CHAPTER THREE

ISOLATION AND CHARACTERISATION OF MUTANTS CARRYING DOMINANT IVERMECTIN RESISTANT MUTATIONS

3.1. INTRODUCTION

Dominant IVM resistance mutations have been observed in populations of trichostrongylid parasites from Australian (2 strains of *Haemonchus contortus* (LeJambre, 1993)) and South African (1 strain of *Haemonchus contortus* (Martin and Turney, 1992)) sheep. These are the only studies that have analysed the genetics of IVM resistant strains isolated from field populations of parasitic nematodes. It appears from these studies that IVM resistant strains occurring due to selection in agricultural situations will most likely carry single dominant mutations (see Chapter 1), a sample of three is small but possibly conclusive.

To monitor the selection of alleles in parasite populations, an assay for mutations at the DNA level would be most desirable. A very sensitive assay is necessary when the mutations being monitored are dominant, as selection can be very rapid (Barnes, Dobson et *al.*, 1995). A DNA assay that can distinguish heterozygotes from homozygotes can be used to obtain data concerning the relative fitness of different genotypes under various anthelmintic treatment regimes and the resultant allele frequencies in parasite populations. Information regarding the relative fitness of homozygotes and heterozygotes in the absence of anthelmintic selection may also be valuable in predicting the persistence of resistance alleles in parasite populations.

The development of a DNA assay requires knowledge of the sequences of the genes involved. The study of dominant IVM resistance mutations in *C. elegans* outlined in this chapter was begun with the aim of identifying genes responsible for dominant IVM resistance in field populations of parasites. It was hoped that candidate genes for parasite resistance would be identified firstly, by determining the frequency of dominant mutations and the number of loci involved and secondly, by genetic mapping of one or more loci in preparation for a cloning effort.

The analysis of dominant mutations may reveal something of the mechanism of resistance to IVM in nematodes. The study of dominant negative (antimorphic) mutations is a useful tool for identification of gene function (Herskowitz, 1987). Furthermore, null (hypomorphic) mutations in genes with redundant or essential function will be mutationally silent or lethal respectively, and the study of such loci may only be facilitated by rare dominant mutations (Park and Horvitz, 1986). For example, if there is a multimeric protein in which extra subunits of type A can substitute for subunits of another type B, in the absence of B. If the gene encoding B becomes non-functional due to a null mutation, there is no resulting phenotype as A can substitute for missing B molecules. If however, the gene encoding B is mutated

so that it still binds to other protein subunits (perhaps including A), but is not capable of performing another function X, then the function of the multimeric protein can be disrupted by incorporation of mutant B molecules. This type of mutation may be dominant, as in the heterozygote the mutant B molecules are expressed and can interfere with the normal function X. Additionally this type of dominant mutation in B has defined a functional region of the protein subunit responsible for X, but not involved in binding of B with other protein subunits (example adapted from (Herskowitz, 1987)).

Two IVM resistance loci (*che-3* Grant, Pers. Comm., 1994: and *osm-3* (Tabish, Siddiqui et *al.*, 1995)) have been recently cloned and found to show sequence similarity with the microtubule motor proteins dynein and kinesin respectively, although it is not yet known whether *che-3* and *osm-3* have molecular motor function. As mutations in these genes share common defects in amphid neuronal morphology with most other identified IVM resistance (Dyf) genes (see Chapter 2) in *C. elegans*, the possibility that other IVM resistance genes might also encode molecular motor proteins or components of motor/microtubule complexes was considered.

Mutations disrupting molecular motor function share a number of genetic characteristics (Knowles and Hawley, 1991; Endow and Titus, 1992), extracopy suppression, synthetic lethality, extragenic noncomplementation, functional redundancy and dominance. As Dyf mutants most commonly have shortened or malformed sensory cilia (Lewis and Hodgkin, 1977; Perkins, Hedgecock et *al.*, 1986; DeRiso, Ristoratore et *al.*, 1994), some of these mutations might also be expected to affect the structural components of cilia. Intergenic noncomplementation between mutations in tubulin genes (Stearns and Botstein, 1988; Hays, Deuring et *al.*, 1989) and between tubulin mutations and mutations in genes encoding microtubule associated proteins (MAP) (Regan and Fuller, 1988; Interthal, Bellocq et *al.*, 1995) has been reported. The products of some Dyf genes may also be MAPs as there is now a great deal of evidence suggesting a role for these proteins in cell morphogenesis and specifically in determining dendritic as opposed to axonal outgrowths from neurons (Lee, 1993; Hirokawa, 1994).

Intergenic noncomplementation has been observed in a number of systems and has been used to imply functional interactions between the genes involved. Intergenic noncomplementation between *smg* informational suppressor mutations in *C. elegans* has led to a proposal that these genes are involved in a complex which regulates RNA degradation in cells (Hodgkin, Papp et *al.*, 1989; Pulak and Anderson, 1993), also

noncomplementation between mutations in a range of yeast loci have identified a number of genes involved in mating-type specificity (Rine and Herskowitz, 1987). Identification of unlinked mutations which failed to complement missense α-tubulin alleles, has led to the identification of testis-specific B-tubulin loci in *Drosophila* (Hays, Deuring et *al.*, 1989), and in yeast interactions between mutations in Sep1 and α-tubulin and B-tubulin, have led to a proposal that Sep1 is a microtubule associated protein (Interthal, Bellocq et *al.*, 1995). Intergenic noncomplementation can also reveal interactions between genes which encode proteins involved in consecutive cellular processes in which the gene products may not have any direct physical interaction. This idea has been used to explain partial intergenic noncomplementation between the kinesin-like genes *ncd* and *nod* in *Drosophila* (Knowles and Hawley, 1991). Mutations at either locus cause chromosome nondisjunction in homozygotes, and in double heterozygotes carrying one mutant allele at each locus, chromosomal nondisjunction is also observed (Knowles and Hawley, 1991). Knowles and Hawley (1991) propose that noncomplementation is due to an accumulative effect of partial defects in two processes: maintenance of nonexchange chromosomes in the karyosome mediated by *nod* and correct organisation of the entire spindle mediated by *ncd*.

Extensive genetic analysis of different alleles at a single locus can yield information which is central to interpretation of molecular data when this becomes available. Therefore in this chapter extensive genetic characterisation of the dominant IVM resistant mutations isolated is described. A good example of the application of genetic information to molecular work is the analysis of *unc-54* mutations in *C. elegans*, summarised in (Waterston, 1988).

By identifying four classes of *unc-54* mutations and analysing muscle ultrastructure in these, it was predicted that different classes of mutation defined different functional domains of the UNC-54 protein, the major myosin heavy-chain molecule found in thick filaments of *C. elegans* body-wall muscle. This idea was confirmed later by sequence analysis of mutants. Myosin molecules have a linear rod region (which has a characteristic coil-coil structure) and a globular head. The whole molecule is divided into three subfragments, the light meromyosin subfragment (LMM) and subfragment 2 (S2) make up the rod and subfragment 1 (S1) the head.

Two weakly dominant alleles of *unc-54* delete a portion of LMM and result in a severe reduction in the number of thick filaments in muscle cells. The thick filaments that are formed often have a larger than normal diameter. Homozygotes carrying

these mutations are almost paralysed and twitching of the surface of the animal can be observed.

A second class of mutations have near normal or abnormal muscle ultrastructure and move slowly. Many of these also show dominant suppression of *unc-22*(s12). These recessive mutations all have been localised to the S1 subfragment. Alleles in this class with normal muscle ultrastructure have alterations adjacent to the ATP binding site or in the SH1 thiol residue, a region also hypothesised to function in ATP-hydrolysis, and alleles which confer altered muscle ultrastructure alter the sequence of the 54-kD fragment central to S1.

Genetic mapping has placed a third incompletely dominant class of mutations in a very small region of the *unc-54* gene. Sequencing of one of these (e1152) has shown a replacement of a Gly-Lys amino acid pair with Arg-Met in a position in the S2 subfragment adjacent to its junction with S1. These mutant animals move slowly as heterozygotes and are severely paralysed or non-viable as homozygotes. They produce normal levels of UNC-54 but the thick filaments are characteristically disorganised and much thicker than in wild type muscle. It seems likely that these mutations result in conformational changes in the protein, probably associated with the orientation of the rod relative to the head regions of the molecule.

Fourthly, recessive null-mutations in *unc-54* cause the number of thick filaments to be reduced by about 75%. These are the most common *unc-54* mutants isolated and sequencing has revealed that these mutations typically are nonsense mutations or frame-shift mutations within the 3' region of the gene, resulting in dysfunctional protein products. These mutants are morphologically distinguishable from other *unc-54* mutants, being recessive and resulting in paralysis, shorter body length and transparent appearance in adults.

Genetic interactions between *unc-54*, *unc-15*, *unc-22* and *unc-45* mutants have also led to predictions about the interactions between their gene products. *unc-54;unc-15* double mutants are more severely uncoordinated than *unc-54* alone, suggesting an additive effect of the two mutations. This is supported by molecular characterisation of these genes identifying the encoded proteins as being separate structural units of muscle thick filaments (*unc-54* = myosin and *unc-15* = paramyosin). In contrast *unc-54;unc-45* mutants are no more uncoordinated than mutants carrying either mutation alone, implying that both mutations affect the same component of muscle fibres. *unc-54* mutations, with defects in the first subfragment of the gene, are able to suppress the twitcher phenotype of *unc-22* mutations, suggesting an interaction between the UNC-22 protein and UNC-54

myosin. The use of antibodies to the UNC-22 protein have allowed it to be localised to the A band of body-wall muscle, apparently supporting the notion that these proteins have a close physical interaction. Analysis of the mode of inheritance and pleiotropic phenotypes of these mutations along with identification of genetic interactions, was essential to the final outcomes of these experiments.

This chapter outlines the identification and characterisation of four dominant IVM resistance mutations from two loci in *C elegans*. Some common characteristics of IVM resistance mutations of *C. elegans* were discussed in Chapter 2. Defects in amphid neuron cilia (Dyf phenotype) and temperature sensitive defects in viability of strains on 5 ng/mL IVM were the principal pleiotropic effects associated with resistance loci. To elaborate on these findings, observations of growth of dominant IVM resistance strains at high temperature were made and the Dyf phenotype of homozygotes and heterozygotes was also established. The resistance of homozygotes and heterozygotes to different concentrations of IVM was also analysed. To anticipate molecular findings, intergenic complementation tests, for the Dyf phenotype, between the dominant IVM resistance alleles and also between these alleles and mutations at other loci were undertaken. Chapter 4 will continue this analysis, investigating interactions between Dyf genes and other mutations. Studies of genes affecting muscle ultrastructure and function have greatly increased understanding of muscle function, and it is hoped that the present study will mark the beginning of similar work which will eventually yield an increased understanding of sensory cilia function in *C. elegans*..

3.2. METHODS

3.2.1. Mutagenesis screening for mutants carrying dominant IVM resistant mutations.

The following protocol was designed to detect IVM resistance mutations from the F_1 progeny of worms mutagenised with ems.
Mutations isolated from a screen such as this are either spontaneous recessive mutations or dominant ems-induced mutations. From any large sample of worms there are also a number which grow to adult on IVM plates but which do not produce any resistant progeny (Kim and Johnson, 1991), such 'non-heritable' resistant isolates are also expected to be found in mutagenesis screens but these are easily eliminated by picking individual worms to IVM plates after screening. Recessive ems mutations would not be observed in the F_1.

