Rol-6 and Unc-46 Rol-6 are expected to be equally represented in the F3 progeny. If the array carries a wildtype copy of the *srl-2* gene then transgenic worms homozygous for

rol-3(s1040ts) should arrest development since the extrachromosomal copies of *srl-2*$^{+}$ should abolish the homozygous *srl-2(s2507)* phenotype. Therefore, if an array being tested "rescues" *srl-2*, the number of expected Unc-46 Rol-6 progeny is reduced to zero.

RESULTS

PCR mediated alignment of the genetic and physical maps within the *rol-3* region.

Prior to this study, there was very little correlation between the genetic and physical maps of LGV (left). A *mec-1* associated Tc1 polymorphism was known, and the molecular location of *her-1* had been determined (PERRY *et al.* 1993). However, *rol-3* and 30 other mutationally identified genes (JOHNSEN AND BAILLIE 1991, see Figure 16) had been mapped to the interval between *mec-1* and *her-1*. Previous studies focusing on the correlation of the genetic and physical maps within the *dpy-20* - *unc-22* region of LGIV (CLARK 1992, JONES 1994, MARRA 1994 AND SCHEIN 1994) and at the *dpy-14* of LGI (McKAY 1993), demonstrated an average density of one mutagenically identified essential gene per cosmid clone in gene cluster regions. As a rough estimate, I extrapolated these findings to the *rol-3* region. As seen in Figure 1 there are 15 essential genes to the left of *rol-3*, and 13 essential genes to the right. Therefore, if a similar metric as seen for these discrete regions of LGI and IV applied to zones 16-21 of LGV, *rol-3* would lie roughly in the center of the portion of the physical map bounded by *mec-1* and *her-1*.

According to ACeDB (R. DURBAN and J. THEIERY-MIEG *unpublished*), the cDNA cm7b4 (WATERSTON *et al.* 1992) maps to the YAC clone Y5C10. The sequence of cm7b4 is also available from ACeDB, and I took advantage of this in order to place cm7b4 onto the genetic map. I utilized a PCR based strategy (BARSTEAD and WATERSTON 1991, WILLIAMS *et al.* 1992) to map the physical location of cm7b4 (and therefore YAC Y5C10) in relation to the breakpoints of the deficiencies *mDf1*, *mDf3*, *sDf29*, *sDf47*, *sDf57* and *sDf71* whose limits were genetically well defined. To this

end I derived the primers 179 and 180 from the sequence of cm7b4. When both primer sites are intact, 179 and 180 amplify a 350 bp product from *C. elegans* genomic DNA. Template was produced from individuals homozygous for one of each of the aforementioned deficiencies. Typically, such individuals arrest development at an embryonic stage. If one or both of the primers derived from cm7b4 was deleted by deficiency in question, no 350bp amplification product would be observed. Conversely if the deficiency does not delete either one or both of the primer sites, PCR amplification would occur and the expected 350 bp product would be produced. The results of this analysis suggested that cm7b4 resides within *mDf3*, but outside of *mDf1*,*sDf29, sDf57, sDf47, sDf57 and sDf71*, which placed it into zone 18B of the genetic map This suggested the overlap portion of the YAC clones Y5C10 and Y75G4 within which cm7b4 maps, resided between the right breakpoint of *sDf57* and the left breakpoint of *sDf29.* Using Southern blot and PCR analysis I positioned the genomic DNA flanked by primers 179 and 180 onto the LGV cosmid clone W03E11. This placement has been confirmed by data from the sequencing consortium which is currently available from ACeDB.

Four other LGV specific primer pairs were constructed and provided by I. GREENWALD. All four of these primer pairs were derived from cDNAs generated by YUGI KOHARA (*personal communication*). Each of these clones has been hybridized to gridded filters carrying the *C. elegans* genome represented in an overlapping set of YAC clones (KOHARA, *personal communication*), and this mapping data is available on ACeDB. The formal identity of the cDNA clones from which these primers were derived is displayed in Table 11. For brevity I use the primer names (yk47, yk100, yk649, yk967)

in place of their formal names. The physical map position of the YACs to which these clones mapped suggested that these primer sets would likely anchor the physical and genetic maps immediately to the right of *rol-3*. Using PCR, I have mapped yk649 to the same interval of the genetic map as cm7b4, namely zone 18B. The yk967 primer pair map into the region defined by the overlap of deficiencies *sDf29*, *sDf47* and *sDf71* (zone 19B), and primers derived from yk49 displayed the same PCR amplification pattern as observed for the yk967 primers, which placed yk49 into zone 19B of the genetic map. Failure of yk100 primers to amplify off of *mDf1*, *mDf3*, *sDf29* or *sDf47* template mapped yk100 into zone 20A.

As expected, the yak primer set has contributed to the correlation of the genetic and physical maps to the right of the *rol-3* locus; deficiency zones 18B, 19B and 20A have each been anchored to the physical map by at least one PCR site. In an attempt to determine the extent of zone 18A, thus establishing size limits on the genomic region expected to contain *rol-3*, primers were designed from the sequence of the cosmids C08D8, WO2D7 and F21F8. Prior to the design and deployment of these primers, cm7b4 was the left most anchor in zone18B. Since the cm7b4 derived primer pair (179 and 180) amplified from template derived from *sDf29* homozygous dead eggs, it was assumed that any primer pair derived from genomic sequence to the left of cm7b4 would also amplify off of sDf29. Therefore, primers from C08D8, W02D7 and F21F8 were not assayed for amplification from *sDf29*, or any deficiency right of *sDf29*.

Primers derived from C08D8, W02D7 and F21F8 failed to amplify off of mDf3 template suggesting that mDf3 extended into F21F8. However, F21F8 primers failed to amplify from *sDf57* template whereas the primer pairs derived from C08D8 and W02D7

did amplify off of *sDf57* template. These results suggested that the cosmid F21F8 (or at least the portion from which the primers were derived) resides within zone 18A.

Figure 19 displays raw data from a PCR reaction testing primers derived from yk967 against a number of deficiency homozygote templates. This figure indicates the criteria used to score positive or negative results with respect to the product formed. Failure of a primer pair to amplify an expected product was scored as a negative "-" while amplification of a PCR product was scored as a positive "+" result. The expected amplification product size for each primer set, reaction conditions and compatible control primers for each reaction are listed in Table 9 (Materials and Methods). The results of the PCR mapping analysis are presented in their entirety in Table 11.

Table 11

PCR mediated physical mapping of deficiency breakpoints.

PCR primers	Primer origin	*mDf1*	*mDf3*	*sDf29*	*sDf57*	*sDf47*	*sDf71*
F21f and r	F21F8	ND	-	ND	-	ND	ND
W02f and r	WO2D7	ND	-	ND	+	ND	ND
C08Df and r	CO8D8	ND	-	ND	+	ND	ND
179, 180	cm7b4	+	-	+	ND	+	+
yk649	yk11d5.5	+	-	+	ND	+	+
yk967	yk26a4.5	+	-	-	ND	-	-
yk49	yk10a9.5	+	-	-	ND	-	-
yk100	yk19h11.3	-	-	-	ND	-	ND

Germline transformation:

Coincident with the PCR deficiency breakpoint analysis, I employed germ-line transformation in an attempt to determine the molecular location of *rol-3*. My strategy was to make transgenic worms carrying one or more cosmid clones and use these animals in complementation tests with strains bearing mutations in essential genes with genetic positions near to *rol-3*. The arrays were constructed by injecting cosmid DNA along with the transgenic marker plasmid pCes1943 into the syncytium making up distal arms of the hermaphrodite gonad (Table 10). In most cases, injections were performed into N2 hermaphrodites. pCes1943 is a modified version of pRF4 which carries the semi-

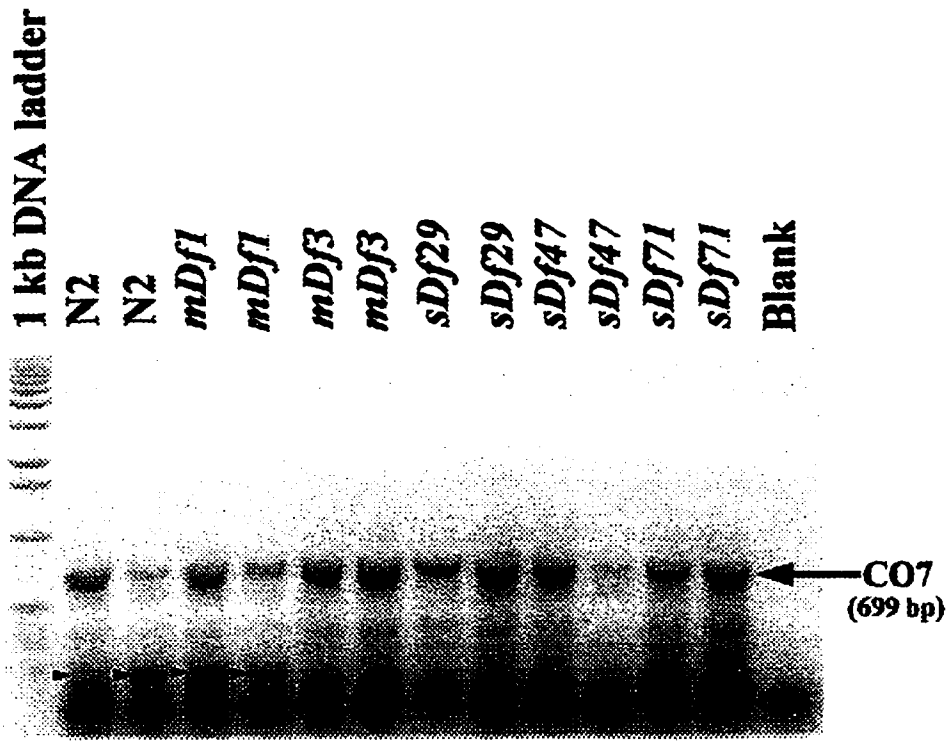
hermaphrodites. pCes1943 is a modified version of pRF4 which carries the semi-dominant allele of *rol-6, (su1006)* (MELLO *et al.* 1991). Worms transgenic for an array carrying *rol-6(su1006)* display the Rol-6 phenotype which is characterized by rolling along their long axis in a right-handed manner, and is manifest in larval development (COX *et al.* 1980, MELLO *et al.* 1991). Any stable line of transgenic animals obtained was subjected to PCR using primers specific for the cosmid vector co-injected (Materials and Methods). The PCR based analysis performed on stably transmitting transgenic lines is a diagnostic test directed at checking the fidelity of the array. Failure to amplify a product with the cosmid vector specific primers indicates that the cosmid co-injected with marker plasmid is likely not present in the array. Conversely, presence of an amplification product suggests that the cosmid vector is present in the array. I made the assumption that the cosmid insert was present concomitant with the vector. The cosmid arrays borne by the transgenic animals are transmittable (Stinchcomb 1985, WAY and CHALFIE 1988) and thus can be used in the standard genetic manipulations governing the use of free duplications. If a strain carrying a particular transgenic array fails to complement an essential gene mutation carried in a test strain; then, in the next generation, homozygous lethal progeny will undergo phenotypic repair and the cosmid is said to rescue the mutation. As a starting point, I generated transgenic animals carrying cosmids residing immediately to the right of the mec-1 *Tc1* associated polymorphism. Some cosmids were obtained as clones from the Sanger Center at Cambridge England, whereas other clones, provided as purified cosmid DNA, were obtained as a gift from CRAIG MELLO and JAMES PRIESS. Detailed protocols regarding both construction of the transgenic arrays,

and complementation testing of essential genes against the arrays, are presented in Materials and Methods.

