CHAPTER FOUR

MUTATIONS SUPPRESSING THE IVERMECTIN RESISTANT PHENOTYPE OF Dyf MUTANTS.

4.1. INTRODUCTION

Analysis of suppressor mutations, which change mutant phenotypes determined at other loci, can be a powerful tool for investigation of gene function and elucidation of genetic and biochemical pathways.

Dominant mutations which result from new or altered gene function (neomorphs) can often be suppressed by second site null mutations at the same locus (Herman, 1988). An example is the neomorphic muscle mutant *unc-93*(e1500); this incompletely dominant mutation causes uncoordinated movement and complete abolition of egg laying ability. Null mutations at this locus are wild type but fail to complement *unc-93*(e1500) for uncoordinated movement and egg-laying defects (Greenwald and Horvitz, 1980). Some wild type alleles (e.g. *unc-93*(n234)) also suppress the uncoordinated and egg-laying defects of *unc-93*(e1500) in *unc-93*(e1500)*unc-93*(n234) double homozygotes (Greenwald and Horvitz, 1980). The isolation of intragenic revertants of *unc-93*(e1500) revealed that the gene had redundant function, as null alleles were wild-type, and the reversion of e1500 by null alleles suggested that the mutant gene was expressed and had neomorphic function (Greenwald and Horvitz, 1980). Intragenic suppressor mutations can also be used to define important functional domains in a gene product. For example the mutations *glp-1*(q224) and *glp-1*(q231) both occur within the tandemly-repeated *cdc10/SW16* motifs of the GLP-1 protein. Seventeen intragenic revertants of either of these mutants defined only five amino acid substitutions in the same *cdc10/SW16* motif region of the sequence (Lissemore, Currie et *al.*, 1993), implying that this region is extremely important to wild type protein function.

A screen for suppressors of *dyf-12*(nr2477) was carried out to further investigate dominant IVM resistance *dyf-12* mutations, it was decided to conduct . This experiment served a number of purposes. Firstly, if a null mutation can act as an intragenic suppressor, then the *dyf-12*(nr2477) mutant allele must be transcribed. This occurence would suggest that the nr2477 allele is hypermorphic or antimorphic, as a second mutation would not be expected to effect a hypomorphic dominant allele. Secondly, the occurrence of intragenic suppression would allow transposon tagging of the gene, looking for transposition events which interrupted *dyf-12* - and so suppressing IVM resistance. Such experiments would be a necessary prelude to cloning the gene by transposon tagging. The third potential outcome of suppressor mutagenesis screens would be identification of extragenic

suppressor loci which may reveal important aspects of IVM resistance by Dyf mutants.

Two broad categories of extragenic suppressors exist; informational suppressors and indirect suppressors. Informational suppressors generally involve changes in the translational machinery of the cell: the best characterised examples are mutations which affect tRNA molecules (Murgola, 1985). Nonsense and missense suppressors both involve changes to particular tRNA molecules. Nonsense suppressor mutations allow an aminoacyl-tRNA molecule to insert an amino acid at a termination codon (amber suppressors recognise UAG, ochre UAA and opal UGA), thus reversing effects of nonsense mutations in translated genes and allowing a normal-length polypeptide to be produced in place of a prematurely-terminated one. In missense suppressors, the specificity of the suppressor tRNA is altered so that the correct (or similar) amino acid is inserted at the site of the original mutation and protein function is restored. At the molecular level, the majority of missense and nonsense suppressors directly affect the tRNA anticodon sequence (Murgola, 1985; Kondo, Makovec et *al.*, 1990). Other suppressor mutations alter the conformation of tRNA molecules or change base modifications in the anticodon region (Murgola, 1985). Frameshift suppressors have also been characterised which create tRNA molecules which recognise four base pair codons rather than three base pair codons. This allows correct translation of frameshift-altered mRNAs by skipping over one base in the sequence and continuing translation in the correct reading frame (Roth, 1981). Frameshift suppressor mutations can also be mediated by changed tRNA molecules which recognise four base pair codons or are conformationally changed so that the first base of the next triplet is unavailable forcing the translational machinery to ignore one base and shift phase (Roth, 1981). Other frameshift suppressor mutations may increase or decrease the amount of particular tRNA molecules, allowing "incorrect" tRNA molecules to recognise codons, at least a proportion of the time (Roth, 1981). Other suppressor mutations which act at the translational level may affect proteins associated with tRNA or rRNA processing or translation itself; so called "omnipotent" suppressors (Kushnirov, Ter-Avanesyan et *al.*, 1988). Examples of these might be changes in aminoacyl transferase genes such that tRNA molecules are created which carry the "wrong" amino acid (Murgola, 1985). Also changes in rRNA sequences have been observed which may suppress missense, nonsense or frameshift mutations (Murgola, 1985).