Progeny of 120 N2 (Wild type) hermaphrodites were grown to adult stage (5 days at 20°C). Animals were washed off the plates and treated with alkaline hypochlorite solution (Wood, 1988) to dissolve the adults but leave eggs *in utero* intact. Eggs were then divided into ten aliquots for inoculation onto ten seeded 10 cm NGM plates. The now synchronous culture of animals was grown to L4 stage (three days at 20°C). L4 larvae were washed off plates and washed once in M9 worm buffer. They were then resuspended in 5 mL 0.05M ems in a 50 mL tube and left at room temperature (approx. 20°C) with agitation for four hours (Brenner, 1974; Wood, 1988). Worms were washed three times to remove ems and divided into ten aliquots which were placed on seeded NGM plates. After allowing recovery overnight, worms were washed off plates, treated with alkaline hypochlorite, washed twice in M9 buffer and resuspended in 5 mL of this buffer. After removing a 10 µL aliquot for determination of egg numbers, the remaining eggs (F_1) were placed on 50 seeded 5 cm IVM plates (5 ng/mL IVM in NGM agar). Worms which grew to adult on IVM plates were picked individually to fresh IVM plates and their progeny retained as resistant isolates. To ensure the independence of IVM resistance mutations selected, only one resistant isolate was kept from each plate. For determination of egg numbers, the 10 µL of egg suspension was added to 290 µL of M9, and this was divided equally and placed onto three seeded NGM plates. By counting the number of eggs which hatched and grew into adult worms from each plate, and multiplying the mean of these by 1500, the number of F_1s screened was calculated.

Three mutagenesis screening experiments, as described above, were undertaken in an attempt to isolate dominant IVM resistance mutations. A control experiment designed to detect spontaneous IVM resistance mutations was also conducted which was identical except that no ems was used.

3.2.2. Confirmation of dominance.

The rate of spontaneous mutation for IVM resistance is high ($2x10^{-5}$) (Kim and Johnson, 1991). The frequency of ems-generated recessive IVM resistance mutations is extremely high ($4.2x10^{-3}$, or 1 in 240 haploid genomes). The screening procedure described above, which is designed to detect dominant IVM resistance mutations, could also detect spontaneous recessive IVM resistance mutations. Because three generations of worms were grown to produce the parental worms used in the mutagenesis experiment (above), and because these generations were produced only by selfing, a spontaneous mutation arising in the first, second or third generation will give rise to many homozygotes in the F_1 of the mutagenesis experiment. To allow for this possibility, strains isolated from the mutagenesis screen were re-screened to confirm dominance.

Because of the large number of strains requiring re-screening, it was decided to evaluate the resistance of heterozygotes in liquid media (S-media, IVM & bacteria). IVM response of wild type worms differed in liquid as compared to in agar. Wild type worms grew at 4 ng/mL IVM in liquid but not at 6 ng/mL. 8 ng/mL was used to evaluate resistance of heterozygotes and some were also grown in media free of IVM as controls.

Resistant strains were crossed to N2 males. Males from this cross (which were heterozygous or hemizygous for the resistant mutation) were crossed to a marked strain (*dpy-13*(*e184*) or *unc-18*(*e81*)) on seeded NGM plates overnight. Mated hermaphrodites were transferred to IVM (8 ng/mL in liquid for *dpy-13*; 5 ng/mL in agar for *unc-18*) where they laid some eggs. Strains were scored as dominant if nonDpy worms grew to be fertile adults within ten days. Strains whose heterozygous progeny were IVM resistant in liquid were re-tested on agar to confirm the genetic dominance of these mutations.

3.2.3. Construction of new strains from the strains isolated in the mutagenesis experiments.

3.2.3.1. Outcrossing dominant IVM resistant strains.

To eliminate any additional mutations that may have been induced in the dominant IVM resistant strains, the strains were outcrossed to the wild type N2 strain in the following manner:

- N2 males were crossed with IVM resistant hermaphrodites.
- Hemizygous (or heterozygous) F_1 males carrying the resistance mutation were then crossed with *unc-18*(e81) hermaphrodites.
- nonUnc F_1 hermaphrodites were transferred to 5 ng/mL IVM plates.
- IVM resistant F_2 were picked individually to NGM plates and progeny of individuals that did not segregate any Unc F_3 were selected as outcrossed homozygous IVM resistant strains.
- This cycle was repeated three times to obtain six times outcrossed nr272, nr2389 and nr2477 strains; and was repeated twice to obtain a four times outcrossed nr2344 strain. (NB - this strategy was developed to ensure homozygosity of X-linked IVM resistance loci by eliminating the *unc-18* allele used in repulsion). For nr2389 however, a number of cycles of selfing on IVM plates was necessary as elimination of *unc-18*(e81) did not ensure homozygosity of nr2389; as this mutation was found to be autosomal.

3.2.3.2. Construction of marked IVM resistant strains.

A number of dominant IVM resistant strains were created which were homozygous for visible mutations. These strains were necessary for detection of heterozygous progeny in crosses.

(nr2389)I/(nr2389)I,*unc-60*(e723)V/*unc-60*(e723)V; (nr272)X/(nr272)X,*unc-60*(e723)V/*unc-60*(e723)V; (nr2344)X/(nr2344)X,*unc-60*(e723)V/*unc-60*(e723)V and (nr2477)X/(nr2477)X,*unc-60*(e723)V/*unc-60*(e723)V strains were made by crossing hemizygous (nr272/0, nr2344/0 or nr2477/0) or heterozygous (nr2389/+) males with *unc-60*(e723)/*unc-60*(e723) hermaphrodites. Wild type F_1 progeny were picked at the L4 stage to seeded NGM plates and moved each day to fresh plates for four consecutive days. Unc F_2 progeny were then picked (6-10 per plate) to 5 ng/mL IVM plates and resistant Unc progeny selected. Resistant Unc worms were selfed for about six generations on 5 ng/mL IVM plates. For these strain constructions hemizygous and heterozygous males were made by crossing N2 males with outcrossed dominant IVM resistant strains.

unc-18(e81) nr272/*unc-18*(e81) nr272 was also made using the above protocol except nr272/+ males for the initial cross with *unc-18*(e81)/*unc-18*(e81) were made by crossing N2 with the original (non-outcrossed) nr272 strain.

dpy-8(e130) (nr2344)/*dpy-8*(e130) (nr2344); *dpy-8*(e130) nr2477/*dpy-8*(e130) (nr2477) and *dpy-8*(e130)X/*dpy-8*(e130)X; (nr2389)I/(nr2389)I strains were constructed by mating hemizygous (nr272/0, nr2344/0 or nr2477/0) or heterozygous (nr2389/+) males with *unc-18*(e81)*dpy-8*(e130)/*unc-18*(e81)*dpy-8*(e130) hermaphrodites. Wild type F_1 progeny were picked at the L4 stage to seeded NGM plates and moved each day to fresh plates for four consecutive days. Dpy F_2 progeny were then picked (6-10 per plate) to 5 ng/mL IVM plates and resistant Dpy progeny selected. Resistant Dpy worms were selfed for about six generations on 5 ng/mL IVM plates. For these strain constructions hemizygous and heterozygous males were made by crossing N2 males with the originally isolated dominant IVM resistant strains.

3.2.4. IVM dose response of homozygotes and heterozygotes.

3.2.4.1. Determination of resistance of homozygotes to different IVM concentrations.

Homozygotes of the strains nr272, nr2344, nr2389 and nr2477 were tested for IVM resistance on 5, 10, 15 and 20 ng/mL IVM plates. The method followed for observation of growth on IVM plates was similar to that described previously (methods - Chapter 2); and both outcrossed and original strains were analysed. Briefly, six to ten adult hermaphrodites were placed onto plates of different drug concentration, and a given strain was determined to be resistant to a particular IVM concentration if fertile adult worms were present after fourteen days at 20°C. Each IVM concentration was tested in duplicate and sensitive N2 worms were also tested for IVM resistance on separate plates at all concentrations as a negative control. If N2 was observed to grow on any plates of a given concentration, the experiment was repeated with a fresh batch of plates.

3.2.4.2. Determination of resistance of heterozygotes to different IVM concentrations.

Mated hermaphrodites from the crosses outlined below were placed onto IVM plates containing 5, 10, 15 or 20 ng/mL IVM. Some of the hermaphrodites used were sensitive to IVM, but outcrossed progeny were generally produced on the plates. Typically, four to six mated hermaphrodites were placed on each

plate and each IVM concentration was tested in duplicate. Sensitive N2 worms were also tested for IVM resistance on separate plates at all concentrations as a negative control. If N2 was observed to grow on any plates of a given concentration, the experiment was repeated with a fresh batch of plates. A given genotype was determined to be resistant to a particular IVM concentration if fertile adult worms were present after fourteen days at 20°C.

Possible maternal effects were examined by comparing heterozygotes which had inherited the resistance allele from the male parent (eg. nr2389^{p}/+^{m}) with those which had inherited the resistance allele from the hermaphrodite parent (e.g. nr2389^{m}/+^{p}). To control for maternal effects of marker mutations, crosses were designed so that the marker mutation used (*unc-60*(e723)) was inherited maternally in both types of crosses.

3.2.4.3. Generation of heterozygotes for dose response experiments.

unc-18(e81) +/+ nr272; *unc-18*(e81) +/+ nr2477; *unc-18*(e81) +/+ nr2344 and *unc-18*(e81)/+,nr2389/+ worms were obtained by crossing hemizygous (nr272/0, nr2344/0 or nr2477/0) or heterozygous (nr2389/+) males with *unc-18*(e81)/*unc-18*(e81) hermaphrodites. For these crosses the original strains isolated from the mutagenesis screen were used to generate males by crossing them with N2 males.

nr2389^{m}/+^{p},*unc-60*(e723)^{m}/+^{p}; nr272^{m}/+^{p},*unc-60*(e723)^{m}/+^{p}; nr2344^{m}/+^{p},*unc-60*(e723)^{m}/+^{p} and nr2477^{m}/+^{p},*unc-60*(e723)^{m}/+^{p} worms were made by crossing N2 males with nr2389/nr2389,*unc-60*(e723)/*unc-60*(e723); nr272/nr272,*unc-60*(e723)/*unc-60*(e723); nr2344/nr2344,*unc-60*(e723)/*unc-60*(e723) and nr2477/nr2477,*unc-60*(e723)/*unc-60*(e723) hermaphrodites respectively. For these crosses, the outcrossed IVM resistant strains were used to generate the marked hermaphrodite strains (as per the method outlined above). This cross generates worms which have inherited both the IVM resistance gene and *unc-60*(e723) maternally.

+^{m}/nr2389^{p},*unc-60*(e723)^{m}/+^{p}; +^{m}/nr272^{p},*unc-60*(e723)^{m}/+^{p}; +^{m}/nr2344^{p},*unc-60*(e723)^{m}/+^{p} and +^{m}/nr2477^{p},*unc-60*(e723)^{m}/+^{p} worms were made by crossing hemizygous (nr272/0, nr2344/0 or nr2477/0) or homozygous (nr2389/nr2389) males with *unc-60*(e723)/*unc-60*(e723) hermaphrodites. For these crosses, the outcrossed IVM resistant strains were used to generate the hemizygous males by crossing them with N2 males: nr2389/nr2389 males were obtained from a male stock created by heat shock and maintained as outlined in (Wood, 1988).

3.2.5. Determination of Dyf phenotype of homozygotes and heterozygotes.

3.2.5.1. Staining of amphid neurons of IVM resistant strains.

Worms were grown on NGM plates at 20°C. Worms of the appropriate genotype were picked to seeded DiO plates (see below) and left in a darkened container in the 20°C incubator overnight: a staining method based on the protocol published by (Hedgecock, Culotti et *al.*, 1985). The next morning, worms were picked from the plates into 5 µL Terasaki plate wells filled with 0.01% NaN_3 in M9 buffer. Animals were then observed under fluorescent light with 40X magnification on a Zeiss compound microscope. Kodak Ektachrome 64T colour reversal film was used for photography of stained worms at 40X magnification using a 60 second exposure time. The proportion of worms showing any staining of amphid neurons (nonDyf) was recorded as a quantitative measure of penetrance of the Dyf phenotype. 95% confidence intervals for proportions of nonDyf worms were calculated from tabulated values given in (Rohlf and Sokal, 1969). Also noted was the intensity of staining of amphid neurons, and photographs are presented in the results section to illustrate differences in staining intensity observed for heterozygote worms.

3.2.5.2. DiO Plates

DiO plates were made by allowing 400 µL of 20 µg/mL DiO (3,3'-dioctadecyloxacarbocyanine perchlorate - (Haugland, 1992)) in M9 buffer to soak into NGM plates (seeded with *E. coli* OP50) for 2 hours in a darkened container.