Figure 19

Agarose Gel Electrophoresis of yk967 Primer Amplifications

Agarose gel electrophoresis of PCR amplification products generated with yk967 primers. Each reaction was performed in duplicate. Templates are labeled above each lane. In all cases, worm genomic DNA templates were obtained by chitinase digestion of unhatched eggs. The "blank" lane illustrates a negative control. All components present in the reaction mixtures are present in the blank with the exception of template DNA. The expected product size for the experimental primers (yk967f&r) is 200 bp. The primer pair C07f&r are specific for the LGIII cosmid clone C07G2, and amplify a 699 bp fragment. C07f&r were included as positive reaction controls. A 1kb DNA ladder is included as size marker. The experimental band is amplified only off of N2 and *mDf1* derived template, whereas the control band is present in all lanes with template. This data suggests that the yk967 priming sites reside within the portion of LGV deleted in *mDf3*, *sDf29*, *sDf47* and *sDf71*, but outside of that deleted by *mDf1*.



► (200 bp product amplified using yk967 forward and reverse primers)

The rescue of *let-406(s514)*:

The *let-406* locus is represented solely by the gamma ray induced allele *s514*. Worms homozygous for *let-406(s514)* normally arrest development at a mid-larval stage. However, when hermaphrodite worms homozygous for *eT1* and carrying an array constructed from F58G4 were mated to males of the genotype *dpy-18(e364)/eT1(III)* ; *unc-46(e177) let406(s514)/eT1(V)*, a number of fertile DpyUncs were seen in the progeny of individually picked F1 Rollers. This result suggested that the cosmid F58G4 is composed of *C. elegans* genomic DNA containing the *let-406+* gene locus including all required cis acting regulatory regions. This result provided me with an unambiguous left most anchor from which I could instigate a transgenic rescue walk towards *rol-3* from the left.

The rescue of *let-434*:

Worms homozygous for *let-434(s19040)* develop to adulthood; but are sterile. No evidence of unfertilized oocytes or germline gonad can be observed in these individuals. Male worms of the genotype *dpy-18(e364)/eT1III* ; *unc-46(e177) let-434(s1904)/eT1V* were mated to *eT1* homozygous worms carrying a transgenic array constructed by the co-injection of ZK307, F25G6 and F01H8 (obtained from C. MELLO and J. PRIESS). F2 DpyUnc progeny of individually picked F1 WT Rollers were tested for fertility and in 4/4 cases were found to be fertile, suggesting a complete reversion of the *let-434(s1904)* phenotype. Since this rescue was accomplished with an array constructed by the co-injection of three cosmid clones, it is not a trivial task to determine if all three cosmids are

present in the array, and if not all three, which of the three. Therefore, *let-434* could reside on either of ZK307, F25G6 or F01H8. Although the rescue of *let-434* may have resulted from a combination of two or all three cosmids in the array it is unlikely since none of the cosmids are contiguous. Furthermore, since my PCR analysis suggests that F01H8 and likely F25G6 are within deficiency zone 19A, while *let-434* resides in zone 18B, it is likely that the presence of ZK307 conferred the rescuing activity. This suspicion was confirmed by a subsequent experiment demonstrating rescue of *let-434* with an array composed solely of ZK307.

Although an exhaustive examination of phenotype was not carried out on the apparently "rescued" DpyUnc homozygous *let-434* individuals, a number of inferences can be made. Although I do not know what role *let-434* plays in somatic and germline gonadogenesis, nor the nature of the mutation, it's clear that DNA carried by ZK307 is able to fully compensate. This suggests that the complete gene complement of *let-434*, as well as the required regulatory regions, are intact on this array.

ZK307 is centered within a large cosmid contig which is currently being sequenced by the *C. elegans* genome sequencing consortium. Since *let-434* had been localized to ZK307, it is possible that other essential genes which map into zone 18 may also reside within this contig. This contig is defined on the left by the cosmid C08D8, and on the right by the cosmid F25G6. DNA from those cosmids spanned by C08D8 and F25G6 (inclusive), which constitute the minimally overlapping clone set sequenced by the *C. elegans* genome sequencing consortium was obtained from Robert Waterston at the St. Louis Genome Sequencing Center. It was expected that since these DNAs represented

aliquots of the DNA stocks actually sequenced by the consortium, they would provide the most interpretable link between the physical map clones and the sequence map. Therefore, these clones were used in the production of transgenic animals, and the arrays formed were used in an attempt to rescue mutations within zone 18 essential genes (*rol-3*, *let-412*, *let-464*, *let-434*). The extrachromosomal arrays constructed are presented in Table 10 (Materials and methods) and the complementation data concerning these arrays Vs LG(V) essential genes is presented in Figure 20.

rol-3 fails to be rescued by all cosmid bearing extrachromosomal arrays tested. This is consistent with the placement of F21F8 into the overlap region of *sDf57* and *mDf3* (zone 18A) by PCR mapping. W02D7 was the closest clone to the zone 18A - 18B boundary tested; however, it maps into zone 18B. Consequently, W02D7 is unable to rescue mutations in *rol-3*. The rescue of *let-433* with an array bearing F25G6 is also consistent with the PCR deficiency mapping data. *let-433* resides within zone 19A which is defined by the left-breakpoint of *sDf29*. I have PCR mapped yk649 into zone 18A, and yk49 into zone 19B. Therefore, F25G6, which maps between yk649 and yk49 on the physical map, is a good candidate clone which genetically maps within deficiency zone 19A. The rescue of *let-433* with F25G6 is in agreement with this placement.

let-464 fails to be rescued by any of the zone 18B genomic clones tested. This most likely indicates that *let-464* resides within the gap spanned by the YAC clone Y68D10. However, the possibility that *let-464* resides within the zone 18B cosmid contig, but cannot be rescued either as a result of the clones used or due to an unfavorable interaction with the transformation marker, cannot be ruled out. Likewise, rescue of

Figure 20

Complementation Data for Extrachromosomal Arrays vs Zone 18 Lethal Mutations.

If a given array rescues the mutation in question, a plus (+) is indicated. Failure of an array to rescue a given mutation is indicated by a minus (-) sign. The "a "in *sEx601, sEx603 sEx605* and *sEx609* vs *rol-3(s1519)* indicates that these arrays were produced by direct injection into a rol-3(s1519) /eT1 balanced strain. Failure to rescue was determined by the presence of Roller-6 progeny, and the complete absence of Dpy Unc progeny.

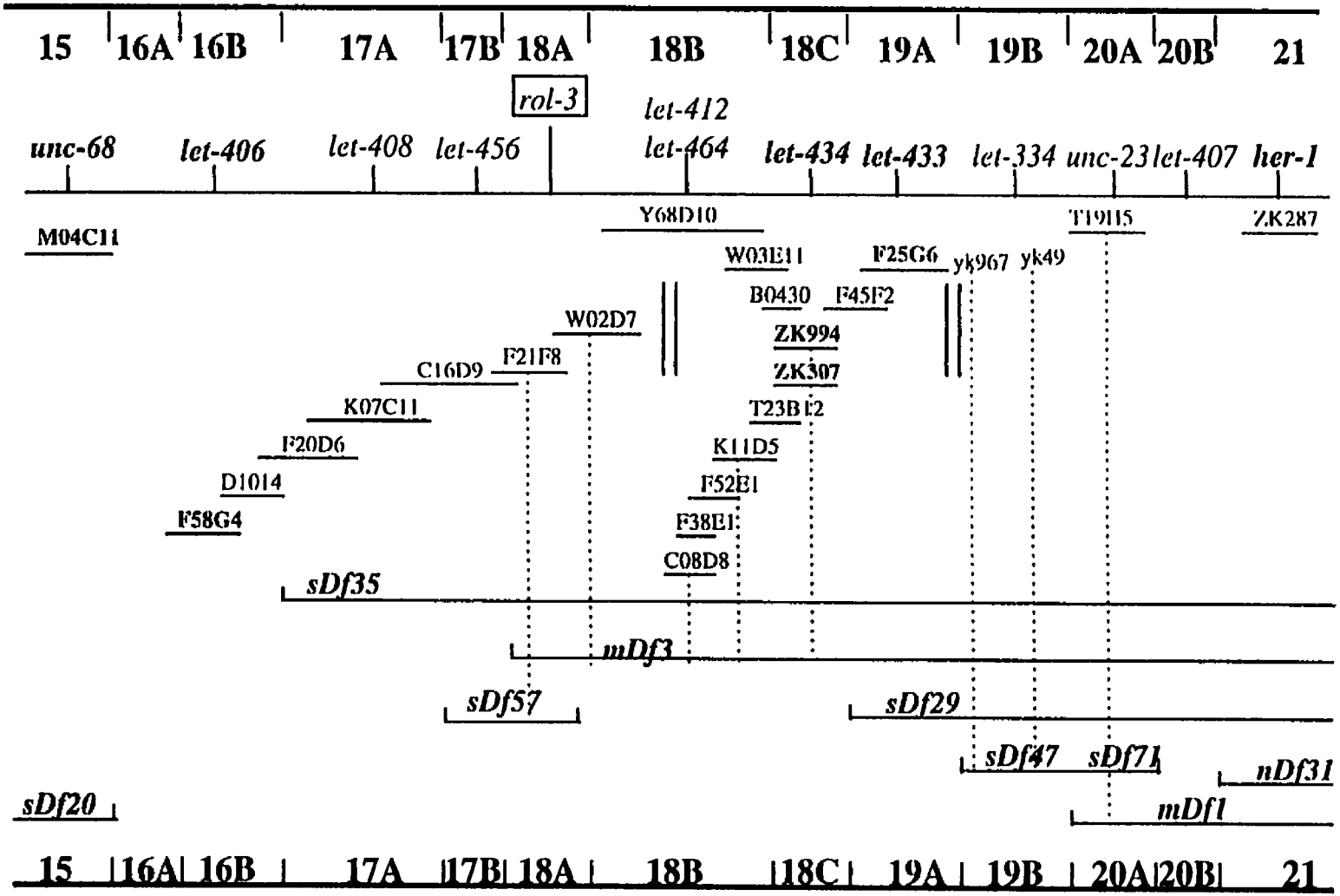
ESSENTIAL GENE		*rol-3*	*rol-3*	*let-412*	*let-434*	*let-464*	*let-433*
ALLELE		*s1040ts*	*s1519*	*s579*	*s1904*	*s1530*	*s950*
ARREST STAGE		Mid *	Early	Mel	Sterile Adult	Early	Sterile Adult
ARRAY NAME	CLONES						
sEx601	W02D7	-	- a	ND	-	-	ND
sEx602	C08D8	-		-	-	-	-
sEx603	C08D8 + F38E1	-	- a	-	-	-	-
sEx604	F38E1	-		-	-	-	-
sEx605	F38E1 + F52E1		- a	-	-	-	-
	F52E1	ND	ND	-	ND	ND	-
sEx606	F52E1 + K11D5	-	ND	-	-	-	-
sEx607	K11D5	-	ND	ND	-	-	-
sEx609	K11D5 + T23B12	-	- a	ND	-	-	-
sEx608	T23B12	-	ND	ND	-	-	ND
sEx610	T23B12 + ZK994	-	ND	ND	-	-	ND
sEx611	ZK994	-	ND	ND	+	-	-
sEx612	ZK994 + F45F2	-	ND	ND	+	-	-
sEx613	F45F2	-	ND	-	-	-	-
	F45F2 + F25G6	-	ND	ND	ND	-	ND
sEx614	F25G6	-	ND	ND	ND	ND	+
sEx101	B0340	-	-	-	-	-	-
sEx104	W03011	-	-	-	-	-	-
sEx103	T24H5	-	-	-		-	-
sEx105	ZK307	-	-	-	+	-	-

Figure 21

Correlation of a Portion of the LGV Genetic Map (Zones 16-21).