The process of informational suppression at the level of translation is usually less than 100% efficient as there are usually

multiple copies of tRNA genes, so only low quantities of wild type peptides are produced in suppressed strains (Waterston and Brenner, 1978; Murgola, 1985). This allows rescue of the mutant phenotype without production of lethal levels of abnormal peptides from other non-mutant genes. Also, tRNA-mediated suppression may not suppress all mutations which have a particular codon change (Kondo, Makovec et *al.*, 1990). For example the amber-suppressible mutation *unc-13*(e450) from *C. elegans* is suppressed by the $tRNA^{Trp}$ amber (ie recognises and inserts tryptophan at UAG rather than AAG) suppressor *sup-7* but not *sup-33* even though both are $tRNA^{Trp}$ amber suppressors with identical anticodon base changes. This aspect of suppression can be explained if there are specific cell types in which certain tRNA genes are expressed: in this case *sup-7* is expressed in the same cell types as *unc-13* whereas *sup-33* is not (Kondo, Makovec et *al.*, 1990).

Another type of informational suppression in eukaryotes is associated with intron splicing rather than translation. snRNA molecules have been mutated *in vitro* to create sequences which can base pair with aberrant splice-recognition sequences, resulting in the production of correctly-spliced mRNA molecules from mutant sequences in which the splice-recognition sequence was previously inoperative (Parker, Siliciano et *al.*, 1987). Another type of informational suppression at the RNA level is mRNA surveillance suppression (or *smg* suppression) (Pulak and Anderson, 1993). In *C. elegans* six *smg* loci have been identified which suppress specific alleles of a range of genes and also cause slightly abnormal morphology of the male tail (hence the acronym *smg* for suppressor, morphological effect on genitalia) (Hodgkin, Papp et *al.*, 1989). Mutations at the six *smg* loci fail to complement one another suggesting that they all affect the same process (Hodgkin, Papp et *al.*, 1989). *smg* mutants can suppress the effects of some alleles by increasing the stability of abnormally large or altered mRNA such that it may act to partially restore wild type function (Pulak and Anderson, 1993). This only applies if the mutant mRNA is able to be translated into a functional or partially functional protein. Recessive nonsense mutations in some alleles of *unc-54* are dominant in *smg* homozygotes, the increased amount of mutant message apparently leading to the mutant phenotype being expressed even in the presence of a wild type copy of the gene (Pulak and Anderson, 1993). Also, some *tra-1* alleles have weakly masculising or no effect in wild type backgrounds but *tra-1,smg* XX worms are feminised (rather than being hermaphrodites); thus the *smg* mutations make animals carrying loss of function or silent *tra-1* mutations appear like animals carrying gain of function *tra-1*

mutations (Zarkower, DeBono et *al.*, 1994). It has been demonstrated that some *smg*-suppressible (or *smg*-influenced) alleles produce unstable transcripts, and that the amounts of these mRNA molecules are greatly increased in *smg* homozygotes (Pulak and Anderson, 1993; Zarkower, DeBono et *al.*, 1994). It has been proposed that the wild type *smg* genes control or are part of a regulatory system that eliminates abnormal mRNA transcripts from the cell, thus protecting the cell from disruptive effects of such mRNAs produced from occasional errors in transcription (Pulak and Anderson, 1993). Therefore, when this system is inactivated in *smg* homozygotes a greater quantity of abnormal transcripts accumulate in the cell, wild type transcripts from other genes however are unaffected. The system may be more complex than this however, as in some *smg* -suppressible alleles of *tra-1*, there is no apparent increase in quantity of abnormal transcripts (Zarkower, DeBono et *al.*, 1994).

The most distinguishing feature of informational suppressors is that they are allele specific and suppress mutations at a range of loci with unrelated functions. Informational suppressors at different loci may also fail to complement one another if they are involved in a common mechanism (e.g. *smg* suppressors - (Hodgkin, Papp et *al.*, 1989)) and may have dominant and/or maternal effects (e.g. amber suppressors - (Riddle and Brenner, 1978; Hodgkin, 1985)) . Informational suppressor mutants also may have no phenotype other than suppression of a set of other mutations, or their phenotype may be associated with a general loss of fitness rather than loss of any particular function.