3.2.5.3. Generation of heterozygotes for staining.

unc-18(e81)+/+nr272, *unc-18*(e81)+/+nr2477, *unc-18*(e81)+/+nr2344 and *unc-18*(e81)/+X;nr2389/+I worms were obtained by crossing hemizygous (nr272/0, nr2344/0 or nr2477/0) or heterozygous (nr2389/+) males with *unc-18*(e81)/*unc-18*(e81) hermaphrodites. Mated hermaphrodites were picked to NGM or 5 ng/mL IVM plates and outcrossed worms collected four days later. For these crosses the original strains isolated from the mutagenesis screen were used to generate males by crossing them with N2 males.

unc-18(e81)nr272/++, *dpy-8*(e130)nr2344/++, *dpy-8*(e130)nr2477/++ and *dpy-8*(e130)/+X;nr2389/+I worms were obtained by crossing N2 males with *unc-18*(e81)nr272/*unc-18*(e81)nr272, *dpy-8*(e130)nr2344/*dpy-8*(e130)nr2344, *dpy-8*(e130)nr2477/*dpy-8*(e130)nr2477 and *dpy-8*(e130)/*dpy-8*(e130)X;nr2389/nr2389I hermaphrodites respectively. Mated hermaphrodites were picked to NGM or 5 ng/mL IVM plates and

outcrossed worms collected four days later. For these crosses the original strains isolated from the mutagenesis screen were used to generate the marked hermaphrodite strains (as per the method outlined above).

nr2389^{m}/+^{p}X;*unc-60*(e723)^{m}/+^{p}V, nr272^{m}/+^{p}X;*unc-60*(e723)^{m}/+^{p}V, nr2344^{m}/+^{p}X;*unc-60*(e723)^{m}/+^{p}V and nr2477^{m}/+^{p}X;*unc-60*(e723)^{m}/+^{p}V worms were made by crossing N2 males with nr2389/nr2389I;*unc-60*(e723)/*unc-60*(e723)V, nr272/nr272X;*unc-60*(e723)/*unc-60*(e723)V, nr2344/nr2344X;*unc-60*(e723)/*unc-60*(e723)V and nr2477/nr2477X;*unc-60*(e723)/*unc-60*(e723)V hermaphrodites respectively. For these crosses, the outcrossed IVM resistant strains were used to generate the marked hermaphrodite strains (as per the method outlined above). This cross generates worms which have inherited both the IVM resistance gene and *unc-60*(e723) maternally.

+^{m}/nr2389^{p}I;*unc-60*(e723)^{m}/+^{p}V, +^{m}/nr272^{p}X;*unc-60*(e723)^{m}/+^{p}V, +^{m}/nr2344^{p}X;*unc-60*(e723)^{m}/+^{p}V and +^{m}/nr2477^{p}X;*unc-60*(e723)^{m}/+^{p}V worms were made by crossing hemizygous (nr272/0, nr2344/0 or nr2477/0) or homozygous (nr2389/nr2389) males with *unc-60*(e723)/*unc-60*(e723) hermaphrodites. For these crosses, the outcrossed IVM resistant strains were used to generate the hemizygous males by crossing them with N2 males: nr2389/nr2389 males were obtained from a male stock created by heat shock and maintained as outlined in (Wood, 1988). The crosses outlined here generate worms which have inherited the IVM resistance gene paternally and *unc-60*(e723) maternally. These were made to allow direct comparison with worms which had inherited resistance genes maternally. (NB. In both cases any maternal influence of the *unc-60* marker is controlled, because both types of heterozygotes have an *unc-60/unc-60* hermaphrodite parent).

3.2.6. Linkage analysis of dominant strains.

3.2.6.1. Sex linkage.

Experiments used to confirm the dominance of the mutations nr272, nr2344, nr2389 and nr2477 were also used to examine sex-linkage of these mutations. Heterozygous or hemizygous males carrying the resistance gene were generated by crossing to N2 and these males were crossed to *unc-18*(e81) hermaphrodites. If the mutations were X-linked, no male progeny would be expected to be resistant to IVM, as these individuals inherit their only X-chromosome from the wild type hermaphrodite parent. In contrast, an autosomal mutation would be passed onto male progeny, and resistant male heterozygotes would be observed.

The absence of male progeny on IVM plates could indicate either sex-linkage or that IVM resistance in heterozygotes is limited to the hermaphrodite sex. The possibility of sex-limitation cannot be ignored when interpreting the results of these experiments.

3.2.6.2. Identification of linkage by construction of IVM resistant Unc strains.

By crossing nr2389/nr2389 males with *unc* hermaphrodites carrying *unc-15*(e73)I, *unc-4*(e120)II, *unc-32*(e189)III, *unc-24*(e138)IV, *unc-60*(e723)V or *unc-18*(e81)X, and picking F_1 progeny to IVM plates, IVM resistant Unc worms can be observed in the F_2. If, however, the *unc* and IVM resistance mutations are linked, very few recombinant F_2 individuals are expected to be seen. The nr2389 mutation was mapped to a linkage group using this method.

3.2.6.3. Construction of doubly-marked strains for three factor mapping.

Dpy, Unc and other visible phenotypes are easy to score and are conferred by mutations at a sufficiently large number of loci to make them extremely useful for three factor genetic mapping. To construct strains doubly homozygous for *dpy* and *unc* mutations and that are useful for mapping, two markers which lie between one and five map units apart are chosen. Three of the IVM resistance mutations (nr272, nr2344 and nr2477) to be mapped were suspected to be X-linked. Strains which carried X-linked *dpy* and *unc* mutations therefore were needed for three factor mapping. *dpy-8*(e130)*unc-18*(e81)/*dpy-8*(e130)*unc-18*(e81) and *dpy-7*(e88)*unc-9*(ec27)/*dpy-7*(e88)*unc-9*(ec27) were constructed. The genetic map distance between *dpy-7* and *unc-9* is larger than ideal (12.85 cM), however there are few visible markers in this region of the X chromosome.

unc-18(e81)/0 males are not capable of mating, however *dpy-8*(e130)/0 males can mate with hermaphrodites occasionally. To construct *dpy-8*(e130)*unc-18*(e81)/*dpy-8*(e130)*unc-18*(e81) hermaphrodites, *dpy-8*(e130)/0 males were made by crossing *dpy-8*(e130) homozygotes with N2 males. These were then crossed with *unc-18*(e81) homozygotes and wild type hermaphrodite progeny were selected at the L4 stage, picked to seeded NGM plates and moved each day to fresh plates for four consecutive days. Many Unc F_2 progeny were selected and picked (4/plate) to NGM plates and after four days *dpy-8*(e130)*unc-18*(e81)/*dpy-8*(e130)*unc-18*(e81) F_3 recombinant worms were selected and their progeny maintained as a hermaphrodite stock for use in three factor genetic mapping.

A *dpy-7*(e88)*unc-9*(ec27)/*dpy-7*(e88)*unc-9*(ec27) strain was constructed in a similar manner, however two P_0 crosses were used: *unc-9*(ec27)/0 males with *dpy-7*(e88)/*dpy-7*(e88) hermaphrodites and *dpy-7*(e88)/0 males with *unc-9*(ec27)/*unc-9*(ec27) hermaphrodites. Both *dpy-7*(e88)/0 and *unc-9*(ec27)/0 males mate occasionally.

3.2.6.4. Three factor mapping of IVM resistance mutations.

The three factor mapping protocol used for mapping dominant IVM resistance mutations is based on the method outlined in (Wood, 1988). The method was used to order IVM resistance (*avr*) mutations with respect to two other mutations (a *dpy* and an *unc* allele) by construction and selfing worms of genotype + *avr* +/*dpy* + *unc*. The F_1 progeny from selfing + *avr* +/*dpy* + *unc* worms are principally DpyUnc or wild type in appearance, but among the progeny are Dpy nonUnc and Unc nonDpy recombinant individuals. To order the *avr* mutation with respect to the *dpy* and *unc* mutations, Dpy nonUnc and Unc nonDpy F_2 worms were picked to seeded IVM plates (1/plate) and their F_3 progeny scored for IVM resistance. It was necessary to score F_3 progeny as some Unc phenotypes are difficult to score on IVM plates and as some Dpy worms grow very slowly on IVM plates. Using this protocol, the proportions of Dpy nonUnc recombinants and Unc nonDpy recombinants that carried the IVM resistance allele could be determined.

Wild type + *avr* +/*dpy* + *unc* worms were made by crossing hemizygous (nr272/+, nr2344/+ or nr2477/+) or homozygous (nr2389/nr2389) males with *dpy unc*/*dpy unc* hermaphrodites. For mapping nr272, nr2344 and nr2477, *dpy-8*(e130)*unc-18*(e81)/*dpy-8*(e130)*unc-18*(e81) and *dpy-7*(e88)*unc-9*(ec27)/*dpy-7*(e88)*unc-9*(ec27) hermaphrodites were generated according to the method outlined above and were used for mapping. For mapping nr2389, *dpy-14*(e188)*unc-29*(e403)/*dpy-14*(e188)*unc-29*(e403) and *dpy-24*(s71)*unc-75*(e950)/*dpy-24*(s71)*unc-75*(e950) hermaphrodites were used for mapping (these were a gift from Dr. A. Rose, University of British Columbia).

3.2.6.5. Interpretation of three-factor mapping data.

If recombinant animals of only one type (Dpy nonUnc or Unc nonDpy) were found to be resistant, then the *avr* mutation was positioned outside of the *dpy-unc* genetic interval on the side closest to the locus in which homozygous mutant animals were all sensitive to the drug. This occurrence also confirms linkage of the IVM resistance gene to the other two markers (random segregation would always make unlinked genes appear to be between the *dpy* and *unc* markers).

The presence of both Dpy nonUnc and Unc nonDpy resistant worms however indicates the IVM resistance loci is positioned between the *dpy* and *unc* markers. In all cases, linkage was also validated by three factor data excluding the IVM resistance mutation from another linked (but not overlapping) interval. Once IVM resistance loci were mapped to a *dpy-unc* genetic interval, the relative position of the *avr* locus was calculated. If we call the *dpy-unc* genetic distance *d* cM, the number of IVM resistant Dpy nonUnc recombinants *D*, the number of IVM resistant Unc nonDpy recombinants *U*, and the total numbers of Dpy nonUnc and Unc nonDpy recombinants scored for resistance *tD* and *tU* respectively; then the position of the IVM resistance locus is:

(*D* /*tD*) X *d* from the *dpy* locus, or
(*U*/*tU*) X *d* from the *unc* locus.

As these map positions are based on proportions of the *dpy-unc* distance, confidence intervals can be calculated by multiplying tabulated confidence intervals for percentages by the *dpy-unc* map distance. 95% confidence intervals were taken from tabulated values given in (Rohlf and Sokal, 1969).

3.2.7. Complementation testing of nr272, nr2344, nr2477 and nr2389 against each other and other Dyf loci for the Dyf phenotype.

3.2.7.1. Staining of worms and quantification of penetrance of the Dyf phenotype.

This was undertaken using the protocol for staining of heterozygotes outlined in section 3.2.5.1.. Confidence limits were also calculated as above.

3.2.7.2. Construction of double heterozygotes for DiO staining.

nr272/0, nr2344/0, nr2477/0 and nr2389/+ males were mated in all combinations with nr272/nr272X;*unc-60*(e723)/*unc-60*(e723)V, nr2344/nr2344X;*unc-60*(e723)/*unc-60*(e723)V, nr2477/nr2477X;*unc-60*(e723)/*unc-60*(e723)V, and nr2389/nr2389X;*unc-60*(e723)/*unc-60*(e723)V hermaphrodites to generate nr272+/+nr2344X;*unc-60*(e723)/+V, nr272+/+nr2477X;*unc-60*(e723)/+V, nr2477+/+nr2344X;*unc-60*(e723)/+V, nr272/+X;nr2389/+I;*unc-60*(e723)/+V, nr2477/+X;nr2389/+I;*unc-60*(e723)/+V, and nr2344/+X;nr2389/+I;*unc-60*(e723)/+V, F_1 progeny for DiO staining. Reciprocal crosses in which the resistant alleles were inherited from the male or hermaphrodite parent were used in order to analyse any maternal effects of one or other of the

mutations used, the marker mutation *unc-60*(e723)V was inherited maternally for all heterozygotes observed.

nr272/0, nr2344/0 and nr2477/0 males were combined with *daf-10*(e1387)/*daf-10*(e1387)IV;*unc-60*(e723)/*unc-60*(e723)V, *dyf-6*(mn346)/*dyf-6*(mn346)X;*unc-60*(e723)/*unc-60*(e723)V, *dyf-7*(m537)/*dyf-7*(m537)X;*unc-60*(e723)/*unc-60*(e723)V and *dyf-12*(sa127)/*dyf-12*(sa127)X;*unc-60*(e723)/*unc-60*(e723)V hermaphrodites to produce double heterozygous nonUnc F_1 progeny for DiO staining.