The genetic map positions of cosmid or EST based PCR primer pairs are indicated by dashed lines. Genetic loci which have been localized to a cosmid are shown in bold type. Physical map positions of *unc-68* (H.KAGAWA personal communication) and *her-1* (Perry *et al.*1993) are available from ACeDB. The cosmids D1014, F20D6, K07C11, and C16D9 have not been positioned relative to the genetic map.

Correlation of the Genetic and Physical Maps of LGV (Zones 16 - 21)



let-412 with a zone 18B cosmid bearing extrachromosomal array has yet to be demonstrated. The cosmid clone W02D7 remains untested for rescue of let-412. The correlation data discussed in this section is presented in Figure 21.

SECTION IIB

LOCALIZATION OF *SRL-2(III)* THROUGH CORRELATION OF THE LGIII GENETIC AND PHYSICAL MAPS

INTRODUCTION

Dr. David Baillie has been actively involved in studying genome organization, the goal of which is to identify and understand the arrangement of genes and other sequences relevant to the development of an organism along the length of the chromosomes. These studies serve to determine the functional basis for this arrangement. As previously discussed, (Introduction, Section 2A) one method for examining genome organization involves the identification of all genes which affect a particular developmental stage or process. Dr. Baillie has focused on the identification and characterization of *C. elegans* genes which can be mutated to give a lethal phenotype, and thus define components essential for development of the organism. A recent addition regarding this body of knowledge work is focused on the *dpy-17 - unc-36* interval of LGIII, balanced by the large free duplication *sDp3 (III ; f)*. (ROSENBLUTH, CUDDEFORD and BAILLIE *et al.* 1985). This region is the site of an intensive study aimed at identifying essential genes. To date, six deficiencies subdividing the 2 m.u. region, and 125 mutations defining 53 complementation groups have been identified (H. STEWART and D. L. BAILLIE, personal communication). Figure 22 is a map on which some of the representative features of this interval are shown. For the sake of clarity, the essential gene content of this region has been omitted from the map. The positions of markers, the 6 deficiencies and the duplications *sDp3* and *sD8* are shown relative to one within this region.

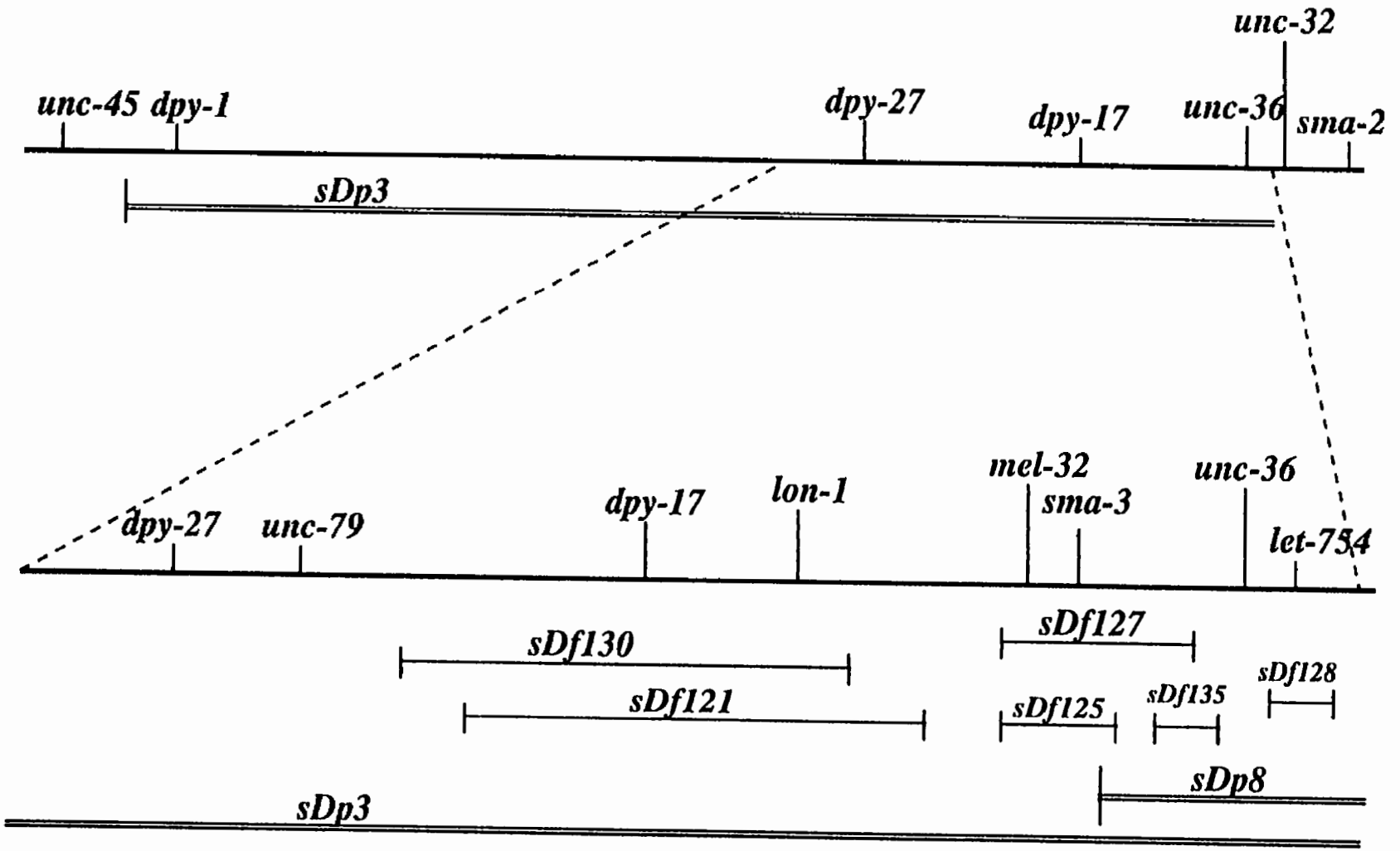
An extension to the generation and identification of essential mutations on LGIII has been the been the construction and application of the *C. elegans* transgenic cosmid library (D. JANKE, J. SCHEIN and D. L. BAILLIE, *personal communication*).

Figure 22

Partial Genetic Map of LGIII

Partial genetic map of LGIII indicating the positions of the visible markers, deficiencies and duplications used in this section. The current state of this map is a result of efforts by D. Janke and H. Stewart (*personal communication*).

Genetic Map of LGIII (left)



As the *C. elegans* Genome Sequencing Consortium progresses through sequencing the genome, all data, including both raw and finished, annotated sequence, is made public domain.

The *C. elegans* sequencing project was initiated on LGIII (WILSON *et al.* 1994). In collaboration with the Genome Sequencing Consortium, cosmid DNA originally used as sequencing stock is immortalized in the form of transgenic arrays carried by *C. elegans*. These transgenic animals, each carrying an extra-chromosomal array containing a cosmid of known sequence, are made available to the *C. elegans* community as a mapping resource. There are currently over 400 strains representing 200 cosmids maintained as extrachromosomal array bearing C elegans strains. Of these, 147 are derived from LGIII physical map clones, 70 of which map to the *dpy-17- unc-36* interval of LGIII.

Two factor mapping data for *srl-2* suggested that it resided within the interval bounded by *dpy-17* and *sma-2*. Therefore, it was likely that *srl-2* would map to the *dpy-17 - unc-36* interval. In this section I report the accurate placement of *srl-2* into the *dpy-17 - unc-36* interval. Having an accurate map location for *srl-2* allowed me to take full advantage of the wealth of LGIII mapping resources made available by researchers in Dr. Baillie's laboratory. A combination of 3-factor, duplication and deficiency mapping was performed which mapped *srl-2* to the right of *sma-3*, within the overlap regions of *sDf127* and *sDf135*.

In collaboration with N. Franz, *srl-2* was positioned onto the physical map on the basis of PCR deficiency breakpoint mapping of *sDf125* and *sDf137* (Materials and methods). Alignment of the physical and genetic maps relative to the breakpoints of

sDf127 and *sDf135* (N. Franz *unpublished results)* enabled me to identify clones which represent the genomic region thought to contain *srl-2*. In order to determine the genome map location of *srl-2* to the resolution of a cosmid clone, I tested a subset of the *C. elegans* transgenic cosmid library representing LGIII for their ability to rescue the srl-2(s2507) phenotype (recessive suppression of *rol-3(s1040*ts). The results of this analysis suggest that *srl-2* resides within the region defined by the overlap of the cosmids B0361 and F56C9.

RESULTS

Accurate placement of *srl-2* onto the LGIII genetic map.

Three factor mapping

srl-2 had been localized to the *dpy-17* - *sma-2* interval on the basis of 2-factor mapping (see Section I). In an attempt to better position *srl-2* onto the LGIII genetic map a combination of 3-factor mapping, complementation mapping against deficiencies and duplications was undertaken. 3-factor mapping was performed as described in Materials and Methods, the results of which are shown in Table 12. Since presence of *srl*-2 was assayed by suppression of the temperature sensitive mid-larval lethal phenotype associated with worms homozygous *rol-3(s1040*ts), care had to be taken in determining which of the recombinant progeny not only carried *srl-2*, but that each recombinant carried the *rol-3* marked chromosome as well. Since the *rol-3* marked chromosome was also marked with *unc-46*, recombination between these two markers was expected to occur and this event was also taken into account (see MATERIALS and METHODS). Dpy recombinants from *dpy-17* + *unc-36/+ srl-2* + worms were approximately 3.75 times more likely to include exclude *srl-2* than to include it. Since the *dpy-17* - *unc--36* distance is 1.82 (HODGKIN, DURBIN and MARTINELLI 1995) this suggests that *srl*-2 maps approximately 0.5 m.u. to the left of *unc-36*. Conversely, Sma recombinants from *sma-3* + *unc-36/+ srl-2* + are 4 times more likely to exclude *srl-2* than to include it. This suggests that *srl-2* maps approximately 0.15 m.u. to the right of *sma-3*.