A second class of extragenic suppressors however are often locus rather than allele specific and typically suppress mutations at loci involved in a common function. Such suppressors often have specific phenotypes and a series of mutants with opposite phenotypes can be used to define a genetic pathway which can lead to an understanding of particular biological processes. Also, carefully designed suppressor screens can result in the discovery of new genes involved in certain functions. An example is the discovery of a new gene, *fem-3*, (a mutation which causes feminisation of hermaphrodites and males) involved in the sex determination pathway of *C. elegans* , which was defined in a screen for extragenic suppressors of *tra-3* (*tra-3* transforms hermaphrodites into males or hermaphrodites with some male characteristics) (Hodgkin, 1986). Analysis of suppressor mutations can also be useful in dissecting multiple functions of genes with a range of pleiotropic effects. Suppressors can reverse some characteristics of a second mutation without affecting other pleiotropic phenotypes associated with the same mutant. This type of analysis can reveal which pleiotropic effects of a mutation

are separable, and can allow analysis of one gene effect in isolation from others. Extragenic suppression of lethal *rol-3* mutations by *srl-1* and *srl-2* , reverses the larval lethal effects of *rol-3* without affecting the adult-specific rolling phenotype (Barbazuk, Johnsen et *al.*, 1994). The allele specificity of suppressor mutations can also reveal information about the function of the wild-type suppressor gene. *sog* mutants which suppress *glp-1* in *C. elegans* only suppress non-null *glp-1* alleles (Maine and Kimble, 1993). This indicates that *sog* mutants do not bypass the animal's requirement for the GLP-1 protein but instead may be involved in the cell-signalling pathway in which *glp-1* is active, or may regulate expression of *glp-1* (Maine and Kimble, 1993).

The type of information illustrated by the examples above can be assembled in detail for a particular biological process in order to elucidate genetic pathways controlling certain events in organisms. One example which has been extensively investigated in *C. elegans* is vulva formation.

In *C. elegans* a set of six vulval precursor cells (VPCs - P3.p, P4.p, P5.p, P6.p, P7.p & P8.p) adopt one of three lineage fates 1°, 2° or 3° (Horvitz, 1988). The progeny of cells which adopt the 1° and 2° fates make up the vulva of the adult hermaphrodite. A range of mutants have been isolated which either fail to form a vulva at all (Vul phenotype) or which form multiple vulva-like protrusions (Muv phenotype). Epistatic suppression, in which an individual homozygous for two mutations with different phenotypes expresses only one of those phenotypes, has been used to order genes with Muv and Vul phenotypes into a genetic pathway which describes the processes leading to particular cell fates in the vulval lineage (Ferguson, Sternberg et *al.*, 1987; Sternberg and Horvitz, 1989). This genetic pathway, along with analysis of temperature sensitive mutants, gain and loss of function mutations at some loci, and with laser ablation of particular cells, has lead to a model for vulva cell differentiation (Horvitz, 1988; Sternberg and Horvitz, 1989). The genes *let-23* and *lin-12* are important components of the vulval formation pathway (Sternberg and Horvitz, 1989; Sternberg and Horvitz, 1991). Both molecules are cloned and possess domains which suggest they are cell surface receptor molecules.

let-23 is a tyrosine kinase gene with homology to mammalian epidermal growth factor receptors (Sternberg and Horvitz, 1991). Although some mutations at this locus are lethal, others selectively interfere with vulva formation leading to a Vul phenotype. The ability of *let-23* (Vul) mutations to epistatically suppress *lin-15* (Muv) mutations, led to the discovery of these vulva specific mutations (Sternberg and Horvitz, 1991): *let-60 ;lin-*

15 animals are Vul. Also rare hyperinduced alleles of *let-23* are Muv, these mutations are unusual in that they are Muv only in the presence of a vulval-inducing cell, the anchor cell. These results along with molecular evidence for interactions between *let-23* and *let-60* (another locus at which both Muv and Vul alleles have been isolated), have led to the proposal that *let-23* is the receptor for an inducing signal from the anchor cell which induces the primary fate in VPCs (Sternberg and Horvitz, 1991).

lin-12 is a locus at which both loss of function (*lin-12* (0)) and dominant (*lin-12* (d)) mutations with different characteristics have been identified (Sternberg and Horvitz, 1989). In the loss of function mutants, only 1° and 3° cell lineages are produced (typically 2-3 of the VPCs adopt a 1° fate), and in the dominant mutants all six VPCs adopt a secondary fate. *lin-12* mutations also have pleiotropic effects on the anchor cell, such that the anchor cell is not present in *lin-12* (d) homozygotes and multiple anchor cells are present in *lin-12* (0) homozygotes (Sternberg and Horvitz, 1989). Therefore the different phenotypes could be a result of the activity of the gene in the anchor cells or in the VPCs. Construction of *lin-12* (0)/*lin-12* (d) animals allowed this possibility to be explored. In some of these animals the anchor cell was present and all VPCs but one adopted the 2° fate, the remaining cell adopting the 1° fate. In other animals of the same genotype the anchor cell was absent and all VPCs adopted the 2° fate. These experiments confirmed the role of the anchor cell in inducing the 1° fate in the cells nearest it and implicated a cell-autonomous role for *lin-12* in the decision between 2° and other developmental fates. To determine the role of *lin-12* with respect to other vulva lineage mutants, doubly mutant strains were constructed to look for epistatic interactions between *lin-12* mutations and other Muv and Vul mutations (Sternberg and Horvitz, 1989). The Vul mutations *lin-3*, *lin-7*, *lin-10* and *lin-2*, eliminated the unusual phenotype of *lin-12* (0) mutants indicating that these genes act in a pathway parallel to *lin-12* or act before *lin-12* in a genetic pathway. The dominant mutations of *lin-12* however suppress the Vul phenotype of *lin-3*, *lin-7*, *lin-10* and *lin-2*, indicating that the two genes must act in separate pathways. Finally, the interactions between *lin-12* mutations and Muv mutations of *lin-15* indicate that *lin-12* acts in paracrine suppression of 1° cell fate by 1° cells on their neighbours, thus inducing these cells to adopt the 2° fate. In support of this idea, *lin-12* (0);*lin-15* (Muv) individuals are Muv but all cells develop according to the 1° fate rather than a mix of both 1° and 2° (as in *lin-15* homozygotes). So *lin-12* is involved in determination of the 2° fate only, whereas *lin-15* influences both 1° and 2° fates (Sternberg and Horvitz, 1989).