Homozygous nr2389/nr2389 males were crossed with homozygous hermaphrodite *che-3*(nr5), *che-3*(e1124), *che-13*(e1805), *mec-8*(e398), *dyf-1*(mn335), *dyf-5*(mn400), *dyf-10*(e1383), *che-10*(e1809), *dyf-13*(e1383), *daf-19*(m86) or *daf-10*(e1387) to create nr2389/+, X/+ double heterozygotes for staining.

3.2.7.3. Interpretation of complementation testing.

The Dyf phenotype is incompletely dominant in nr272, nr2344 and nr2477. Complementation in double heterozygotes involving these mutations is indicated when the proportion of Dyf double heterozygotes is not-significantly higher (p=0.05) than for nr272/+, nr2344/+ or nr2477/+ alone. Failure of complementation is indicated when 100% of the double heterozygotes are Dyf. Partial noncomplementation was indicated in some instances when the number of Dyf double heterozygotes was less then 100% but significantly more than for nr272/+, nr2344/+ or nr2477/+.

Dyf is completely recessive in nr2389, therefore 100% of double heterozygotes will be nonDyf for mutations which complement nr2389. The presence of any Dyf double heterozygotes is an indication of partial noncomplementation; complete noncomplementation is indicated by 100% Dyf double heterozygotes.

3.2.8. Growth of dominant IVM resistance strains at 27°C.

N2, *che-3*(nr5), nr272, nr2344, nr2389 and nr2477 hermaphrodites were grown at 20°C on NGM plates seeded with *E. coli* OP50. Eggs were isolated by dissolving adults in alkaline hypochlorite solution. The eggs were allowed to hatch and grow on 5 ng/mL IVM or NGM plates pre-incubated at 27°C. After three days (NGM), or four days (IVM), animals (P_0) which grew were transferred to one of four treatments:

- IVM at 20°C;
- IVM at 27°C;
- NGM at 20°C;
- NGM at 27°C.

The presence of F_1 worms was scored fourteen days after transfer of the P_os. The development of F_1 worms, and their F_2 progeny when necessary, was observed.

Due to faulty temperature control on the incubator used, this experiment was completed at 27°C rather than 25°C, at which *C elegans* grows well and the formation of dauer larvae is facilitated (Golden and Riddle, 1984). The results of this experiment therefore are difficult to interpret, although wild type controls grew well at 27°C.

3.3. RESULTS

3.3.1. Mutagenesis screen for Dominant IVM resistance mutations.

$1.2x10^6$ F_1 genomes were screened in three ems mutagenesis experiments and 20 independent strains, resistant to 5 ng/mL IVM, were obtained from the F_1 progeny of mutagenised hermaphrodites. These isolates were inbred by selfing individual IVM resistant worms for approximately six generations. Heterozygous males were made by crossing the isolated strains with N2 males. By mating these males with non-resistant hermaphrodites and looking for IVM resistant F_1 worms, the dominance of the mutation was confirmed. For all twenty mutant strains, the IVM resistant phenotype was found to be inherited recessively, so these probably carried spontaneous recessive mutations.

In a further attempt to isolate dominant IVM resistant mutations, 106 isolates were obtained from a mutagenesis screen undertaken by Dr. C. Johnson (Nemapharm U.S.A., 1992). In a secondary screen similar to that outlined above, five of these 106 strains were found to carry dominant IVM resistance mutations. If the rate of recovery of false positives was equivalent in the screen undertaken here and the screen undertaken at Nemapharm, then we could assume that the number of haploid F_1 genomes screened to obtain the 106 isolates was at least; $[106/20] \times [1.2x10^6] = 6.4x10^6$. The mutation rate for dominant mutations is therefore at least as low as $[5/(6.4x10^6)] = 7.9 \times 10^{-7}$.

The spontaneous rate of mutation for IVM resistance was also examined by conducting an identical screen of non-mutagenised worms. In this screen, twenty independent strains were isolated from $1.2.x10^6$ haploid genomes to give a mutation frequency of $1.7x10^{-5}$.

Table - 3.1. Dose response of outcrossed homozygotes and heterozygotes of four dominant IVM resistance mutations.

Genotype tested	IVM concentration (ng/mL)			
	5	10	15	20
nr272m/nr272m	+	+	+	-
nr272m/nr272m,*unc-60* m/*unc-60* m	+	+	-	-
nr272m/+p,*unc-60* m/+p	+	-	-	-
nr272p/+m,*unc-60* m/+p	+	+	-	-
nr2344m/nr2344m	+	+	-	-
nr2344m/nr2344m,*unc-60* m/*unc-60* m	+	+	-	-
nr2344m/+p,*unc-60* m/+p	+	+	-	-
nr2344p/+m,*unc-60* m/+p	+	+	-	-
nr2389m/nr2389m	+	+	+	+
nr2389m/nr2389m,*unc-60* m/*unc-60* m	+	+	-	-
nr2389m/+p,*unc-60* m/+p	-	-	-	-
nr2389p/+m,*unc-60* m/+p	+	-	-	-
nr2477m/nr2477m	+	+	+	-
nr2477m/nr2477m,*unc-60* m/*unc-60* m	+	+	-	-
nr2477m/+p,*unc-60* m/+p	+	+	-	-
nr2477p/+m,*unc-60* m/+p	+	-	-	-

m - chromosome inherited from hermaphrodite (maternal) parent
p - chromosome inherited from male (paternal) parent

The table shows results of analysis of heterozygotes and homozygotes of nr272, nr2344, nr2389 and nr2477 for IVM resistance at 5, 10, 15 and 20 ng/mL. Heterozygotes which have inherited the drug resistance gene maternally or paternally are shown separately. + indicates growth of all worms on IVM and - indicates failure of all worms to reproduce on that drug concentration. The homozygous strains used in the experiments are outcrossed to N2 six times, except for nr2344 which was outcrossed four times, selecting for IVM resistance in the heterozygote in every second generation.

protocol but with the outcrossed strains (Compare table 3.1. with results presented in appendix B.1.1. - The disorientation of sensitive worms on IVM makes the use of an uncoordinated mutant parent necessary to ensure F_1 eggs are laid on the plates and mated hermaphrodites are not lost on the plate edge (see methods). Different *unc* mutations were used in the generation of heterozygotes in table 3.1. and those in appendix B.1.1. and it is possible that thedose response differences were due to a dominant effect of one of the *unc* mutations. Table 3.1. also shows that *unc-60* can partially suppress IVM resistance of nr272, nr2389 and nr2477 homozygotes. To investigate the possibility of a maternal effect on dominance of IVM resistance, worms heterozygous for dominant IVM resistance alleles were generated. Two crosses were used to generate worms that had inherited the IVM resistance gene paternally or maternally; the IVM dose response of these is shown in table 3.1. In both cases the worms are heterozygous for maternally inherited *unc-60*(e723). The nr2477 heterozygotes were more resistant to IVM when the nr2477 allele was inherited maternally rather than paternally and the IVM resistance of nr272 and nr2389 heterozygotes was greater when the alleles were paternally inherited. Heterozygous nr2389/+ worms which inherited nr2389 maternally were not resistant to 5 ng/mL IVM, so expression of resistance in nr2389/+ appears to be dependent on paternal inheritance of the resistance mutation. Alternatively, these animals might be only slightly more IVM resistant than wild type controls. nr2344 heterozygotes were resistant to 10 ng/mL IVM and sensitive to 15 ng/mL IVM irrespective of the mode of inheritance of the nr2344 allele.

3.3.3. Dyf phenotype of the dominant ivermectin resistant Dyf alleles nr272, nr2344, nr2477 and nr2389 as homozygotes and heterozygotes.

3.3.3.1. Dyf phenotype

It is necessary to define the terms wild type, Dyf and nonDyf as used in this report. Animals were scored as Dyf if **no** amphid neuron staining was seen. If faint staining was observed and/or staining of a subset of amphid neurons was seen, these animals were scored as nonDyf. The term wild type has been avoided in the context of amphid neuron staining because a continuum of staining intensity in nr272, nr2344 and nr2477 heterozygotes from faint to very bright was seen. In order to quantitate these observations in terms of the number of animals in which the Dyf phenotype was completely penetrant, the term nonDyf has been used to describe worms in which any amphid neuron dye filling

was observed, regardless of intensity. The penetrance of the Dyf phenotype for heterozygotes therefore refers to the proportion of animals which are Dyf as opposed to nonDyf.

3.3.3.2. Original Strains (Appendix B)

The Dyf phenotype is commonly associated with IVM resistance in *C. elegans*, especially resistance above 5 ng/mL (see Chapter 2). The dominant IVM resistance strains isolated here were assayed for dye-filling of amphid neurons with DiO (see methods). Both heterozygotes and homozygotes were analysed. Homozygotes were 100% Dyf whether grown on IVM media or NGM media (data not shown). The Dyf phenotype of heterozygotes was however, incompletely dominant in most cases. Results of dye-filling experiments for animals from the original strains are given in Appendix B. It is clear that Dyf is incompletely dominant in nr272, nr2344 and nr2477 heterozygotes, whereas the Dyf phenotype is almost completely recessive in nr2389 heterozygotes. From this data it is clear that the Dyf phenotype is incompletely dominant for nr272, nr2344 and nr2477, but recessive for nr2389.

There is no significant maternal effect on the penetrance of the Dyf phenotype in heterozygotes although there was a trend for more nr272/+ worms to be nonDyf when the nr272 allele is inherited maternally. With this data, however, it is not possible to separate maternal effects from any *cis* (as opposed to *trans*) effects of the linked *unc-18*(e81) allele which is also heterozygous in these individuals.

Another observable trend is that the penetrance of the Dyf phenotype is increased in nr272, nr2344 and nr2477 heterozygotes when the worms are grown on IVM plates. The sample size is not large enough to tell whether this trend is significant.

3.3.3.3. Outcrossed strains (Table 3.2.)

nr272, nr2344, nr2389 and nr2477 homozygotes remained Dyf after outcrossing, but the incomplete dominance of Dyf in nr272/+, nr2344/+ and nr2477/+ worms was reduced (compare table 3.2. with table B.1.2. - appendix B). There was no difference in the proportion of Dyf to nonDyf heterozygotes for maternally or paternally inherited alleles when using the outcrossed strains, and the results shown in table eight are combined data for both crosses. No heterozygotes were grown on IVM for dye-filling analysis when the outcrossed strains were used.

3.3.3.4. Intensity of staining.

nr272/+, nr2344/+ and nr2477/+ heterozygotes which had any amphid neuron staining were scored as nonDyf, regardless of the intensity of staining, but there was considerable variation in intensity of staining in nonDyf worms. Figure 3.1. shows staining of N2, nr2477/nr2477 and nr2477/+ worms. The nr2477/+ worm shown was scored as nonDyf but the staining is clearly fainter than wild type.

Table - 3.2. Dyf phenotype of the dominant ivermectin resistant Dyf alleles nr272, nr2344, nr2477 and nr2389 as homozygotes and heterozygotes (outcrossed strains).