Table 12

Three factor mapping data for *srl-2(s2507)*.

Parental genotype	Recombinant phenotype selected	Possible recombinant chromosomes	Number of each class observed	Adjusted value.*
sma-3 + unc-36 / + *srl-2* +	Sma	*sma-3 srl-2 +* and *sma + +*	12 44	11 43
dpy-17 + unc-36 / + *srl-2* +	Dpy	*dpy-17 srl-2 +* and *dpy-17 + +*	33 11	30 8

*Adjusted value is corrected for falsely classified recombinants as a result of recombination between unc-46(V) and rol-3(V) (See Materials and Methods). Total number of worms screened fro Sma recombinants = 6771, Total number of worms screened for Dpy recombinants=9384

<u>Mapping *srl-2(s2507)* to the duplication *sDp8(III ; I)*</u>

sDp8 is a duplication of the central cluster of LGIII which is linked to LGI. I expect that most alleles of *srl-2* are loss of function alleles (see RESULTS, Section I). If the portion of the genome reiterated in *sDp8* spans the *srl-2* locus, then I expected *srl-2/srl-2 sDp8* worms to fail to suppress the recessive temperature sensitive mid-larval lethal

arrest phenotype of *rol-3(s1040ts)*. *sDp8* carries a wildtype copy of the *unc-36* gene, therefore, *eT1(III) ; eT1(V) ; sDp8* worms appear phenotypically wildtype. In order to bring *sDp8* into a homozygous *srl-2* background, I mated *eT1(III) ;eT1(V) sDp8(III ; I)* males to *srl-2(s2507) ; unc-46(e177) rol-3(s1040ts)* hermaphrodites. F1 wildtype hermaphrodites were set up individually, and those of the genotype *sDp8(III ; I) ; srl-2(s2507)/eT1(III) ; unc-46 rol-3(s1040ts)/eT1(V)* were recognized by the presence of Unc-46 Roller-3 F1 progeny. Any line which failed to segregate Unc-46 Roller progeny was discarded (genotypically *eT1(III) ; eT1(V) ; sDp8 (III ; I)*. I observed 868 Wild types : 99 Unc-36 : 31 Unc-46 Rol-3 indicating that srl-2 resides within the portion of LGIII reiterated by *sDp8*.

Deficiency mapping srl-2(s2507) to sDf121, sDf125, sDf127 and sDf135

srl-2 was complementation tested with the deficiencies *sDp121, sDf125, sDf127* and *sDf135*. The scheme used for these complementation tests is outlined in the materials and methods. It is expected that the majority of *srl-2* alleles represent knock-out or loss of function mutations in *srl-2* (see section I). Based on this assumption I expect that *srl-2(s2507)*, in *trans* to a deficiency, would suppress the lethal phenotype of *rol-3(s1040*ts) in much the same way observed for homozygous *srl-2(s2507)*. If the deficiency fails to complement *srl-2* then I expect to observe a 2 : 1 ratio of Unc to DpyUnc in the self-cross progeny of hermaphrodites of the genotype *dpy-18(e364) srl-2(s2507)/dpy-17(e164) sDf Unc-32(e189)* ;

unc-46(e177) rol-3(s1040)/+ +. If the deficiency complements *srl-2*, I expect to see Unc progeny only rarely. These would be the result of recombination between the *unc-46* and *rol-3* loci. Such recombinant progeny would be represented less than once in 100 progeny. The data obtained for the deficiency mapping of *srl-2* is presented in Table 13. *srl-2*(s2507) was found to complement *sDf121* and *sDf125*, but failed to complement *sDf127, sDf135*. The summation of the three factor, deficiency and duplication mapping data places the *srl-2* locus to the right of *sma-3*, within the interval shared by the overlapping deficiencies *sDf127* and *sDf135*. (Figure 14).

Table 13

Complementation data for *srl-2* Vs LGIII deficiencies

Df	wildtype	Dpy	Unc	DpyUnc	Complementation +/-
sDf121	923	313	12	47	+
sDf125	1447	424	2	50	+
sDf127	742	248	86	39	-
sDf135	543	202	62	27	-

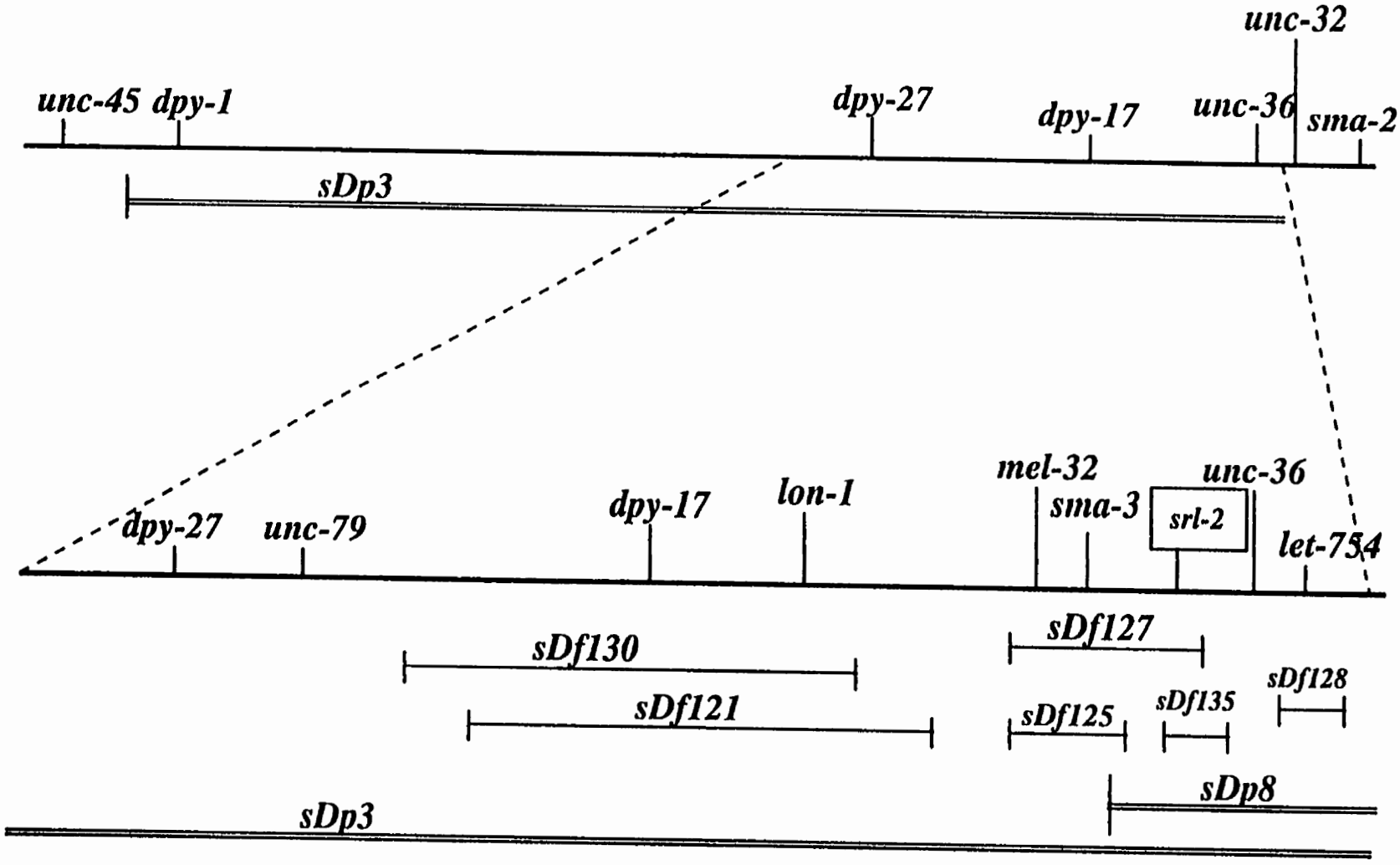
Polymerase Chain Reaction mediated alignment of the physical maps around *srl-2*.

Initial placement of *srl-2* on the physical map was done via a PCR mediated low resolution alignment of the genetic and physical maps of LGIII. Over 30 LGIII specific primer pairs, constructed by Norm Franz, currently exist. These primer pairs were

Figure 23

The Placement of *srl-2*(III) onto the Partial Genetic Map of LGIII.

Genetic Map of LGIII (left)



developed from the annotated sequence of select members of the minimally overlapping set of LGIII cosmids available from ACeDB and the Washington University Genome Sequencing Center World WideWeb site. Each primer pair can be used to assay whether or not the physical region from which one or both members of a primer pair resides maps within or outside a given deficiency. Norm Franz had positioned the left breakpoint of *sDf127* to the right of the cosmid F42A10, and that the deficiency extended rightward to the cosmid T20B12. Since the *srl-2* maps within the overlap region of *sDf127* and *sDf135*, effort was made to identify that portion of the physical map corresponding to the overlap region. The data relating the overlap region of *sDf135* and *sDf127* is presented in Table 14, and the results of the analysis are shown in Figure 24.

Table 14

PCR Based alignment of *sDf127* and *sDF135* with the LG(III) genetic map.

	Cosmid origin of the primer pairs					
	C06E8	*ZK418*	*T20H4*	*B0361*	*T20B12*	*K07D8*
sDf127	-	-	-	-	-	+
sDf135	+	+	+	+	-	+

The results suggest that left breakpoint of *sDf135* resides to the right of the 3' end of the reverse primer B03R, and the deficiency extends to a position somewhere between the 5'

end of the forward primer T20f and the 3' end of the reverse primer K07r. From the physical map of this region (Figure 23) it is clear that the YAC Y50B11 overlaps with T20B12 and extends left to ZK418, covering 11 members of the minimally overlapping set of cosmids making up the sequenced set in this region. It was expected that the physical position of *srl-2* would likely be within the region defined by Y50B11.

Identification of cosmid clones containing srl-2 through transformation rescue.