The current model of wild type vulval induction consists of an inductive signal produced by the anchor cell, which acts via *let-23* and *let-60* to induce either 1° or 2° fate, and involves another paracrine signal which acts via *lin-12* to induce the 2° fate (Sternberg and Horvitz, 1989; Sternberg and Horvitz, 1991). Investigations of genetic interactions between loci with opposite functions therefore have revealed much in regard to the vulval induction developmental pathway in *C. elegans*.

One of the aims of defining suppressors of IVM resistance is also to help elucidate the mechanisms of IVM resistance in Dyf animals. The identification of extragenic suppressors described in this chapter along with the identification of mutations imparting super sensitivity to IVM in chapter 2, provide a starting point for further elucidation of an IVM resistance mechanism in *C. elegans*.

4.2. METHODS

4.2.1. Mutagenesis screen for suppressors of the Avr phenotype of *dyf-12*(nr2477)d.

4.2.1.1. Experiment 1.

NS2477 (*dyf-12*(nr2477)/*dyf-12*(nr2477) hermaphrodites - see appendix 1.) animals were mutagenised as described in chapter 3.2.1., allowed to recover overnight then treated with alkaline hypochlorite to obtain an egg suspension. The eggs obtained were placed on seeded NGM plates. After five days 3060 F_1 adult hermaphrodites were picked (20 per plate) to 153 IVM (5 ng/mL) plates and allowed to lay eggs overnight before being removed. On the third day following the overnight brood, F_2 worms which had not developed past the L1 stage (arrested larvae) were picked to seeded NGM plates and used in subsequent rounds of screening.

4.2.1.2. Experiment 2.

The second suppressor screen was conducted in the same manner as the first except only 1500 F_1 animals (10/plate on 150 plates) were used and the screen was undertaken using 3 ng/mL IVM plates.

4.2.1.3. Re-screening of putative suppressed strains.

The same re-screening protocol was used for putative suppressed strains from both mutagenesis experiments. Sensitive F_2 individuals were allowed to recover on NGM plates and were then picked at the L4 stage to individual NGM plates and brooded; this step was necessary to eliminate any larval lethal mutations recovered in the screen (these would not reach the L4 stage) and also eliminated any sterile strains produced as these did not lay viable eggs on NGM. F_2 animals were then allowed to lay on IVM plates (5 ng/mL). Strains which grew on NGM but not on IVM were retained for the next round of screening. Strains obtained from experiment one were re-screened three times and strains from experiment two were re-screened twice.

4.2.1.4. Phenotypic Characterisation of Suppressor of Avr (*sav*) mutants.

The *sav* strains obtained in the mutagenesis experiments were further characterised using three criteria before genetic analysis. Firstly, obvious morphological phenotypes were noted. Secondly, the dose response of the strains to various sublethal (with respect to Wt) and lethal concentrations of IVM was established, following the procedure outlined in Chapter 2.2.1. Finally, all strains were stained with DiO to establish whether the Dyf phenotype of the *dyf-12*(nr2477) was suppressed with the Avr phenotype. Staining was carried out according to the procedure outlined in Chapter 2.2.5.1.

4.2.1.5. Mode of inheritance of *sav* mutants.

The mode of inheritance of the *sav* mutations obtained in the two mutagenesis experiments was investigated by evaluating the Avr phenotype of the F_1 produced by mating N2 males (+/+,+/o) with the strains isolated (*sav/sav,dyf-12*(nr2477)*/dyf-12*(nr2477)). The cross produces males heterozygous for *sav* and hemizygous for *dyf-12* (*sav /+,dyf-12/*o) and hermaphrodites heterozygous at both loci (*sav /+,dyf-12/dyf-12*). As *dyf-12*(nr2477) heterozygotes are Avr at 5ng/mL (chapter 3), animals heterozygous at both loci should be Avr if the *sav* mutation is recessive. Male F_1s will also be resistant providing the *sav* mutation is autosomal and recessive.