Genotype	Fraction of nonDyf individuals [fraction] % {95% C.I.}
nr272/nr272	$[^{0}/_{60}]$ **0%** {0.0-8.4}
nr272/+, *unc-60* /+	$[^{114}/_{116}]$ **98%** {93.5-99.7}
nr2344/nr2344	$[^{0}/_{58}]$ **0%** {0.0-8.7}
nr2344/+, *unc-60* /+	$[^{106}/_{118}]$ **90%** {84.7-96.8}
nr2477/nr2477	$[^{0}/_{58}]$ **0%** {0.0-8.7}
nr2477/+, *unc-60* /+	$[^{121}/_{124}]$ **98%** {93.7-99.6}
nr2389/nr2389	$[^{0}/_{54}]$ **0%** {0.0-9.3}
nr2389/+, *unc-60* /+	$[^{93}/_{93}]$ **100%** {94.9-100}

Numbers in each cell represent the percentage of nonDyf animals (ie. animals exhibiting any uptake of the lipophilic dye DiO into their amphid neurons when stained overnight (see methods)). The number of worms exhibiting staining over the total number scored is shown in square brackets and confidence intervals for the percentage of nonDyf worms are given in brackets.

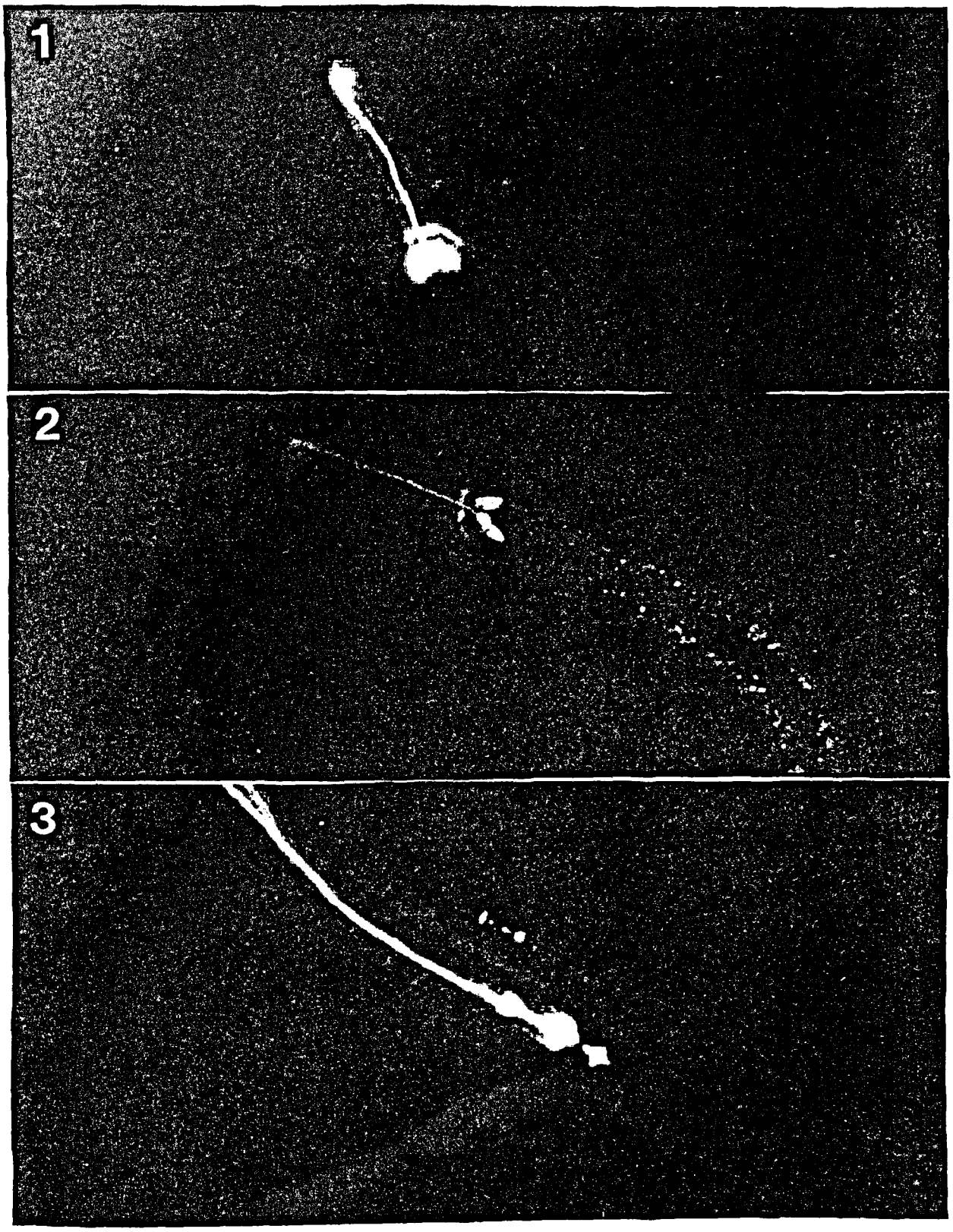

Figure 3.1. - DiO staining of amphid neurons. **1** wild type (N2) worm showing bright staining of amphid neuron cell bodies (40 X magnification). **2** nonDyf heterozygote (nr2477/+) showing faint staining of amphid neuron cell bodies (40X). **3** Dyf (nr2477/nr2477) worm showing no staining of amphid neuron cell bodies.

3.3.4. Genetic mapping and complementation testing of dominant ivermectin resistance mutations.

The mutations *dyf*-?(nr272), *dyf*-?(nr2389), *dyf*-?(nr2344) and *dyf*-?(nr2477) were originally assigned to the X-chromosome on the basis that male progeny from the cross between *dyf*-?/0 males and +/+ hermaphrodites were not resistant to IVM. This result is supported for three of four alleles by the results of the cross between *dyf*-?/0 males carrying the mutations nr272, nr2344 and nr2477 with hermaphrodites homozygous for nr272, nr2344 or nr2477 respectively, in which 100% of progeny were Dyf (ie. were homozygous - table 3.3.). In contrast, nr2389 shows autosomal linkage; when male nr2389 heterozygotes were made by crossing N2 males with nr2389 homozygotes; the F_1 progeny of a cross between these males and nr2389 homozygotes consisted of 50% wild type animals and 50% Dyf worms (table 3.4.). This observation is in contrast to the observation that IVM resistance is not apparent in male heterozygote progeny in the above crosses. The discrepancy arises when the Dyf phenotype is used rather than IVM resistance, this will be discussed later.

Three factor genetic mapping of nr272, nr2344 and nr2477 excluded these mutations from the *unc-18-dpy-8* interval, this result confirms X-linkage of these mutations (an unlinked mutation would recombine with both markers). nr272, nr2344 and nr2477 all map to the interval between *dpy-7* and unc-*9* (table 3.5.) and fail to complement one another for the Dyf phenotype (table 3.3.), indicating that these mutations are allelic.

To establish linkage of nr2389; nr2389/nr2389 males were made by heat shock of an outcrossed nr2389 strain. nr2389 males successfully mated when the male:hermaphrodite ratio was high in contrast to some Dyf homozygote males which do not mate (Perkins, Hedgecock et *al.*, 1986; Starich, Herman et *al.*, 1995). Homozygous nr2389 males were mated to five separate strains, each carrying an *unc* mutation on a different autosome, and one strain carrying an X-linked *unc* mutation. Mated Unc worms were transferred to 5 ng/mL IVM plates to select F_1 animals heterozygous for the *unc* mutation and nr2389. IVM resistant F_1 worms were allowed to produce F_2 progeny on 5 ng/mL IVM plates. The presence of IVM resistant Unc worms in the F_2 was taken as an indication of the *unc* mutation segregating independently of nr2389. The only *unc*/*unc*, nr2389/+ genotype that did not appear in the F_2 was *unc-15*(e73)I/*unc-15*(e73)I, nr2389/+: an indication that the nr2389 locus was on linkage group I. Three factor genetic mapping data excluding nr2389 from the *dpy-24* - *unc-75* interval (table 3.5.) confirms linkage of nr2389 to linkage group I.

The Dyf phenotype of the dominant IVM resistance mutations is incompletely dominant or recessive (table 3.2.)., this allows complementation testing between Dyf alleles. Twenty seven Dyf loci have been described in *C. elegans*; complementation testing between the dominant IVM resistance alleles and other Dyf mutations with similar map positions showed that nr272, nr2344 and nr2477 were allelic with *dyf-12*(sa127), and that nr2389 was allelic with *dyf-10*(e1383). nr2389 fails to complement *dyf-10*(e1383) (table 3.4.), and maps between *unc-29* and *dpy-14*(table 3.5.), a map position which is consistent with the reported map position of *dyf-10* (Starich, Herman et *al.*, 1995). nr272, nr2344 and nr2477 fail to complement *dyf-12*(sa127) (table 3.3.), and map between *unc-9* and *dpy-7* (table 3.5.), a map position which is close to the reported map position of *dyf-12* (Starich, Herman et *al.*, 1995). The complementation, and three factor mapping data suggest that nr2389 is an allele of *dyf-10* and that nr272, nr2344 and nr2477 are alleles of *dyf-12*.

To determine if *dyf-10*(e1383) and/or *dyf-12*(sa127) were dominant for IVM resistance, dominance testing was undertaken as outlined in the methods for screening putative dominant mutations outlined above. No IVM resistant *dyf-10*(e1383)/+ or *dyf-12*(sa127)/+ worms were observed on 5 ng/mL IVM plates.

dyf-10(nr2389)I fails to complement *dyf-12*(nr272)X, *dyf-12*(nr2344)X or *dyf-12*(nr2477)X. This unusual observation is not unique amongst the data presented in tables 3.3 and 3.4. There is also partial failure of complementation (where the number of Dyf double heterozygotes is significantly greater than for either single heterozygote) between: *dyf-12*(nr272)X and *daf-10*(e1387)IV, *dyf-6*(mn346)X and *dyf-7*(m537)X; *dyf-12*(nr2344)X and *daf-10*(e1387)IV, *dyf-6*(mn346)X and *dyf-7*(m537); and between *dyf-12*(nr2477)X and *daf-10*(e1387)IV. Additionally, there were some non-significant differences in penetrance of the Dyf phenotype observed between *dyf-10*(nr2389)I/+,*unc-60*/+ and double heterozygotes with *che-3*(e1124)I, *che-13*(e1805)I, *mec-8*(e398)I, *dyf-1*(mn335)I, *che-10*(e1809)II, *dyf-13*(e1383)II and *daf-19*(m86)II.

Table - 3.3. Complementation tests (for the Dyf phenotype) of the dominant ivermectin resistant Dyf alleles nr272, nr2344 and nr2477 against alleles of previously defined Dyf loci.

Hermaphrodite Parent	Male Parent: Wt §	Male Parent: nr272/0 X	Male Parent: nr2344/0 X	Male Parent: nr2477/0 X
+/+, *unc-60/unc-60*	100% {95.7-100} [84/84]	98% * {93.5-99.7}* [59/59]	90% * {84.7-96.8}* [51/59]	98% * {93.7-99.6}* [58/61]
nr272/nr272, *unc-60/unc-60*	98%* {93.5-99.7}* [55/57]	0% {0.0-9.2} [0/38]	0% {0.0-5.7}* [0/35]	0% {0.0-4.5}* [0/55]
nr2344/nr2344, *unc-60/unc-60*	90% * {84.7-96.8}* [55/59]	0% {0.0-5.7}* [0/28]	0% {0.0-16.0} [0/21]	0% {0.0-4.3}* [0/34]
nr2477/nr2477, *unc-60/unc-60*	98%* {93.7-99.6}* [63/63]	0% {0.0-4.5}* [0/24]	0% {0.0-4.3}* [0/49]	0% {0.0-12.7} [0/27]
daf-10 /daf-10 , unc-60/unc-60 [a]	100% {92.3-100} [46/46]	73% {60.1-83.5} [44/60][c]	54% {40.9-66.7} [33/61][c]	80% {67.9-89.0} [49/61][c]
dyf-6 /dyf-6 , unc-60/unc-60	100% {90.0-100} [35/35]	72% {58.1-82.6} [41/57][c]	87% {75.1-94.1} [53/61]	90% {79.3-96.2} [52/58]
dyf-7 /dyf-7 , unc-60/unc-60	100% {90.9-100} [38/38]	80% {67.7-89.2} [47/59][c]	72% {58.2-83.3} [39/54][c]	95% {86.1-98.9} [57/60]
dyf-12 /dyf-12 , unc-60/unc-60	100% {93.9-100} [58/58]	0% {0.0-5.8} [0/62][d]	0% {0.0-5.9} [0/61][d]	0% {0.0-6.0} [0/59][d]

a - *daf-10*(e1387)IV was included as an unlinked control. **c** - significantly different (P=0.05) from the single heterozygote (nr272/+, nr2344/+ or nr2477/+) **d** - not significantly different from the (nr272/nr272, nr2344/nr2344 or nr2477/nr2477) homozygote. * - these confidence intervals and percentages are for combined data from both reciprocal crosses (ie. maternally and paternally inherited alleles) where there were no significant differences between the two sets of data. Actual data are shown in square brackets. § - ie N2 males.