Y50B11 is a YAC clone present in the array upEx1, which is maintained extrachromosomaly in an otherwise genetically wild type background (E. BUCHER and B. GATEWOOD, *personal communication*). This strain, along with eight members of the transgenic cosmid library, each carrying an extrachromosomal array consisting of a single cosmid comprising a discrete portion of the physical map represented by Y50B11, were tested for the ability to repair the phenotype of *srl-2*. Since rescue of *srl-2* would result in the loss of suppression of the *rol-3(s1040*ts*)* phenotype, rescued individual were expected to arrest development. Such an event would be difficult to assay directly, so I developed a scheme which allowed for the accurate determination of the number of Srl Rol-3 animals expected in the case of an extrachromosomal array failing to rescue *srl-2*. Therefore, any decrease in the number of these animals would be immediately obvious. The scheme used is described in the Materials and Methods, the data is presented in Table 15. The scheme is dependent on the construction of an animal which is quadruple heterozygous for *srl-2(III), rol-3(V)*, a marker linked to *rol-3*, the reciprocal translocation *eT1 (III ; V)*, and also carries an extrachromosomal array which is to be tested. As

previously discussed, *eT1* acts as a recombination suppressor over the right portion of LG(III) and the left portion of LGV; and, as a result of anuploidy, markers on LG(III)(right) and LGV(left) are pseudo-linked (see Material and methods). Since *srl-2* falls outside of the recombinationally balanced portion of LGIII, recombination between itself and the *eT1(III)* breakpoint is expected. In practice, I observe the actual degree of recombination observed between *srl-2* and *eT1(III)* to be quite low. Therefore, the F1 progeny of this quadruple heterozygote should approximate a 1 : 4 : 1 ratio of Srl Rol-3 : Wildtype : Unc-36 animals. An extrachromosomal array present in the quadruple heterozygote Po was expected to segregate into each of the three progeny classes with equal frequency. Therefore, if the extrachromosomaly maintained DNA does not repair the *srl-2* phenotype I expected to see approximately equal numbers of Unc-36 Rol-6 and

Unc-46 Rol-6 worms. If, however, the DNA carried in the extrachromosomal array was capable of repairing the defect in *srl-2*, a significant decrease in the number of Srl Unc-46 Rol-3 animals relative to the number of Unc-36 Rol-3 observed was expected. Two features of the data are immediately evident. The first is that the data is obviously dichotomous. Of the nine extrachromosomal arrays assayed for their coverage of *srl-2*, six resulted in Unc46 Roller6 and Unc36 Roller6 animals with approximately equal frequencies. This suggests that these six cosmid containing arrays (sEx78: F37C12, sEx32: T20H4, sEx69: T20B12, sEx67: R151I, sEx73: F08F8, sEx163 ZK418) are incapable of repairing the Srl-2 phenotype implying that these clones do not contain the wildtype *srl* locus. On the other hand, F1 progeny from the self-cross of transgenic P0s

carrying *sEx93*: F56C9, *sEx238*: B0361 or *upEx1*: Y50B11 display a significant reduction in the numbers of UNC-46 ROLLER-6 progent relative to UNC-36 ROLLER-6 progeny. This suggests that the wildtype *srl-2* locus is present on each of these three overlapping clones.

TABLE 15

Germ-line transformation rescue of srl-2

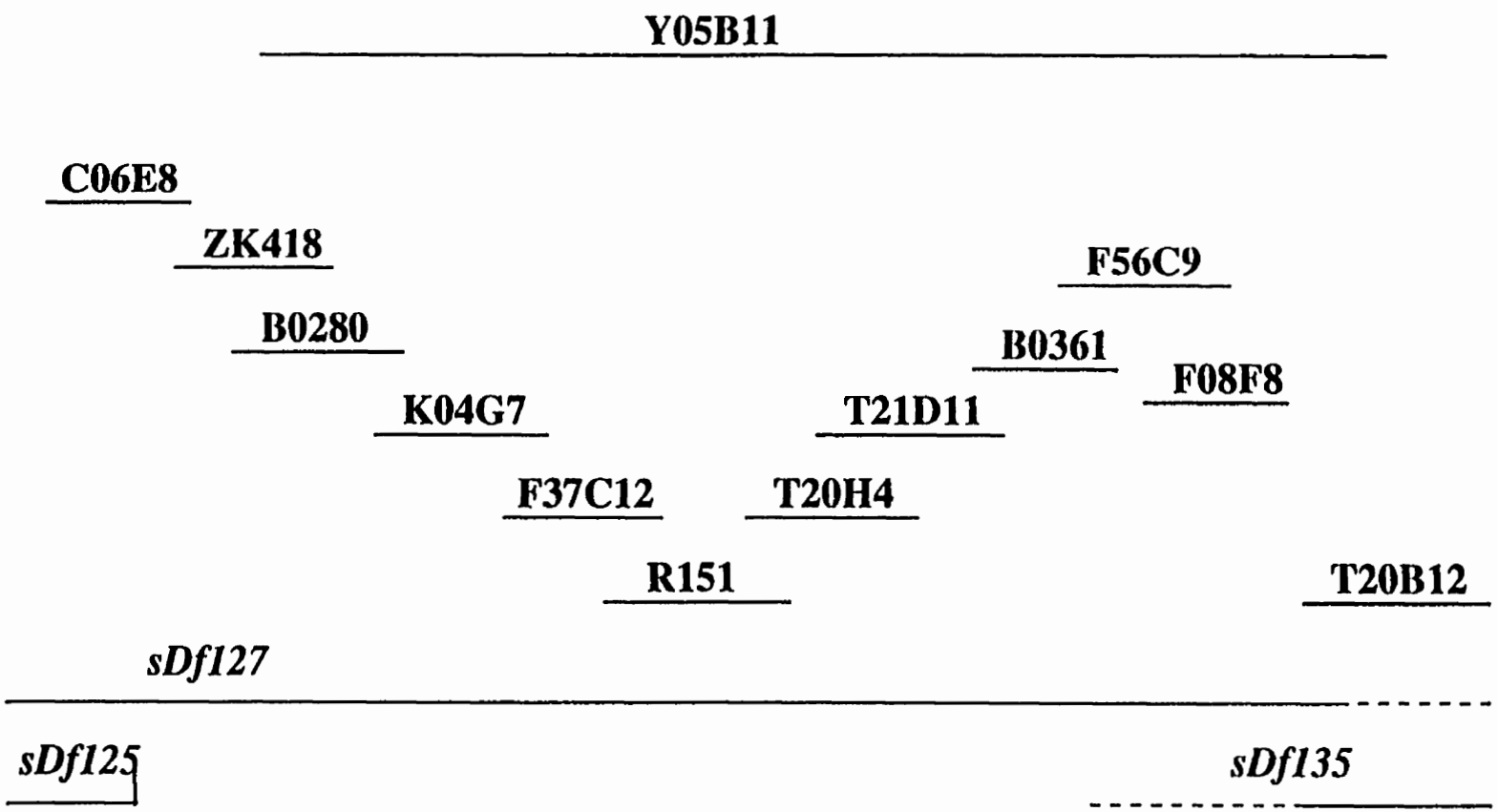
ARRAY	CLONE	ROLLER	UNC-36 ROL	UNC-46 ROL	# WORMS	UNC-46 ROL / UNC-36 ROL
upEx1	Y50B11	69	18	4	153	0.22
sEx238	B0361	62	15	2	284	0.13
sEx93	F56C9	105	16	5	284	0.31
sEx163	ZK418	103	17	18	208	1.06
sEx73	F08F8	112	16	17	329	1.06
sEx67	R151	105	21	18	299	0.85
sEx69	T20B12	31	6	5	122	0.83
sEx32	T20H4	93	16	13	239	0.81
sEx78	F37C12	84	15	9	226	0.60

The second feature of table 15 concerns the three arrays carrying clones thought to rescue *srl-2*. It was expected that total repair of the *srl-2* phenotype would lead to *rol-3(s1040*ts) mediated larval arrest. However, in no case was a complete absence of Unc46 Roller6 animals observed. This likely reflects the mosaic nature of inheritance of extrachromosomal arrays rather than phenomenon related to a partial rescue event (see discussion).

Figure 24

Relationship between the LGIII Cosmid Clones used in the Transformation Rescue of *srl-2,* and their Position on the LGIII Genetic Map as a Result of PCR *Df* Breakpoint Mapping.

PCR-Mediated Placement of the LGIII Deficiency Breakpoints of *sDf127* and *sDf135* on LGIII



Analysis of the overlap region of B0361 and F56C9

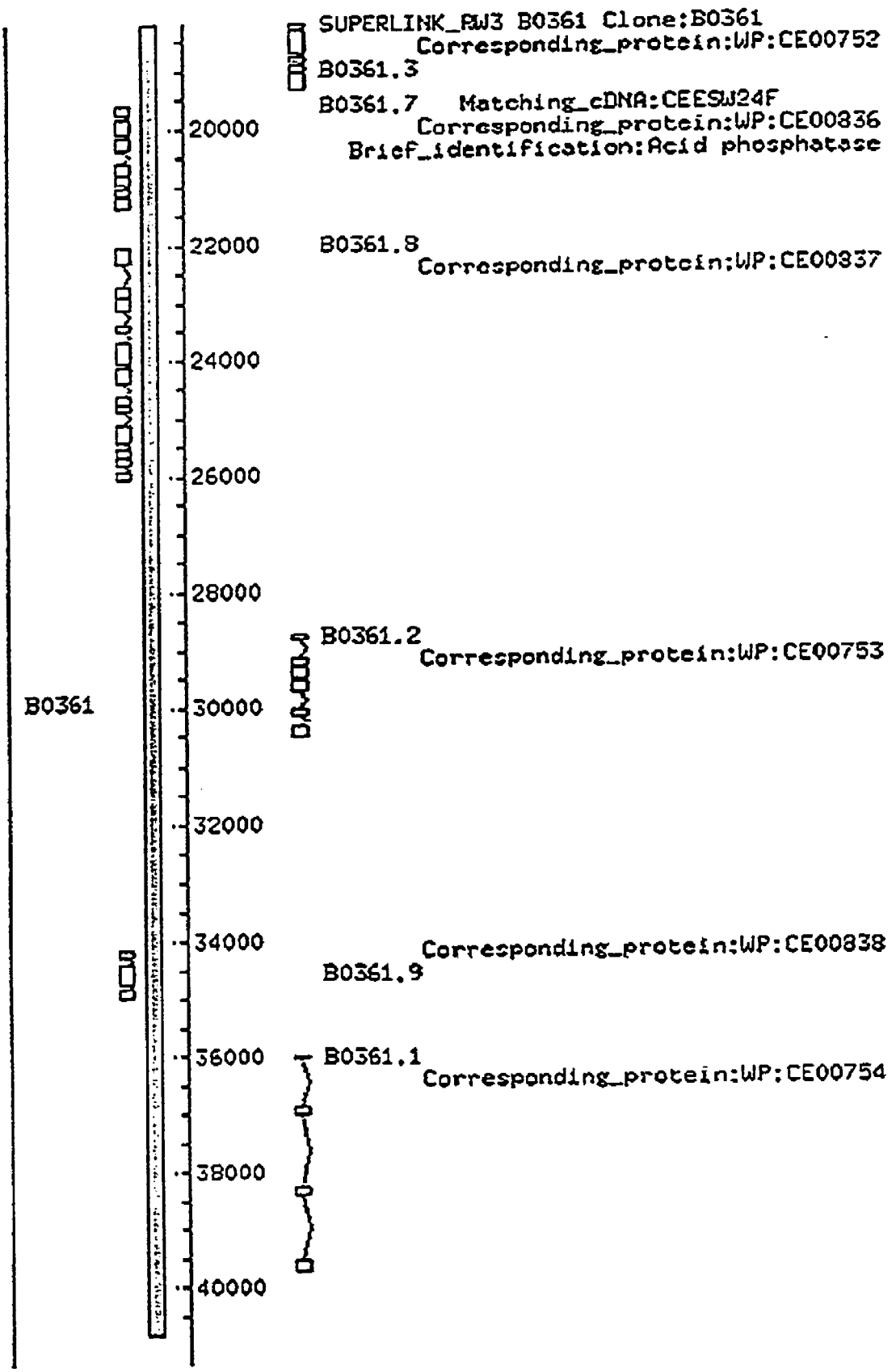
The Genbank entry for the cosmid clone B0361 indicates the cosmid is 42528 bp in length The 3' overlapping cosmid sequenced by the *C. elegans* sequencing consortium is F56C9, and nucleotide 1 of F56C9 corresponds to position 33927 of F56C9. This indicates that the region of overlap between B0361 and F56C9 is 8601bp in length, and therefore, the sequence element conferring phenotype repair of *srl-2* is likely restricted to this 8.6 Kb fragment. A pictorial representation of the 8.6kb overlap region is displayed in Figure 25. The clone, B0361, has been sequenced by the *C.elegans* sequencing consortium and the sequence is annotated and available from Genebank or from ACeDB. There are two genefinder (FAVELLO, HILLIER and WILSON 1995) predicted proteins within the region defined by the overlap of B0361 and F56C9. Figure 24 is a representation of an ACeDB screen print out on which the two complete genes residing on the 8.6kb overlap region are displayed. B0361.1 is composed of four small exons, and is predicted to code for a 145aa polypeptide. The BLAST algorithm (ALTSCHUL *et al.* 1990) was used to conduct searches between the B0361.1 product and both the non redundant data base and data base of expressed sequence tags (dbest) at the National Center of Biotechnology Information (NCBI). These searches failed to indicate significant similarity between the B0361.1 gene product and any other identified protein thus failing to assign a known function to the B0361.1 gene product. I have also failed to detect a significant match with any member of the WORMPEP 11 databank of *C.elegans* proteins. The second putative coding element residing within the overlap is B0361.9. Evidence that B0361.9 represents an authentic gene exists in the form of experimental evidence. The cDNA yk103E shares sequence identity over a large portion