4.2.1.6. Complementation testing of au7 with X-linked dpy mutations.

dpy-3(e27), *dpy-7*(e88) and *dpy-8*(e130) males were mated to *sav*(au7) hermaphrodites and the presence of male outcross Dpy F_1 worms was used as an indication of a successful cross. The presence or absence of wild type hermaphrodite progeny in successful crosses was recorded as complementation or noncomplementation respectively.

4.2.1.7. Linkage analysis of *sav* mutations.

a) *sav* mutants with no visible morphological phenotype.

To identify the linkage group on which *sav* mutations were located, males heterozygous for an *unc* mutation (see appendix B.2.) and hemizygous for *dyf-12*(nr2477), were crossed with the strains isolated from the mutagenesis experiments (*sav/sav,dyf-12*(nr2477)/*dyf-12*(nr2477)). The F_1s from this cross were brooded and Unc progeny were picked to IVM plates (5 ng/mL) and their progeny scored for Avr on day 10 or 12. The expectation when the *sav* mutation is unlinked to the *unc* mutation is that 1/4 of all Unc F_2s will be homozygous for *sav* and so will have no resistant progeny despite being homozygous for *dyf-12*(nr2477) (see figure 4.1.). Linkage is indicated by a complete absence of susceptible Unc F_2 progeny, assuming that cross over between linked *unc* and *sav* mutations will be very rare in the F_1. The fraction of sensitive Unc F_2s was compared to the expected proportions using chi-squared analysis. It is possible that some *unc* mutations may interact with *sav* mutations and alter the outcome of these experiments. For example, if any of the suppressors isolated were informational suppressors which could also suppress the Unc phenotypes of any of the markers used, then no Unc,Sav animals would be observed and this could erroneously be interpreted as linkage between the *unc* and *sav* loci. Also it is feasible that an interaction between *unc* and *sav* alleles might result in *sav* heterozygotes being sensitive to IVM (as for *sav* homozygotes). In this case the expected outcome is 3/4 of the F_2 will be sensitive to IVM. In order for the expected 3/4 ratio to be observed it is necessary that *sav* heterozygotes have a maternal effect suppression in the *unc* background. Where the number of Unc F_2s with sensitive progeny was significantly higher ($P<0.05$) than 1/4, the result was also compared to 3/4.

An alternative protocol to the one above was also used in linkage analysis of *sav* mutations (protocol B - figure 4.1.). In the alternative protocol males heterozygous for *sav* and hemizygous for *dyf-12*(nr2477) were crossed with hermaphrodites homozygous for an *unc* mutation and *dyf-12*(nr2477). In this case an absence of linkage between the unc and the sav was indicated when 1/8 of the F_2 Uncs had only IVM sensitive progeny; linkage is again indicated by a complete absence of susceptible Unc F_2 progeny. If the ratio of sensitive Unc progeny was high, the ratio was compared to a ratio of 3/8 resistant progeny using chi-squared test to investigate the possibility of a dominant maternal Sav effect in the Unc background.

b) *sav* mutants with a Dpy phenotype.

The *sav* mutants au7, au19 and au20 were observed to have a Dpy (see appendix A.1.) pleiotropic phenotype. By observing recombination between au19 and au20 and *unc-13* (e51)I, *unc-4*(e120)II, *unc-32*(e189)III, *unc-24*(e138)IV and *unc-60*(e723)V, the linkage group on which au19 and au20 were located was deduced. When a particular Dpy/Unc combination did not appear in the F_2, the *dpy* mutation was assumed to reside on the same linkage group as the *unc* mutation. The Dpy phenotypes of au19 and au20 are extremely severe, effectively masking most Unc phenotypes in double homozygotes. Recombination, between the Dpy *sav* mutants au19 and au20 and the *unc* mutants, was observed by progeny testing F_2 Unc worms, looking for Dpy animals in their progeny (selected F_2 worms must be *unc*/*unc*,*sav*/+ for Dpy animals to be observed in the F_3). Failure of an *unc* marker and the *sav* mutation to recombine in the F_2 (as tested in the F_3), indicates linkage between the *unc* and the *sav*.

Figure 4.1. Linkage analysis of *sav* mutations.

Protocol A.