Numbers in each cell are the percentage of nonDyf animals (ie. animals exhibiting any uptake of the lipophilic dye DiO into their amphid neurons when stained overnight (see methods)). The number of worms exhibiting staining over the total number scored is shown in square brackets and confidence intervals for the percentage of nonDyf worms are given in brackets. The genotype of the animals represented in each cell is heterozygous for the allele at the head of the column and heterozygous for the allele at the beginning of the row. All individuals were also heterozygous for *unc-60*(e723)V which was donated by the hermaphrodite parent in the cross.

Table - 3.4. Complementation tests (for the Dyf phenotype) of the dominant ivermectin resistant Dyf allele nr2389 against alleles of previously defined Dyf loci.

Genotype of stained animals	Dyf phenotype of F_1 progeny (% nonDyf worms [No. nonDyf/n])
nr272/+X;nr2389/+I;*unc-60*/+V	$[^{0}/_{48}]$ **0%** {0.0-7.4}
$^{1}/_{2}$nr272/+X;nr2389/+I;*unc-60*/+V $^{1}/_{2}$ nr272/+X;+/+I;*unc-60*/+V	§$[^{11}/_{62}]$ **18%** {9.5-29.7}*
nr2344/+X;nr2389/+I;*unc-60*/+V	$[^{0}/_{31}]$ **0%** {0.0-11.2}
$^{1}/_{2}$ nr2344/+X;nr2389/+I;*unc-60*/+V $^{1}/_{2}$ nr2344/+X;+/+I;*unc-60*/+V	§$[^{12}/_{61}]$ **20%** {11.0-32.1}*
nr2477/+X;nr2389/+I;*unc-60*/+V	$[^{0}/_{14}]$ **0%** {0.0-23.4}
$^{1}/_{2}$ nr2477/+X;nr2389/+I;*unc-60*/+V $^{1}/_{2}$ nr2477/+X;+/+I;*unc-60*/+V	§$[^{8}/_{57}]$ **14%** {6.3-25.7}*
nr2389/nr2389I;*unc-60*/+V	$[^{0}/_{68}]$ **0%** {0.0-5.3}
$^{1}/_{2}$ nr2389/nr2389I;*unc-60*/+V $^{1}/_{2}$ nr2389/+I;*unc-60*/+V	§$[^{55}/_{117}]$ **47%** {37.8-56.3}
che-3(nr5) +/+ nr2389	$[^{58}/_{58}]$ **100%** {93.9-100}
che-3(e1124) +/+ nr2389	$[^{57}/_{60}]$ **95%** {86.1-98.9}
che-13(e1805) +/+ nr2389	$[^{60}/_{61}]$ **98%** {90.6-99.9}
mec-8(e398) +/+ nr2389	$[^{57}/_{59}]$ **97%** {88.9-99.6}
dyf-1(mn335) +/+ nr2389	$[^{57}/_{59}]$ **97%** {88.9-99.6}
dyf-5(mn400) +/+ nr2389	$[^{60}/_{60}]$ **100%** {94.1-100}
dyf-10(e1383) +/+ nr2389	$[^{0}/_{59}]$ **0%** {0.0-6.0}
che-10(e1809)/+,nr2389/+	$[^{57}/_{60}]$ **95%** {86.1-98.9}
dyf-13(e1383)/+,nr2389/+	$[^{59}/_{60}]$ **98%** {90.5-99.9}
daf-19(m86)/+,nr2389/+	$[^{56}/_{60}]$ **93%** {83.4-97.9}
daf-10(e1387)/+,nr2389/+	$[^{48}/_{48}]$ **100%** {92.6-100}

* - Significantly different (p=0.05) from the expected 50%.

§ - These data represent the progeny of nr2389/+ males with nr272/nr272, nr2344/nr2344, nr2477/nr2477 or nr2389/nr2389 hermaphrodites. It is expected therefore that only 50% of these worms should be heterozygous for nr2389 as well as the IVM resistance allele donated by the hermaphrodite.

Numbers in each cell are the percentage of nonDyf animals (ie. animals exhibiting any uptake of the lipophilic dye DiO into their amphid neurons when stained overnight (see methods)). The number of worms exhibiting

staining over the total number scored is also shown in square brackets and 95% confidence limits for percentages are presented in brackets.

Table - 3.5. Three Factor Genetic Mapping of the dominant IVM resistant alleles nr272, nr2344, nr2477 and nr2389.

Genotype of heterozygote	Phenotype of selected recombinant	Genotype of selected recombinant (with respect to *trans* IVM resistance (*dyf*) marker)	Comments
nr2389 + +/+ *dpy-24 unc-75*	Unc Dpy	9/11 *dyf*/+ 0/8 *dyf*/+	nr2389 lies to the left of *dpy-24*. The apparent presence of IVM sensitive Unc animals may be due to errors in scoring *unc-75*.
+ nr2389 +/*dpy-14* + *unc-29*	Unc Dpy	10/21 *dyf*/+ 12/21 *dyf*/+	Map position for nr2389 = 2.25 (95% C.I. = 1.98-2.79) (*dpy-14* = 1.47),(*unc-29* = 3.19)*
nr272 + +/+ *unc-18 dpy-8*	Unc Dpy	0/25 *dyf*/+ 23/23 *dyf*/+	nr272 lies to the right of *unc-18*
+ nr272 +/*unc-9* + *dpy-7*	Unc Dpy	5/30 *dyf*/+ 24/30 *dyf*/+	nr272 lies between *unc-9* and *dpy-7*, but closer to *unc-9* Map position = 9.04 (95% C.I. = 7.27-10.49) (*dpy-7* = -1.50),(*unc-9* = 11.35)*
nr2344 + +/+ *unc-18 dpy-8*	Unc Dpy	0/25 *dyf*/+ 22/26 *dyf*/+	nr2344 lies to the right of *unc-18* *dpy-8* suppresses IVM resistance of some nr2344 individuals.
+ nr2344 +/*unc-9* + *dpy-7*	Unc Dpy	8/27 *dyf*/+ 20/27 *dyf*/+	nr2344 lies between *unc-9* and *dpy-7*, but closer to *unc-9* Map position = 7.75 (95% C.I. = 5.98-9.20) (*dpy-7* = -1.50),(*unc-9* = 11.35)*
nr2477 + +/+ *unc-18 dpy-8*	Unc Dpy	0/25 *dyf*/+ 14/24 *dyf*/+	nr2477 lies to the right of *unc-18* *dpy-8* suppresses IVM resistance of some nr2477 individuals.
+ nr2477 +/*unc-9* + *dpy-7*	Unc Dpy	6/30 *dyf*/+ 21/29 *dyf*/+	nr2477 lies between *unc-9* and *dpy-7*, but closer to *unc-9* Map position = 8.27 (95% C.I. = 6.50-10.49) (*dpy-7* = -1.50),(*unc-9* = 11.35)*
+ *dyf-12* +/*unc-9* + *dpy-7*	Unc Dpy	19/87 *dyf*/+ 65/86 *dyf*/+	Combined data for all three alleles. Map position = 8.38 (95% C.I. = 7.51-9.16) (*dpy-7* = -1.50),(*unc-9* = 11.35)*

* - Map positions from CGC *C. elegans* genetic map (1991).

3.3.5. Growth of dominant IVM resistance strains at 27°C.

The results of this experiment are given in table 3.6. As the temperature used was not optimum for the growth of *C. elegans*, the value of these results may be questionable, however N2 grew similarly at 27°C and 25°C, exhausting the food supply and producing dauer larvae. The results for the other strains tested indicate that there may be some temperature sensitive effects on fertility for IVM resistance strains, and that IVM resistance is decreased at higher temperatures. These results are discussed below:

P_0 worms were grown at 27°C from hatching to adult on NGM (N2, nr5, nr272, nr2344 and nr2389) or on 5 ng/mL IVM plates (nr5, nr272, nr2344 and nr2389). nr2477 is exceptional in that these worms do not develop to be adults in the P_0 generation at 27°C on either IVM or NGM plates (ie it is temperature sensitive for growth).

P_0 N2 worms grown at 27°C and maintained on NGM at this temperature produced fertile F_1, F_2 and subsequent generations, so that plates were starved and many dauer larvae were present after 14 days. True dauer larvae are resistant to 1% SDS, sometimes larvae form which are not SDS resistant but have a dauer-like appearance. In a separate experiment (data not shown) larvae that appeared to be dauers were shown to be SDS resistant. nr272 F_1s also grow and produce an F_2 generation on NGM at 27°C, providing the P_0 were not exposed to IVM. Many nr272 F_1 and F_2 developed into dauer larvae despite the presence of a thick bacterial lawn on the plates, these larvae were also shown to be SDS resistant in a separate experiment. As might be expected from a strain which produces dauer larvae constitutively, the plates on which nr272 was growing did not become starved in the fourteen days of the experiment. nr5, nr2389 or nr2344 P_0's grown at 27°C did not produce any F_1 progeny (ie. no eggs were produced) at 25°C irrespective of exposure of the pattern of exposure of P_0 worms to IVM.

Except for nr2477, (which did not develop into adults) all P_0 worms grown on NGM at 27°C produced viable F_1 and subsequent generations of offspring when transferred to NGM at 20°C. nr5, nr272, nr2344 and nr2389 also produced viable F_1 and subsequent generations of offspring when transferred to IVM at 20°C.

In contrast, nr2344 F_1 and F_2s were not produced from worms grown on IVM at 27°C and transferred to NGM at 20°C. Neither nr2344 or nr2389 F_1s were produced from worms grown on IVM at 27°C and transferred to IVM at 20°C.

table 3.6. Temperature sensitive sterility and temperature sensitive IVM resistance of dominant IVM resistant mutants.

Growth of:		
P_o	F_1	F_2

NGM @ 27°C
3N2
3nr5
3nr272
3nr2344
3nr2389
5nr2477

NGM @ 27°C	
3N2	3N2
3nr272	3nr272
5nr2389	
5nr5	
5nr2344	

IVM @ 27°C	
5N2	
5nr272	
5nr2389	
5nr5	
5nr2344	

NGM @ 20°C	
3N2	3N2
3nr5	3nr5
3nr272	3nr272
3nr2344	3nr2344
3nr2389	3nr2389

IVM @ 20°C	
5N2	3nr5
3nr5	3nr272
3nr272	3nr2344
3nr2344	3nr2389
3nr2389	

Growth of:		
P_o	F_1	F_2

IVM @ 27°C
5N2
3nr5
3nr272
3nr2344
3nr2389
5nr2477

NGM @ 27°C	
5nr272	
5nr2389	
5nr5	
5nr2344	

IVM @ 27°C	
5nr272	
5nr2389	
5nr5	
5nr2344	

NGM @ 20°C	
3nr5	3nr5
3nr272	3nr272
3nr2389	3nr2389
5nr2344	

IVM @ 20°C	
3nr5	3nr5
3nr272	3nr272
5nr2389	
5nr2344	

Legend: table 3.6.