of the predicted coding sequence of B0361.9, suggesting that yk103E represents the transcriptional product. The B0361.9 gene is oriented with opposite transcriptional polarity to B0361.1. B0361.9 is composed of three exons, and is predicted to code for a 194aa polypeptide product. BLAST (ALTSCHUL *et al.* 1990) searches of the predicted B0361.9 protein product against the non redundant protein data bank and dbest indicate only one significant match. B0391.9 matches the hypothetical 74.4 KD protein C30D11:09 from Chromosome I of *Schizosacchoromyces pombe* with a blast score of 174 and a smallest sum probability of 7.5e-17. B0361.9 also fails to match any entry in WORMPEP 11.

Both B0361.1 and B0361.9 predicted protein products were subjected to the BLOCKS v 1.6 (HENIKOFF and HENIKOFF 1994) searcher which compares protein sequence to the current database of protein blocks. Blocks are short multiply aligned ungapped segments representing the most highly conserved regions of proteins. The rational behind such a search is that the information from multiply aligned sequences is available in concentrated form; and, that searching a data base thus formatted greatly reduces the amount of background noise thus increasing sensitivity to distant similarities. BLOCK search results for both B0361.1 and B0361.9 were uninformative since neither detected significant similarity to any of the highly conserved protein domains constituting the current data base.

Figure 25

Screen Capture of an ACeDB Screen Display Illustrating the Structures of the two Genefinder Predicted Genes which Reside within the 8.6 kb Overlap Region of B0361 and C56C9.


SUPERLINK_RW3 B0361 Clone:B0361
Corresponding_protein:WP:CE00752
B0361.3
B0361.7 Matching_cDNA:CEESW24F
Corresponding_protein:WP:CE00836
Brief_identification:Acid phosphatase
B0361.8
Corresponding_protein:WP:CE00837
B0361.2
Corresponding_protein:WP:CE00753
B0361
Corresponding_protein:WP:CE00838
B0361.9
B0361.1
Corresponding_protein:WP:CE00754
20000
22000
24000
26000
28000
30000
32000
34000
36000
38000
40000

DISCUSSION

Alignment of the physical and genetic maps by PCR mapping and germline transformation rescue.

This analysis was directed at aligning the genetic and physical maps at a portion of LGV(left) for the purpose of identifying a region of the physical map where the *rol-3* locus was most likely to reside. My correlation data was generated by two independent methods, PCR mapping deficiency breakpoints to the physical map, and germline transformation. I attempted to PCR map the end points of genetically well defined deficiencies against primer pairs that had been derived from LGV physical map clones. The yk set of primers (yk49, yk100, yk649, yk967) were received as gifts from I. Greenwald, others were constructed from LGV cosmid sequence available on ACeDB using the oligo 4.1 primer analysis software. Correlation of the genetic and physical maps involves a mapping protocol in which I assay for the ability of PCR primers to amplify off of genomic template derived from embryos homozygous for a deficiency (BARSTEAD and WATERSTON 1991, WILLIAMS *et al.* 1992). Failure of a PCR primer pair to amplify off of a given deficiency implies that the genomic site from which the primers were derived resides within the genomic portion deleted in the homozygous *Df* embryo. Therefore, this analysis serves to interface physical map data with the recombination based map often generated through mutational analysis of a given chromosomal region.

Prior to the start of this thesis a great deal of work aimed at identifying and characterizing essential genes within the *eT1* balanced region of LGV had been accomplished (ROSENBLUTH, CUDDEFORD and BAILLIE, 1983; 1985;

ROSENBLUTH *et al.*, 1988; JOHNSEN, 1990; JOHNSEN and BAILLIE, 1991). These analyses resulted in the identification of 101 essential genes, generation of 33 deficiencies which subdivide the 23 m.u. *eT1* left into 23 zones, and a comprehensive deficiency map on which the existing *eT1*V genes were assigned to these zones.

The comprehensive deficiency map which defines the *eT1* balanced region is an excellent substrate upon which to base a PCR based correlation of the genome and genetic maps (LGV) The *rol-3* locus resides within the deficiency zone 18A, which I have anchored to the physical map at the site of the cosmid clone F21F8. The neighboring clone, W02D7, maps within *mDf3*, but outside *sDf57*. This provides a right most limit to zone 18B. The site which maps the furthest from W02D7, yet still resides within zone 18B, is yk967. Based on the ACeDB physical map, I estimate this site to lie close to the cosmid ZK994 (see Figure 21). These two points are separated by a six cosmid contig, and a YAC gap. Since one of the cosmids (T23B12) has yet to be sequenced, the distance represented from C08D8 to ZK994 can only be estimated. From ACeDB, the distance represented by the C08D8 - T23B12 interval is 114kb. The size of ZK994 is 24kb, and D. L. Baillie assessed T23B12 at 30kb which is the average size for a *C. elegans* cosmid read (personal communication). Therefore, the distance from C08D8 to ZK994 is 144kb. Estimating the size of YAC gaps is problematic; however, when subject to FIGE analysis, Y68D10 co-migrates with yeast chromosome IV (data not shown) suggesting that it represents 290KB of genomic DNA. Since Y68D10 overlaps with W02D7 and C08D8, the actual physical size of zone 18B is 290kb + 80kb + 30kb =24kb = 424kb. Using the physical size metric of BARNES et al. (1995), which is 1.83

kb per fingerprint band, the same region is 312kb (based on 1.83 kb/fingerprint band + 100 fingerprint bands /YAC gap). In this case, the metric of 1.8kb/fingerprint band results in an underestimate of the physical size of zone 18B. The PCR anchor points also allow the physical size of zones 19A to be determined. The physical limits of 19A are bounded on the left by the mapping of yk649 into zone 18B and the placement of yk967 into zone 19B. Therefore zone 19A must be within the region bound by ZK994 on the left and F25G6 on the right. The size estimate for this region is 54kb (from ACeDB) . Therefore, this zone represents a small physical interval as well as representing a small genetic locus as well. Currently, zone 19A is represented by the single mutation, *let-433*. Zones 19B and 20A are anchored by the PCR primer pairs corresponding to yk49, and yk100 (T19H5) respectively.

I have attempted to anchor essential gene loci to the physical map using the technique of germ line transformation rescue. Toward this end, J. Schein, D. Janke and myself constructed 19 extrachromasomal LGV cosmid array bearing strains. I subsequently used these to rescue three essential genes which reside close to *rol-3* on the genetic map. In the transgenic worm, the cosmid arrays behave as free duplications so that, in the case of an array constructed with a single cosmid clone, the free duplication has a physical size of approximately 40 kb. Therefore, correlation of mutationally identified genes to these small duplications will provide a high resolution refinement to the existing genetic map. As expected, my rescuing experiments have yielded a much higher resolution map of the *rol-3* region then had existed previously (Johnsen and Baillie 1991). Specifically, the zone 16 gene cluster has now been split into a minimum of two

sub-clusters as a result of anchoring *let-406* to the cosmid F58G4. However, until the remaining zone 16 genes are tested for rescue by F58G4, the distribution of the remaining zone 16 essential genes into either zone 16A or zone 16B is unknown. The zone 17 and zone 18 gene clusters have each been split as a result of genetic mapping during the course of this work. l*et-433* and *let-434* were both thought to map into zone 18. However, I positioned *let-433* into zone 19A by demonstrating that it is separated from *let-434* by the left breakpoint of the deficiency *sDf29*. Germline transformation rescue places *let-433* on the cosmid F25G6, which must therefore be in zone 19A. Zone 19B is anchored by PCR primers derived from yk49. Based on the physical map available in ACeDB, yk49 and F25G6 are physically close, and may even overlap. However, based on the genetic map, these sites must be separated by *sDf29*. Therefore, the zone 19A - 19B boundary, and thus the left breakpoint of *sDf29*, must reside somewhere in the vicinity of F25G6.

I divided zone 18 into zones 18A and 18B upon determining that *rol-3(s501)* failed to complement mutations in the zone 17 gene, *let-456*. (rol-3(s501) was later renamed sDf57. B. Johnsen (1990) suggested that *let-412* and *rol-3* may be alleles of the same gene since *let-412* homozygotes arrest as sterile, weak left-roller adults, and they mapped to the same genetic map zone (JOHNSEN 1990). However, I observe complementation between all alleles of *rol-3* tested against *let-412*. Furthermore, *sDf57* complements *let-412* which indicates that the *rol-3* and *let-412* loci are separable by a deficiency breakpoint. I have demonstrated that *let-434* maps to cosmid ZK994 by germline transformation rescue. Since no other zone 18B locus is rescued by ZK994, this

result subdivides zone 18B into two portions, B and C. Therefore, zone 18A is defined by *rol-3*, 18B by *let-464* and *let-412*, and 18C is defined by *let-434*.