+/+, +/o X *unc/unc,dyf-12/dyf-12*	→	unc/+,*dyf-12*/o
unc/+,*dyf-12*/o X *sav/sav,dyf-12/dyf-12*	→	*sav/+,unc/+,dyf-12/dyf-12* or *sav/+,+/+,dyf-12/dyf-12*

↓ (selfed)

Unc progeny if *sav* is linked to *unc.* **approx. 100% have Avr self progeny** (*unc/unc, +/+,dyf-12/dyf-12*)
Unc progeny if *sav* is unlinked to *unc.* **1/4 have only non-Avr self progeny*** (*unc/unc,sav/sav,dyf-12/dyf-12*) **2/4 have Avr◊ and non-Avr self progeny** (*unc/unc,sav/ +,dyf-12/dyf-12*) **1/4 have only Avr self progeny** (*unc/unc, +/+,dyf-12/dyf-12*)

* - These worms are not Unc (and therefore not observed) if the sav suppressor in an informational suppressor which suppresses the *unc* mutation used.
◊ - These worms are nonAvr if the *sav* mutation has a dominant maternal effect in the presence of the *unc* mutation used.

Protocol B.

+/+, +/o X *sav/sav,dyf-12/dyf-12*	→	*sav/+,dyf-12/o*
sav/+,dyf-12/o X *unc/unc,dyf-12/dyf-12*	→	*sav/+,unc/+,dyf-12/dyf-12* or *+/+,unc/+,dyf-12/dyf-12*

↓ (selfed)

Unc progeny if *sav* is linked to *unc.* **approx. 100% have Avr self progeny** (*unc/unc, +/+,dyf-12/dyf-12*)
Unc progeny if *sav* is unlinked to *unc.* **1/8 have only non-Avr self progeny*** (*unc/unc,sav/sav,dyf-12/dyf-12*) **2/8 have Avr◊ and non-Avr self progeny** (*unc/unc,sav/ +,dyf-12/dyf-12*) **5/8 have only Avr self progeny** (*unc/unc, +/+,dyf-12/dyf-12*)

* - These worms are not Unc (and therefore not observed) if the sav suppressor in an informational suppressor which suppresses the *unc* mutation used.
◊ - These worms are nonAvr if the *sav* mutation has a dominant maternal effect in the presence of the *unc* mutation used.

4.2.2. Effect of *dpy* mutations on the Avr phenotype of Dyf strains.

4.2.2.1. Construction of *dpy/dyf* double homozygotes.

dpy/dpy,dyf/dyf strains were made in one of four ways.

1) In the first method, *dyf*/o males were crossed with *dpy/dpy* hermaphrodites and F_1 worms were brooded. Many F_2 Dpy animals were placed on 3 ng/mL IVM plates and individuals which grew were selected and maintained. *dyf-12*(nr2344)*dpy-8*(e130)/*dyf-12*(nr2344)*dpy-8*(e130) and *dyf-12*(nr2477)*dpy-8*(e130)/*dyf-12*(nr2477)*dpy-8*(e130) were made using this protocol.

Po	*dyf-12*/o	X	*dpy-8/dpy-8*	on NGM
F1	*dyf-12 +/+ dpy-8*			selfed on NGM plates
F2	*dyf-12 dpy-8/+ dpy-8* & *+ dpy-8/+ dpy-8*			placed on 3 ng/mL IVM plates as adults.
F3				Avr/Dpy worms selected (these were later tested for Dyf and were confirmed to be homozygous for *dyf-12*.

2) The second method of strain construction was to select Dpy nonUnc recombinant animals from the F_2 of a cross between *dyf/+* or *dyf*/o males and a *dpy unc/dpy unc* strain, this method was used to take advantage of recombinant animals generated during mapping experiments. These recombinants were then selected for growth on 3 ng/mL IVM plates. This method was used to create doubly mutant strains between *dyf-10*(nr2389) and *dpy-8*(e130) and *dpy-7*(e88) and between *dpy-7*(e88) and three alleles of *dyf-12* nr2344, nr272 and nr2477.

Po	*dyf-12*/o or *dyf-10/+*	X	*dpyunc/dpyunc*	on NGM
F1	1/2 *dyf-10/+; dpy unc/++* and 1/2 *+/+; dpy unc/++* or 100% *+ dyf-12 +/dpy + unc*			selfed on NGM plates
F2	*+ dyf-12 dpy/unc + dpy*			placed on 3 ng/mL

	&	IVM plates as adults.
	+ + *dpy/unc* + *dpy*	
	or	
	dyf-10/+; *dpyunc/dpy* +	
	&	
	dyf-10/dyf-10; *dpyunc/dpy* +	
	&	
	+/+; *dpyunc/dpy* +	
F3		Avr/Dpy (nonUnc) worms selected (these were later tested for Dyf and were confirmed to be homozygous for *dyf-12*.