This figure shows the effects of temperature and IVM on the growth of N2, four dominant IVM resistant strains (nr272, nr2344, nr2389, nr2477) and one recessive strain *che-3*(nr5). Worms were grown at 20°C and then were treated with alkaline hypochlorite solution to isolate eggs. The eggs were allowed to hatch and grow on IVM or NGM plates pre-incubated at 27°C (Column 1). After three days (NGM), or four days (IVM), animals (P_0) which grew (3) were transferred to one of four treatments: IVM at 20°C; IVM at 27°C; NGM at 20°C; or NGM @ 27°C (Columns 2,3). The presence of F_1 worms was scored over fourteen days after transfer of the P_0s (3 indicates presence of F_1, 5 indicates the absence of F_1 - column 2). During this time some F_1 worms developed into adults, layed eggs and F_2s developed; the presence of fertile F_2 worms is also given in the figure (column 3).

3.4. DISCUSSION

3.4.1. Mutations conferring resistance to 5 ng/mL IVM in heterozygotes are rare.

Kim and Johnson (1991) have found the rate of ems-generated IVM resistance mutations to be 1 in 240 mutagenised genomes ($4x10^{-3}$). For dominant mutations however the rate of mutagenesis is at least as low as 7.9×10^{-7} (see section 3.3.1.). 20 recessive IVM resistance strains were isolated in the screen for dominant IVM resistance mutations. This observation can be explained by comparison to the spontaneous rate of IVM resistance mutations which has been reported previously as $2.0x10^{-5}$ (Johnson, 1991), and which was $1.7x10^{-5}$ under identical laboratory conditions to the ems-screen for dominant mutations in this work. All of the 20 recessive isolates obtained in the mutagenesis screen for dominants are probably spontaneous recessive mutations which were present in the N2 starting population.

3.4.2. nr272, nr2389 and nr2477 heterozygotes are less resistant to IVM than homozygotes.

nr272, nr2344, nr2389 and nr2477 homozygotes and heterozygotes were tested for resistance to 5, 10, 15 and 20 ng/mL IVM shortly after isolation from dominance screening. Using these drug concentrations no difference was seen between

homozygotes and heterozygotes (appendix B). The experiment was repeated after outcrossing the strains to N2 to remove any additional mutations. The repeated experiment showed that nr272, nr2389 and nr2477 heterozygotes were less resistant to IVM than the homozygotes (ie Avr is incompletely dominant - table 3.1.). nr2344 heterozygotes were, however, as resistant as nr2344/nr2344 homozygotes.

In addition to changes in the dominance of Avr, the level of IVM resistance in homozygotes and heterozygotes differed in the outcrossed strains. Outcrossing might be expected to remove mutations which reduce IVM resistance, this is apparent for nr272 and nr2389 homozygotes but not for nr2344 or nr2477 homozygotes. Outcrossed nr2344 homozygotes were less resistant than the non-outcrossed strain, this might be due to removal of an IVM resistance enhancing mutation.

IVM resistance for the four heterozygote genotypes was reduced by outcrossing. One explanation for this might be that the *unc-60* marker mutation used in these experiments may have had a deleterious maternal effect on resistance of heterozygote progeny, different marker mutations were used in experiments with the non-outcrossed strains. *unc-60*(e723) reduces IVM resistance in homozygotes as well (table 3.1.).

nr272 and nr2389 heterozygotes were more resistant when the resistant alleles were inherited paternally, whereas nr2477 heterozygotes were more resistant when nr2477 was inherited from the hermaphrodite parent. There was no influence of mode of inheritance on resistance of nr2344 heterozygotes. These observations suggest that there could be a maternal influence increasing resistance in nr2477 heterozygotes. Paternal effects on IVM resistance in heterozygotes are not easily explainable, there is some evidence that imprinting of gamete genomes does not occur in *C. elegans*, and covalent alterations to DNA during development have not been detected (Hodgkin, 1994). There remains the possibility however, that imprinting might occur at some loci in *C. elegans* by an unknown mechanism, and that it might influence IVM resistance in *dyf-12* and *dyf-10* heterozygotes.

These experiments reveal four important points concerning IVM resistance as conferred by nr272, nr2344, nr2389 and nr2477. Firstly, there are qualitative differences in dose response between the strains, confirming the independence of the mutations obtained in the mutagenesis screen. Secondly, IVM resistance may be dominant or incompletely dominant depending on genetic background and the allele involved. Thirdly, genetic background may have an affect on resistance of homozygotes and *unc-60*(e723) has been identified as one mutation capable of

reducing resistance of nr272, nr2389 and nr2477 homozygotes. Finally, IVM resistance of heterozygotes may be influenced by maternal or paternal effects.

3.4.3. Mutants isolated for dominant IVM resistance are Dyf, but the Dyf phenotype is incompletely penetrant in nr272, nr2344 and nr2477 heterozygotes, and Dyf is recessive for nr2389.

The results discussed in chapter 2 clearly indicate that Dyf worms are resistant to IVM at 5 ng/mL. While these were not the only mutations found to be responsible for IVM resistance, they were the major class of mutations responsible for resistance to 5 ng/mL IVM (30 loci). Rare high level resistance mutations which are pharmacologically distinct from Dyf mutations also occur at an additional 5 loci. Such high level resistance mutations are rare alleles at these loci (Johnson and Clover, 1995).

It was expected therefore that the dominant IVM resistance alleles isolated in the mutagenesis screen outlined above might confer a Dyf phenotype. The results given in table 8, clearly show that nr272, nr2344, nr2389 and nr2477 homozygotes are Dyf. The fifth dominant mutation obtained in the screen, nr109 is also Dyf (chapter 2).

The Dyf phenotype of nr272, nr2344, nr2389 and nr2477 heterozygotes was also investigated. nr2389/+ animals were wild type ie. the Dyf phenotype for this mutation is therefore recessive and this is in contrast to the IVM resistance phenotype which is incompletely dominant. An exception may be indicated by the observation from table 8 that a small number of nr2389/+ worms are Dyf in the *dpy-8*(e130)/+ background. This effect is not statistically significant, but may indicate a dominant enhancer effect of *dpy-8*(e130) on the Dyf phenotype of nr2389/+ animals.

For nr272, nr2344 and nr2477 heterozygotes the Dyf phenotype is incompletely dominant (table 3.2.). There also is some variation in staining intensity observable between nonDyf heterozygotes.

The apparent dominance of Dyf in nr272/+, nr2344/+ and nr2477/+ was decreased dramatically after outcrossing the parental strains. For nr2344/+ and nr2477/+, the decrease in Dyf dominance was observed in conjunction with decreased resistance of heterozygotes on outcrossing. This appears to support a direct link between Dyf and IVM resistance. However, the complete recessive nature of the Dyf phenotype in nr2389 (table 3.2.), and failure of nr272/+ heterozygotes to be reduced in resistance after outcrossing (despite a reduction in penetrance of Dyf - table 3.1.) suggest that the links between Dyf and IVM resistance are

complex. Similarly, no maternal or paternal influence was apparent for the Dyf phenotype of nr272/+ or nr2477/+ (table 8), whereas paternal and/or maternal influences were clearly observable for IVM resistance of heterozygotes (table 7). Marker mutations used to distinguish cross progeny from self progeny differed between experiments using outcrossed and original strains so differences in effect of these markers may explain the change in penetrance of the phenotype in heterozygotes. Either explanation provides compelling evidence that the Dyf and IVM resistance phenotypes of nr272/+, nr2344/+ and nr2477/+ vary in different genetic backgrounds.

The first set of experiments examining penetrance of Dyf in heterozygotes also analysed differences between heterozygotes grown on IVM and those grown in the absence of IVM. Despite there being no significant differences in penetrance, the number of Dyf worms was higher for the IVM growth conditions in all cases. It is unlikely that IVM interferes with access of DiO to amphid neurons, as worms were grown on IVM and transferred to drug-free media for staining. This leaves a direct effect of IVM on amphid morphology as an explanation for these observations. An effect of IVM on development of amphids or amphid neurons would be novel, and this experiment warrants repeating with larger sample sizes and using outcrossed as well as original strains, to further investigate this issue.

3.4.4. Dominant IVM resistance mutations define two genetic loci, *dyf-10* I and *dyf-12* X.

Genetic mapping and complementation studies presented here (tables 9, 10 & 11) show that nr2389 is an allele of *dyf-10* and that nr272, nr2344 and nr2477 are alleles of *dyf-12*. Complementation tests made use of the recessive or incompletely dominant Dyf phenotypes of these mutations; failure of complementation was indicated by a significant increase in the number of Dyf worms compared to the nr2389, nr272, nr2344 or nr2477 heterozygotes. Complementation tests of nr2389 with *dyf-10*(e1383) (table 3.4.) and of combinations of nr272, nr2344 and nr2477 with each other and *dyf-12*(sa127) (table 3.3.), all yielded double heterozygotes which were 100% Dyf (0% nonDyf), clearly indicating that these two groups of mutations are alleles at separate loci. Three factor mapping experiments, using the IVM resistance phenotype (table 3.5.), also positioned nr2389 close to the reported map position for *dyf-10* (between *unc-13* and *dpy-5* (Starich, Herman et *al.*, 1995)) and positioned nr272, nr2344 and nr2477 in the region of the reported map position of *dyf-12* (on the left arm of chromosome X (Starich, Herman et *al.*, 1995)).

The map position calculated above for *dyf-12* is probably less accurate than indicated by the confidence interval presented in table 3.5. The large genetic distance (12.85 cM) between *dpy-7* and *unc-9* makes it likely that double recombination events between these loci may have occurred in this experiment; the number of double recombination events are assumed to be negligible for these calculations.

A number of characteristics distinguish recessive alleles of *dyf-12* and *dyf-10* from other Dyf mutations (Starich, Herman et *al.*, 1995). Both *dyf-12*(sa127) and *dyf-10*(e1383) are indistinguishable from wild-type for dauer larvae formation, unlike many other Dyf strains which are dauer formation defective. Both loci had previously been defined by only one Dyf allele; as amphid defective mutants have been the object of study in a number of laboratories for over fifteen years, this observation suggests that these alleles represent rare mutational events and that null alleles at these loci may be mutationally silent, lethal or convey some phenotype other than Dyf (Starich, Herman et *al.*, 1995). The fact that neither *dyf-12*(sa127) or *dyf-10*(e1383) are resistant to 5 ng/mL IVM as heterozygotes, indicates that there are at least two classes of Dyf mutations at these loci; dominant IVM resistance mutations and recessive IVM resistance mutations. *dyf-12*(sa127)/*Df* animals are unusual in that they are non-Dyf, despite being hemizygous at the *dyf-12* locus (Starich, Herman et *al.*, 1995). The report presented by (Starich, Herman et *al.*, 1995) suggested that two copies of *dyf-12*(sa127) must be present for a Dyf phenotype to result, explaining the unusual result for *dyf-12*(sa127)/*Df* worms. In contrast, the incomplete dominance of Dyf for *dyf-12*(nr272), *dyf-12*(nr2344) and *dyf-12*(nr2477) suggests that this is not the case for these alleles. Also *dyf-12*(sa127) is wild type with respect to dauer formation (Starich, Herman et *al.*, 1995), whereas *dyf-12*(nr272) is dauer formation constitutive at high temperature (Results - this chapter). Growth of *dyf-12*(nr2477) is arrested when eggs are hatched at high temperature, whereas *dyf-12*(nr272) and *dyf-12*(nr2344) grew to adults in the same experiment. *dyf-12*(nr2344) is completely dominant for IVM resistance (heterozygotes and homozygotes were indistinguishable by dose response) distinguishing it from incompletely dominant alleles *dyf-12*(nr2477) and *dyf-12*(nr272). All four alleles of *dyf-12* therefore have unique characteristics in terms of dauer-formation, growth at high temperature and IVM resistance.

The results presented above also distinguish *dyf-12* and *dyf-10* as the only Dyf loci for which dominant IVM resistance alleles have been identified. The fact that three alleles at the *dyf-12* locus have been isolated in these experiments suggests that

dominant IVM resistance mutations are more common at *dyf-12* than at other loci. The rarity of these alleles may indicate that *dyf-12* and *dyf-10* might be the only loci for which dominant IVM resistance mutations are possible. A series of large mutagenesis screens would be necessary however to establish the number of Dyf loci which can be mutated to impart dominant IVM resistance.