The physical size of the immediate *rol-3* region, zone 18A and B, is greater that 400kb, yet contains few identified essential genes relative to the surrounding regions Johnsen and Baillie 1991). Perusal of the annotated sequence data for this region, although incomplete, suggests that a minimum of 40 genes are present in this region alone. To date, mutations have identified *rol-3* and three other essential genes in this region. This may suggest that this region is no where near saturated for essential genes. Alternatively, this may reflect the proportion of *C. elegans* genes which are essential. Park and Horvitz (1986) estimate that upwards of 50% of *C. elegans* genes may be phenotypically wildtype when mutated, and Schein et al. (1993) have demonstrated that there are no genes between the *let-56* and *unc-22* loci on LGIV which are required for development under laboratory conditions, although coding elements exist in this interval.

A summary of the alignment data for the genetic and physical maps of LGV is presented in Figure 21. It is likely that the anchors I've made between the genetic and physical maps in zones 16 - 20 will aid future molecular characterizations of genes in this region.

Assessment of a transgenic based approach to correlation of the genetic and physical maps.

The experimental approach followed during the course of my transgenic rescue experiments relied on the initial generation of a marked transgenic array within the canonical wildtype strain of *C. elegans*. This methodology has two distinct advantages.

over injecting directly into mutant bearing strains. First, since transgenic arrays behave like free duplications (STINCHCOMB *et al.* 1985; WAY and CHALFIE 1988), an array can be transmitted into the outcross progeny produced between a strain carrying a mutation which is to be tested for rescue and a transgenic strain. This method is very rapid since the injection procedure does not have to be repeated for every mutation to be tested for rescue by a specific cosmid clone. Rather, the rate at which a number of mutation bearing strains can be tested for rescue by any given cosmid array is limited only by the time required to produce an initial stable array carrying strain, the number of matings a given group of researchers can set up and maintain and the *C. elegans* life cycle. The second advantage in generating all arrays in an N2 background is related to the properties of N2 itself. N2 worms are very healthy and easily mated. This robustness serves to increase viability during the injection procedure and is favorable in maintaining the array bearing strains. Furthermore, since N2 is a canonical Wildtype strain it is unlikely that it carries an interacting mutation which may modify the mutation to be tested for rescue. Therefore, rescue results are likely to be easily interpreted. The use of transgenic arrays as free duplications has been successfully applied to the *dpy-5* - *unc-13* region on LGI (McDOWALL 1996), the *unc-22* region on LGIV (SCHEIN 1994), and is currently under intensive use in the *dpy-17* - *unc-36* region of LGIII (D. L. BAILLIE *personal communication*).

Although transgenic rescue has proven to be a rapid and effective method for correlating the genetic and physical maps, the results of my analysis reveal certain limitations of this technique which need to be considered when undertaking a similar

experimental approach. From the genetic map of the *rol-3* region (Figure 16) it is apparent that the mutations *rol-3 let-412* and *let 464* reside within the interval defined by F21F8 and the anchor point of *let-434* (F25G6). Despite the fact that I have generated transgenic arrays composed of contiguous cosmid clones spanning the region between the physical map location of W02D7 and F25G6, I have failed to repair the phenotype of either *let-412* or *let-464*. Since a gap occurs in the cosmid contig defined by these clones it is possible and quite likely that all three of these loci reside within the area of the physical map defined by this gap.

There are, however, a number of limitations to cosmid rescue which may also account for the failure to rescue an apparently well positioned gene. First, it is possible that the gene in question may lie across end points of the cosmids which make up a set of arrays. Therefore, the only way to achieve rescue would be to co-inject two or more overlapping cosmid clones. A related point is that as many as 26% of of *C. elegans* genes may occur as operons which produce polycistronic mRNA precursors that are processed by transplicing (ZORIO *et al.* 1994). Therefore, the regulatory elements required for proper expression may be some distance from the gene in question. The problem with constructing all arrays by injecting cosmid mixtures is that it is difficult to determine whether or not all cosmids present in the mixture are represented in the array.

A second possible reason for failure to rescue a given gene relates to the copy number of the cosmid within the arrays. The ability of a gene product to be correctly temporally and spatially expressed from an extrachromosomal array may vary from gene to gene; and, this variability may be manifest in an apparent cosmid dosage requirement

by the gene in question. It is possible that certain genes have different dosage requirements with respect to the number of cosmids needed to be present within a given array to effect rescue. As such, two neighboring genes, both of which are present within a clone, may require different copy numbers of cosmid to achieve rescue; thus, only one apparent rescue may be observed. Since the array once formed is not thought to undergo any further modifications from generation to generation (Mello *et al.* 1991) it is presumed static with respect to cosmid copy number. One way to determine if incorrect dosage is responsible for the failure to rescue a given gene is to perform cosmid injections directly into balanced strains heterozygous for either mutation. Array production within strains carrying the mutation to be rescued have that added advantage of providing an internal selection for such variables as correct cosmid dosage.

A third factor which may play a role in the failure to rescue a particular gene is related to mosaicism. In *C. elegans*, transgenic arrays are not transmitted into each daughter cell following the mitotic process. Accordingly, the cells of a transgenic *C. elegans* are actually mosaic for the array (MELLO et al 1991; KRAUSE 1995). If a gene is under strict spatial and temporal regulation it is possible that those cells requiring an extrachromosomal copy of the wildtype gene may often not receive an array. In this case, even though the parent transgenic strain may be considered stable, the proper segregation yielding a rescue event within a mutant background may be rare.

The marker chosen as a positive control for transformation may also affect the ability to rescue certain mutations. Currently there are two frequently used positive transformation markers, *rol-6(su1006)* and *dpy-20*$^{+}$. *rol-6(su1006)* has the advantage of

being a dominant transformation marker. Therefore, arrays incorporating *rol-6(su1006)* can be moved into virtually any strain, and easily assayed for presence or absence. However, *rol-6(su1006)* encodes a mutant collagen. Mutations in some collagen genes have been found to suppress some temperature sensitive alterations in the transmembrane receptor produced by the *glp-1* gene (MAINE and KIMBLE 1989). The current theory for this suppression is that the accumulation of mutant collagen may induce the expression of genes encoding heat shock like chaperones which may act to stabilize mis-folded or otherwise mutant proteins (JOHNSON and KRAMER 1993). Its possible that high copy numbers of *rol-6(su1006)* in transgenic arrays may elicit a similar effect, which may result in difficult to interpret interactions between the array and the endogenous genetic background, or may even result in false rescue. *dpy-20*$^{+}$ (CLARK and BAILLIE 1992) is also used as a transformation marker. Arrays formed with dpy-20^{+} DNA tend to have lower copy numbers of the co-injected cosmids relative to *rol-6* containing arrays (D. L. BAILLIE, personal communication). Therefore, genes that are sensitive to dosage may be rescued by arrays containing *dpy-20*$^{+}$, but not arrays composed of *rol-6(su1006)*. There also appears to be differences in the tissue expression of arrays composed of the two types of markers. It has been suggested that *rol-6* arrays are not properly expressed in germ-line tissue (GRANT and GREENWALD 1996)

Transformation rescue of *srl-2*

I determined that the *srl-2* locus resides approximately 0.5 mu to the right of *sma-3* on LGIII, within the region of overlap between *sDf127* and *sDf135*. This has resulted in two findings. First, the placement of *srl-2(s2507)* into a deficiency interval relied on the assumption that *srl-2(s2507)* was a loss of function mutation (see Materials and Methods). In this context, *srl-2(s2507)* behaved exactly as predicted suggesting that it represents a loss of function allele of *srl-2*. Since *srl-2(s2507)* exhibits similar phenotypes to other *srl* mutations, it is likely that most srl- mutation also represent loss of function mutations.

Second, this genetic map position has ultimately resulted in placement of *srl-2* onto the physical map, at the overlap of the cosmid clones B0361 and F56C9. Rescue of *srl-2(s2507)* was also demonstrated with an array carrying the YAC clone Y50B11, known to overlap with B0361 and F56C9.

Although it was expected that extrachromosomal array induced rescue of the *srl-2* phenotype would lead to *rol-3* mediated larval arrest (see MATERIALS and METHODS), in no case was the complete absence of Unc-46 Rol-6 animals observed (should be *phenotypically srl-2+ ; srl-2(s2507) ; rol-3(s1040*ts). It is possible that this phenomena reflects only a partial rescuing ability of each of the extrachromosomal arrays on *srl-2*. If this is the case, a partial rescue could be due to the absence of critical regulatory regions within the cosmid clones making up the extrachromosomal array. sEx238: B0361 and sEx93: F56C9 overlap by approximately 9 kb, within which 2 small

genefinder predicted genes, B0361.1 and B0361.9, reside. The most obvious interpretation is that one of these two coding elements is *srl-2*. Furthermore, It is possible that *srl-2* requires 3' and 5' regulatory regions for correct temporal and spatial expression, and neither of these are present in the overlap region. Therefore, both cosmids would have to be present simultaneously in order to confer proper *srl-2* function. This explanation would be reasonable in the absence of the upEx1: Y50B11 result. The upEx1 array contains a YAC clone approximately 350kb in size. B0361 and F56C9 are well covered by Y50B11, yet the upEx1 array also fails to completely eliminate the presence Unc-46 Rol-6 animals in the assay. Therefore, the absence of a distal regulatory region within the extrachromosomal array is likely not responsible for the apparent leakiness of the rescue event. Furthermore, it is unlikely that the presence of some Unc-46 Roller-6 animals represents a partial rescue of *srl-2* by the transgenic arrays. In most cases the compensation for absence of a wildtype gene by a transgenic array which contains only a partially functioning or mis-expressed gene product usually results in a notable difference from the expected mutant phenotype to something which is better, but not quite wildtype. In the case of an essential gene, this is usually manifest as a shift from one blocking stage, to another occurring later in development. Examples of this phenomena are observed for seven essential genes in the *dpy-5 - unc-13* region of LGI (McDowall 1996). In the case of *srl-2*, complete rescue of *srl-2* via the introduction of a functional *srl-2* gene product is expected to remove the suppression of *rol-3* lethality thus resulting in arrest. If a partially functioning *srl-2* gene product was extrachromosomaly supplied then I would expect to see a shift in phenotype resulting in an animal which was sick relative to the suppressed

animal. Development in such an animal may arrest, but would likely arrest at a stage later than that observed for *rol-3(s1040*ts) in the absence of suppression by *srl-2*. Alternatively, such animals may have decreased brood sizes or slow growth. The observation that the presence of a sEx238, sEx93 or upEx1 array is coincident with a dramatic decrease, but not a complete absence in the number of expected Unc-46 Roller-6 animals suggests that the rescue event is leaky, rather than partial. A more likely explanation for the leakiness associated with transformation rescue of *srl-2* concerns the nature of the extrachromosomal array itself, rather than the absence of a regulatory region from the clone being assayed. It is questionable that regulation of genes present as extrachromosomal arrays will be identical to the regulation of those same genes within the chromosome. Furthermore, since the arrays are thought to be multi-copy, and constructed through recombination events (MELLO *et al.* 1991), it is possible that long repetitive stretches occur within which a regulatory region, or an incomplete portion of a gene are reiterated. The former case may serve to titrate an essential trans-acting regulator, while the later may result in the production of a partially or improperly functioning gene product which may impede the function of a correctly formed gene product. These cases may result in occasional mis-function of the array in a small sub-set of animals carrying it and lead to the leaky rescue observed. It should be noted that such an event occurring in the transformation rescue of an essential gene may not be noticed. Leakiness in this case would lead to an arrested animal which may be indistinguishable from one in which the transgene was absent.