3) Double mutant strains were made between the *dpy* suppressor *dpy-?*(au20)II and *dyf-12*(nr2477) and *dyf-10*(nr2389). Firstly the *dyf-12*(nr2477) mutation had to be removed from the original strain. This was done by backcrossing the strain twice to N2 and selecting F_2 Dpy nonDyf animals from the F_2 of the second round of outcrossing. *dyf-12*(nr2477)/o males were then made by crossing N2 males with WG132 (6 times outcrossed *dyf-12*(nr2477) strain - see appendix 1.) and crossed with the outcrossed *dpy-?*(au20)II strain, Dpy Dyf animals were then recovered from the F_2. The same procedure was followed using *dyf-10*(nr2389)/+ males made from the outcrossed *dyf-10*(nr2389) strain WG76. A similar procedure was used to construct doubles between *dpy-7*(au7) obtained from the suppressor screen and three alleles of *dyf-12* nr2344, nr272 and nr2477 and *dyf-10*(nr2389), except Dpy Dyf animals were chosen from the F_3 for the construct between the two X-linked genes *dpy-7* and *dyf-12*. Construction of *dyf-10*(nr2389)/*dyf-10*(nr2389); *dpy-?*(au20)/*dpy-?*(au20) is outlined below:

Po	*dyf-10*/+ X	*dpy-?*(au20)/*dpy-?*(au20)
F1	*dyf-10*/+; *dpy-?*(au20)/+	
	or	(selfed)
	+/+; *dpy-?*(au20)/+	
F2	*dyf-10* /+; *dpy-?*(au20)/*dpy-?*(au20)	
	&	
	dyf-10 /*dyf-10* ; *dpy-?*(au20)/*dpy-?*(au20)	
	&	
	+/+; *dpy-?*(au20)/*dpy-?*(au20)	
F3	Progeny of Dpy/Dyf worms selected by staining Dpy F2 worms with DiO.	

4) A fourth protocol was necessary for the construction of doubles using the Dpy suppressor *dpy-?*(au19)III. Outcrossing

this strain to remove the *dyf-12*(nr2477) mutation was not possible as the number of progeny produced from *dpy-?*(au19)III homozygotes is extremely low. To make doubles between *dpy-?*(au19)III and *dyf* mutations, the *dyf* mutation *dyf-12*(nr2477) was removed by selecting males from the cross between N2 and *dpy-?*(au19)/*dpy-?*(au19),*dyf-12*(nr2477)/*dyf-12*(nr2477). These males (*dpy-?*(au19)/+,*dyf-12*(nr2477)/o) were then crossed to *dyf* homozygotes (outcrossed strains carrying *dyf-12* nr2344, nr272 or nr2477 or *dyf-10*(nr2389)). The males from these crosses (*dpy-?*(au19)/+ or +/+ and heterozygous for *dyf-10* or hemizygous for *dyf-12*) were then backcrossed to the appropriate *dyf* strain. Dpy progeny from the final backcross to *dyf-12* alleles are homozygous at *dpy-?*(au19) and *dyf-12*. For the cross involving *dyf-10*(nr2389), Dyf nonDpy worms were selected and any Dpy progeny were kept as *dpy-?*(au19), *dyf-10*(nr2389) homozygotes. As an example, construction of *dpy-?*(au19)/*dpy-?*(au19); *dyf-12*(nr272)/*dyf-12*(nr272) is outlined below.

1) +/+; +/0 X au19/au19;*dyf-12*(nr2477)/*dyf-12*(nr2477)

2) au19/+; *dyf-12*(nr2477)/0 X +/+; *dyf-12*(nr272)/*dyf-12*(nr272)

3) au19/+; *dyf-12*(nr272)/0 X +/+; *dyf-12*(nr272)/*dyf-12*(nr272)
&
+/+; *dyf-12*(nr272)/0

4) Select Dpy progeny (all will be homozygous for both nr272 and au19)

4.2.2.2. Dose response of *dpy/dyf* strains to IVM.

Strains doubly homozygous for *dpy* and Dyf mutations were tested for drug resistance at a variety of concentrations of IVM. The method followed was identical to that outlined in Chapter 2.

4.2.3. Effect of *unc* mutations on the Avr phenotype of Dyf strains.

Two *unc* loci (*unc-104* and *unc-116*) have been sequenced and shown to have homology to sequences encoding the microtubule motor protein kinesin (Otsuka, Jeyaprakash et *al.*, 1991; Patel, Thierry-Mieg et *al.*, 1993). The effect of *unc-104* and *unc-116* mutations on IVM resistance of various Dyf mutants was analysed because the Dyf loci *che-3* and *osm-3* had homology to dynein (Grant and Whitington, Pers comm, 1994) and kinesin (Tabish, Siddiqui et *al.*, 1995) respectively, and it is common for there to be interactions between motor protein genes (Endow and

Subsequent screening of putative suppressed strains from both experiments (at 5 ng/mL IVM) revealed that there had been a high number of false positives obtained in the initial screens. The final number of suppressed strains obtained from experiment one was 10 (after 3 subsequent screens) and 6 (after two additional screens) from experiment two (table 4.1.). The rate of recovery of truly suppressed strains therefore was approximately 7% of the strains picked in the first screen for both mutagenesis experiments.