3.4.5. Male nr2389/+ heterozygotes are less resistant to IVM.

When male nr2389/+ worms are mated with a marked wild type strain, only hermaphrodite F_1 progeny are resistant to 5 ng/mL IVM. This factor led to the mutation nr2389 being erroneously assigned to the X-chromosome. When the Dyf phenotype of the progeny between nr2389/+ males and nr2389/nr2389 hermaphrodites was examined (table 3.4), it became clear that the nr2389 mutation was segregating as an autosomal mutation: 50% of the progeny were Dyf and 50% were non-Dyf.

The sex-limited nature of resistance in nr2389/+ worms was readdressed by repeating the above experiment using the following crosses:
nr2389/0X;+/+V males X +/+X;*unc-60/unc-60*V hermaphrodites, and
+/0X;+/+V males X nr2389/nr2389X;*unc-60/unc-60*V hermaphrodites.
Again, no male nr2389/+ progeny were found to be resistant to 5 ng/mL IVM.

Why should nr2389/+ males be sensitive to IVM when nr2389/+ hermaphrodites are resistant? Why are nr2389/+ hermaphrodites only resistant when the nr2389 allele is inherited from the male parent (table 3.1). Further analysis of this unusual mutation may reveal the answers to these questions and provide an insight into the mechanism of resistance to IVM in *C. elegans*. By cloning and sequencing this locus; identifying the site of mutation in nr2389; and by analysing the expression of the gene in homozygotes and heterozygotes, in male and hermaphrodite heterozygotes and in hermaphrodite heterozygotes that have inherited the mutation paternally or maternally, the cell types important for IVM resistance may be identified. Genetic mosaic analysis using *dyf-10*(nr2389), analysis of *dyf-10*(nr2389)/*Df*, *dyf-10*(nr2389)/*dyf-10*(nr2389)/*Dp* and *dyf-10*(nr2389)/+/*Dp* constructs and *him,dyf-10*(nr2389) animals with respect to IVM resistance might also reveal something of the nature of the nr2389 mutation. Specifically, where is the wild-type gene

expressed, where is the mutant gene expressed, are there differences in expression between males and hermaphrodites and is the mutation neomorphic, hypermorphic, antimorphic or hypomorphic? Ultrastructural analysis of different nr2389 constructs could also prove useful.

3.4.6. Intergenic noncomplementation between *dyf-10* and *dyf-12*.

As two IVM resistance loci (*che-3* Grant, Pers. Comm., 1994: and *osm-3* (Tabish, Siddiqui et *al.*, 1995)) have been recently cloned and identified as encoding proteins with homology to the microtubule motor proteins dynein and kinesin respectively; and as intergenic noncomplementation is a distinguishing genetic feature of mutations in molecular motor genes (Knowles and Hawley, 1991; Endow and Titus, 1992); analysis of double heterozygotes carrying unlinked Dyf mutations was deliberately conducted in this set of experiments.

The only example of unlinked mutations exhibiting total failure of complementation observed was between *dyf-10*(nr2389)I and *dyf-12*(nr272)X, *dyf-12*(nr2344)X and *dyf-12*(nr2477)X (table 3.4). As these genes also are the only loci at which dominant IVM resistance mutations have been observed, it seems likely that they share some common function. Intergenic noncomplementation between *dyf-12* and *dyf-10* might suggest that the complementation data which was used to imply that nr272, nr2344 and nr2477 were alleles of *dyf-12* is in doubt. It is likely however that the total number of Dyf loci have been identified and the four mutants nr272, nr2344, nr2477 and *dyf-12*(sa127) fail to complement each other but do not complement *dyf-7*(m537) or *dyf-6*(mn364) which are also located on the X chromosome near to *dyf-12*. Unlinked noncomplementation has often been cited as evidence for physical interactions between genes (Homyk and Emerson, 1988; James, Ranum et *al.*, 1988; Regan and Fuller, 1988; Stearns and Botstein, 1988; Hays, Deuring et *al.*, 1989; Knowles and Hawley, 1991; Endow and Titus, 1992; Interthal, Bellocq et *al.*, 1995). An example of intergenic noncomplementation between mutations has been recently reported for the Sep1 gene and tubulin genes from yeast. Mutant alleles of the *sep1* gene from *Saccharomyces cerevisiae* have been isolated in screens for defects in recombination, exoribonuclease activity and plasmid stability and in a screen for enhancement of nuclear fusion defects in *kar1-1* mutants (Interthal, Bellocq et *al.*, 1995). The array of phenotypes associated with mutations at the sep1 locus suggested that the gene might be involved in microtubule assembly, stability or function. To investigate this

idea, Interthal et *al.* (1995) made double heterozygote diploid yeast strains carrying one mutant allele of sep1 and one mutant allele at the *TUB2* B-tubulin locus. Mutations at the two unlinked loci failed to complement for a spore viability defect, providing good evidence that the *sep1* gene product interacts with microtubules in some way. This observation led the authors to try in vitro assembly of microtubules using Sep1; Sep1 was able to enhance in vitro microtubule assembly using porcine brain or *S. cerevisiae* tubulin as did microtubule associated proteins (MAPs) from porcine brain. Intergenic noncomplementation has revealed interacting genes in a number of instances and it seems likely that the protein products of *dyf-10* and *dyf-12* also might interact in some cellular process. It is most likely that both genes affect amphid neuron cilia structure, as do other Dyf genes, although ultrastructural analysis would be needed to confirm this. Nevertheless, it is tempting to speculate on the possible nature of the proteins encoded by these genes. Tubulin genes, molecular motor proteins and microtubule-associated proteins all are presumed to play a role in neuronal function and differentiation (see introduction); intergenic noncomplementation has also been observed between mutations in the genes encoding these proteins. *dyf-10* and *dyf-12* could conceivably be molecular motor protein or microtubule-associated protein genes; but the answer must await molecular analyses of these loci. If the cloned genes have no known homologues then the data presented here represents a starting point for analysis of the function of the novel proteins.

Intergenic noncomplementation between dominant *dyf-10* and *dyf-12* alleles occurs, but the recessive alleles *dyf-10*(e1383) and *dyf-12*(sa127) have not been analysed in these experiments. To investigate the allele specificity of intergenic interactions, these recessive IVM resistant alleles should be included in further analysis of interactions between *dyf-10* and *dyf-12*.

Partial intergenic noncomplementation was also observed between *dyf-12*(nr272) and *daf-10*(e1387), *dyf-6*(mn346) and *dyf-7*(m537); *dyf-12*(nr2344) and *daf-10*(e1387), *dyf-6*(mn346) and *dyf-7*(m537); and between *dyf-12*(nr2477) and *daf-10*(e1387) (table 3.3.). There was also some evidence of partial intergenic noncomplementation between *dyf-10*(nr2389) and *che-3*(e1124), *che-13*(e1805), *mec-8*(e398), *dyf-1*(mn335), *che-10*(e1809), *dyf-13*(e1383) and *daf-19*(m86) (table 3.4.). The connotations of partial intergenic noncomplementation are unclear, however it is likely that as all these genes have some function in the correct assembly of amphid cilia, they have some interaction at the molecular level. As many of these mutations may be null, it is possible that a partial reduction in copy number of these proteins increases the chance of nr2389/+, nr272/+,

nr2344/+ or nr2477/+ worms developing shortened or malformed amphid cilia, thus increasing the penetrance of the Dyf phenotype in double heterozygotes. An alternative explanation for the partial non-complementation between non-allelic mutations might be maternal effects of *che-3*(e1124), *che-13*(e1805), *mec-8*(e398), *dyf-1*(mn335), *che-10*(e1809), *dyf-13*(e1383), *daf-19*(m86), *daf-10*(e1387), *dyf-6*(mn346) and *dyf-7*(m537) as these alleles were inherited maternally in all instances. This idea could be tested by creating double heterozygotes which have inherited the *dyf-10*(nr2389) or *dyf-12* alleles maternally and the other mutations paternally. This is difficult however as the chemotaxis defects associated with a number of these mutations prevent male worms from mating.

3.4.7. IVM resistance of dominant mutations is temperature sensitive and also is associated with allele specific temperature effects on hermaphrodite viability.

As for the other Dyf mutants analysed in chapter 2, IVM resistance of *dyf-12*(nr272), *dyf-12*(nr2344), *dyf-12*(nr2477) and *dyf-10*(nr2389) is temperature sensitive. All these strains grow well on both IVM and NGM plates at 20°C, but worms grown at 25°C or 27°C do not produce progeny on 5 ng/mL IVM plates. N2 worms grow and reproduce well at both 25°C and 27°C. *dyf-12*(nr2477) homozygotes arrest at L3 or L4 when transferred as eggs from 20°C to 27°C on NGM or IVM (5 ng/mL) plates (figure 3). *dyf-12*(nr2344), *dyf-10*(nr2389) and *che-3*(nr5) grow to adulthood when transferred as eggs from 20°C to 27°C on NGM or 5 ng/mL IVM plates, but produce no eggs.

dyf-12(nr272) homozygotes and N2 grow and reproduce on NGM at 27°C but produce no progeny when adults are transferred to 5 ng/mL IVM: *dyf-12*(nr272) worms grew slowly in comparison to N2, and dauer larvae were produced even when there was plentiful supply of bacteria on the plates. The formation of dauer larvae in wild type cultures is increased by high temperature, crowded conditions (high pheromone levels) and absence of food (bacteria) (Albert, Brown et *al.*, 1981; Golden and Riddle, 1982; Golden and Riddle, 1984; Golden and Riddle, 1984; Golden and Riddle, 1984). *dyf-12*(nr272) therefore is dauer formation constitutive, producing dauer larvae even under non-inducing conditions; in a separate experiment, dauer-like larvae formed by *dyf-12*(nr272) at 25°C were proven to be true dauer larvae by establishing their resistance to 1% SDS.

The apparent sterility of *dyf-12*(nr2344), *dyf-10*(nr2389) and *che-3*(nr5) worms grown at 27°C on NGM and of *dyf-12*(nr272) grown at 27°C on 5 ng/mL IVM is reversible when

adults are transferred from 27°C to NGM or 5 ng/mL IVM plates at 20°C. When grown at 27°C on 5 ng/mL IVM and transferred to NGM or 5 ng/mL IVM plates at 20°C, *dyf-12*(nr272) and *che-3*(nr5) adults recover and produce viable offspring. Sterility of *dyf-12*(nr2344) worms grown on 5 ng/mL IVM at 27°C is not reversible by transfer to 20°C and *dyf-10*(nr2389) worms recover fertility when transferred from 5 ng/mL IVM at 27°C to NGM at 20°C but not when transferred to IVM at 20°C.

Temperature induced sterility seems therefore to be increased by exposure to 5 ng/mL IVM and for *dyf-12*(nr2344) and *dyf-10*(nr2389), IVM can also make temperature induced sterility irreversible. *dyf-12*(nr2477) is late-larval stage lethal when grown at 27°C, and this effect is irreversible by transferral to 20°C. By considering the combined effects of IVM and temperature on growth, fertility and the reversibility of these effects, it is possible to order the strains used in this experiment from most severely affected to least affected. The order is:

1. *dyf-12*(nr2477), **2.** *dyf-12*(nr2344), **3.** *dyf-10*(nr2389), **4.***che-3*(nr5), **5.***dyf-12*(nr272).

This allelic series is not paralleled by the IVM resistance of homozygotes carrying these mutations; in that series *dyf-10*(nr2389) is most resistant (20 ng/mL +) followed by *dyf-12*(nr2477) and *dyf-12*(nr272) (15-20 ng/mL), *dyf-12*(nr2344) (10-15 ng/mL) and lastly *che-3*(nr5) (5-10 ng/mL). An explanation for this apparent discrepancy may be that IVM resistance and temperature/drug effects on viability and fertility are pleiotropic effects of the same mutations separable on the basis of their effects on different cellular processes or different cell types.

A search for mutations imparting non-temperature sensitive drug resistance may be helpful in the dissection of these two phenotypes. Whatever their relationship, the observation of temperature effects on viability and fertility provides yet another level of complexity in the analysis of Dyf mutations affecting, amphid structure, dauer formation, chemotaxis and IVM resistance.