A second interpretation for the apparent leakiness associated with transformation rescue of *srl-2* can be attributed to mosaicism. If *srl-2* is expressed in only a subset of cells, and the array is lost frequently during mitosis, then in some cases, a given transgenic animal may not carry a transgenic array within a cell or subset of cells normally expressing *srl-2*. In the case of using an extrachromosomal array to repair a lethal phenotype, absence of the array in the required tissue as a result of mosaicism will result in arrest. Such animals will likely be indistinguishable from those individuals carrying no array at all. If, however, an extrachromosomal copy of a wildtype gene results in arrest, as is the case for rescue of *srl-2* in an *unc-46 rol-3*(s1040ts) background, the absence of the array in the required tissues may be evidenced by Unc Rol-6 animals. In the present case, the presence of rare Unc Rol-6 Unc-46 animals may represent mosaic animals carrying the extrachromosomal array but not in the tissues expressing *srl-2*.

GENERAL DISCUSSION

The goals of this study were two-fold. First, was the genetic characterization of mutations in the *rol-3* gene. On the basis of the mutation *rol-3(e754)*'s visible effect on gross morphology the *rol-3* gene was assumed to encode a cuticle component, possibly a collagen (HIGGINS and HIRSH 1977, COX et al. 1980). However, the generation of mutations in *rol-3* which result in lethality during larval development suggest that the *rol-3* gene product performs an essential function. I have demonstrated that the visible allele, *e754*, is a weak loss of function allele, whereas the null alleles result in developmental arrest. Furthermore, demonstration that an adult cuticle is not required for the expression of *e754*'s adult specific visible rolling phenotype suggests that *rol-3* does not encode a cuticle component. It is possible that *rol-3* produces a product which performs an essential function during early larval development, and that this function has repercussions later, after the final moult, which may indirectly effect cuticle morphology. I was successful in identifying two loci that, when mutated, are able to suppress the lethality associated with some lethal alleles of *rol-3*. Mutations at these loci, *srl-1(II)* and *srl-2(III)*, appear to effect male tail morphology suggesting that they may normally play a role in formation of this structure.

The *srl-2* locus was chosen for further study based largely on its tractability. I have shown that mutations in *srl-2* are loss of function alleles, and that these are able to suppress a number of hypomorphic *rol-3* alleles, but have no effect on null alleles. An interesting observation is that *srl-2(s2507)* is able to correct ,at least partially, the twisted body wall muscle associated with the adult visible rolling phenotype. *rol-3(s1040)* is a temperature sensitive mutation which has a recessive lethal phenotype at 20 °C, and a

visible Roller phenotype at 15 °C. At 15 °C, *unc-46 rol-3(s1040*ts) worm have twisted body wall muscles, whereas the body wall muscles in *srl-2(s2507), unc-46 rol-3(s1040*ts) animals are not twisted; furthermore, these animals do not display the characteristic rolling movement most obvious in Roller mutants.

The *rol-3* and *srl-2* gene products have not yet been identified; therefore, It is unclear at this point as to the function of these genes products at the molecular level. It is possible that *rol-3* and *srl-2* may function in the basement membrane. Since basement membrane separates the body wall muscles from those of the hypodermis, alterations here could effect both muscle and cuticle simultaneously. Although, only two basement membrane collagens (type IV) have been discovered in *C. elegans* (GUO and KRAMER 1989, GUO, JOHNSON and KRAMER 1991) immunoflourescent studies have failed to detect either of these in areas of the *C. elegans* body wall basement membrane not associated with the body wall muscles (KRAMER *personal communication*). This may indicate that their are other, as yet unidentified, components of *C. elegans* basement membrane. J. Austin (*personal communication*) has determined that the *sma-1* gene of *C. elegans* encodes a Spectrin like protein. Since some mutations in *sma-1* are associated with a Rol phenotype, this argues that defects in cytoskeleton formation can also elicit a Rol phenotype. An interesting observation made by D. Hall, (*personal communication*) is that overexpression of SPARC (an extracellular matrix protein found in basement membrane) can suppress the Rol phenotype of *rol-6(su1006). rol-6* is known to encode a cuticle collagen, and therefore, likely no direct involvement in basement membrane.

However, this result suggests that the basement membrane can have a dramatic effect on the cuticle.

The second portion of this thesis was concerned with alignment of the genetic and physical maps at the *rol-3* locus of LGV, and the *srl-2* locus of LGIII. I have anchored the LGV genetic map zone 18A to the physical map. Since *rol-3* is known to map to 18A, it is likely that *rol-3* resides near the anchor point, which is at the clone F21F8 off the LGV physical map. I have also aligned map zones 18B, 19A, 19B, and 20 during the course of this work. Using germline transformation techniques I have determined that physical locations of the *let-406, let-433*, and *let-434*, as well as have defined the portion of the physical map within which *let-412*, and *let-464* (zone 18B), and *let-416, let-409, let-340, let-334* (zone 19B) must reside. A summary of this data is presented in Figure 21.

Through correlation of the genetic and physical maps around the *srl-2* region I was able to identify two overlapping cosmids, B0361 and F56C9, which rescue the Suppression of Rol-3 phenotype associated with *srl-2(s2507)*. This result suggests that the rescuing activity is associated with the 8.6kb region common to both clones.

While attempting to rescue *srl-2* with cosmid bearing extrachromosomal arrays, I observed a small number of homozygous *srl-2 ; rol-3(s1040ts)* adult animals which also carried the transgenic array. The presence of a cosmid containing *srl-2*$^{+}$ within the array should have resulted in larval arrest since the Suppression of Rol phenotype associated with *srl-2* would be rescued. This likely reflects mosaicism resultant from the mitotic behavior of extrachromosomal arrays (STINCHCOMB *et al.* 1985, MELLO *et al.* 1991).

If mosaicism of the extrachromosomal array is responsible for the apparent leakiness associated with transgenic rescue of *srl-2*, then this raises a point of interest. The *rol-6* gene encodes a cuticle collagen. The extracellular cuticle of *C. elegans* is composed primarily of collagen which are likely secreted from the hypodermal cells. I suspect that in order for *rol-6(su1006)* to express the Rolling phenotype, the transgene must be present within some of the cells which normally express *rol-6*. Since the leaky Unc Rol-6 animals seen express the Rol-6 phenotype it is plausible that *rol-6* and *srl-2* are not expressed in the same cells.

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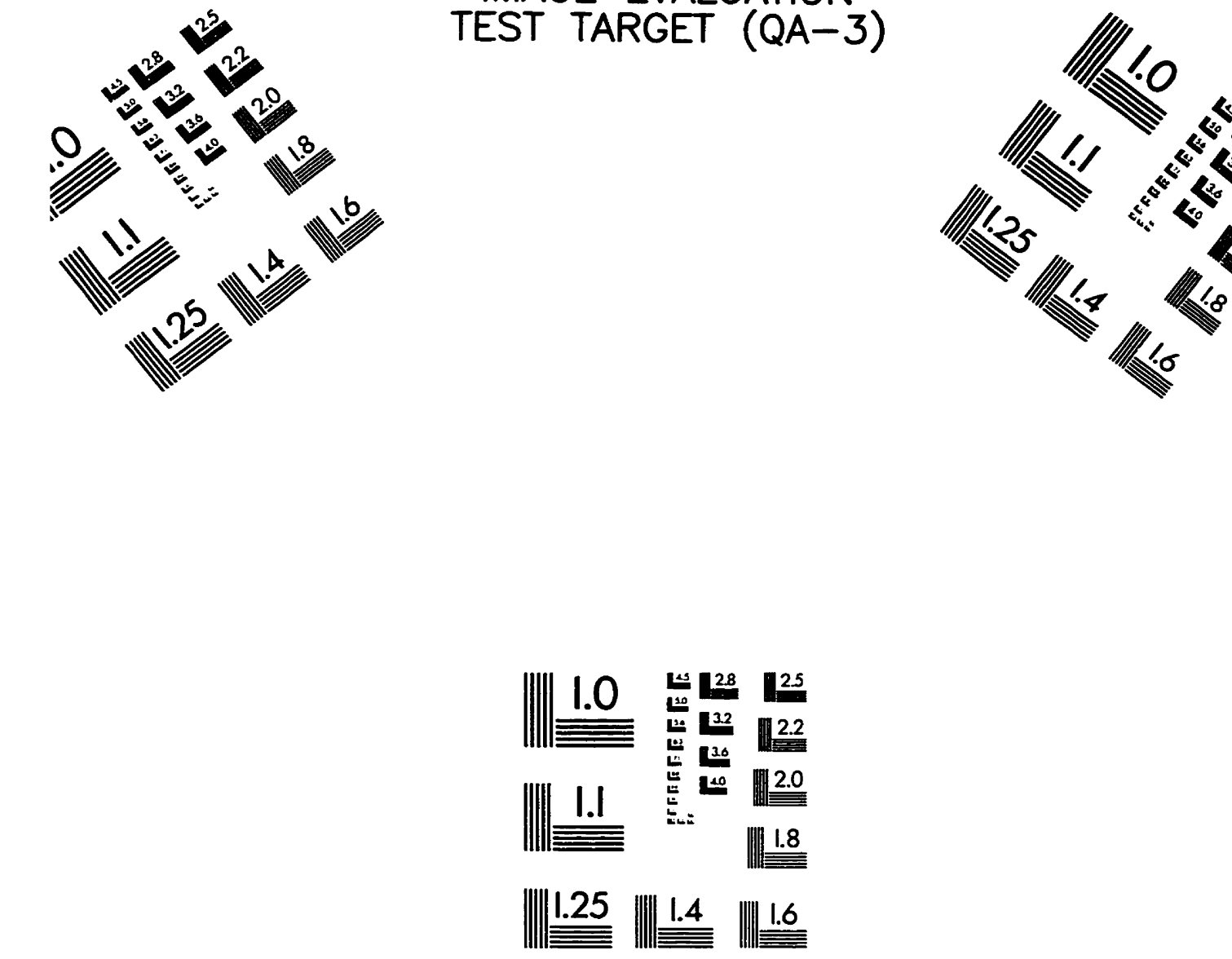

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