The frequency of ems-induced mutations suppressing IVM resistance was 0.0039 mutations per mutagenised genome in experiment one and 0.0040 mutations per mutagenized genome for experiment two. Experiment two had a more stringent initial screen but the mutation frequency was not lower than for experiment 1. The most probable explanation for this apparent discrepancy is that the mutation frequency in experiment one was underestimated due to difficulties encountered in picking all arrested larvae present in the initial screen. Alternatively, there may be a threshold drug concentration below which all mutants of a certain type can suppress IVM resistance: this threshold may be 5 ng/mL or higher, therefore making the expected mutation rate the same for both screens. The second argument is less likely given that a range of mutations were obtained which reduced drug resistance of *dyf-12*(nr2477) to different levels between 1.25 ng/mL and 5 ng/mL IVM.

A proportion of the recovering arrested larvae picked in the initial screen in both mutagenesis experiments were sterile. It is interesting to note that the proportion of sterile mutants obtained in the first experiment is much higher (36%) than for the second experiment (10%) (see also table 12).

4.3.2. Phenotypic Characterisation of *sav* (Suppressor of Avermectin resistance) mutants.

A number of the *sav/sav*; *dyf-12/dyf-12* strains isolated in the mutagenesis experiments had visible phenotypes. Six of the fourteen strains obtained were Unc and five were Dpy. Only three Dpy strains segregated distinct classes of F_2 Dpy and nonDpy offspring when outcrossed to N2 males. These were the Dpy alleles au19, au20 and au7. These results are summarized in table 4.2. The other two Dpy strains produced F_2 progeny which showed a continuous range of lengths from wild type to Dpy, these strains (au18 and au15) are not referred to as Dpy in table 4.2. for this reason.

Table 4.1. Results of two ems mediated mutagenesis screens and subsequent rounds of screening for mutants suppressing ivermectin resistance conferred by *dyf-12*(nr2477).

Expt. No.		screen 1	screen 2	screen 3	screen 4
1	sensitive (suppressed)	146	32/146	11/32	10/11 (10 strains) [a]
	resistant (not suppressed)	N/A	62/146	21/32	1/11
	sterile	N/A	52/146	-	-
	Total	3060	146	32	11
2	sensitive (suppressed)	70	12/70	6/12 (6 strains)	not done.
	resistant (not suppressed)	N/A	51/70	6/12	
	sterile	N/A	7/70	-	
	Total	1500	70	12	

a - Two of these strains were later lost due to poor reproductive performance.

The table shows recovery of mutations which suppress *dyf-12* IVM resistance. Experiment one detected mutants suppressing ivermectin resistance to 5 ng/mL IVM in agar, and screen two was a more stringent screen designed to detect suppression of resistance to 3 ng/mL IVM in agar. All secondary screens were carried out at 5 ng/mL IVM in agar (see methods). Numbers in each column show the outcome ofround of rescreening. The first figure is the fraction of isolates sensitive to IVM, the second is the fraction resistant to IVM and the third number is the fraction that were sterile; the total number of isolates examined in each screen is given at the bottom of the cell. The number of strains finally isolated is given in parentheses in the last column.

All of the strains isolated in the mutagenesis experiments (*sav/sav*, *dyf-12/dyf-12*) were sensitive to 5 ng/mL IVM, but most retained resistance to 3 ng/mL IVM. Three of the strains selected for sensitivity to 3 ng/mL IVM in the initial screen of experiment two grew slowly on 3 ng/mL IVM. This is probably due to the method of selection in the first screen allowing slow growing mutants to be selected along side 'true' *sav* mutants (ie - mutants incapable of completing the life cycle at 3 ng/mL IVM). Screens subsequent to the initial screen used 5 ng/mL IVM and so selected only strains sensitive to 5 ng/mL IVM. Three of the *sav* mutations, au1, au3, and au20 suppressed IVM resistance to wildtype levels in *sav/sav;dyf-12/dyf-12* worms (growth at 1.25 ng/mL but not at 3 ng/mL), and another two alleles (au18 and au19) suppressed resistance to below 1.25 ng/mL IVM: ie - a concentration at which N2 worms will grow, it is not known whether these mutations confer super-sensitivity to IVM in the absence of *dyf-12*(nr3477). These results are summarised in table 4.2.

All fourteen *sav/dyf-12*(nr2477) strains remained Dyf, indicating that the *sav* mutations suppressed the Avr phenotype of *dyf-12*(nr2477) without suppressing the Dyf phenotype. This result confirms the presence of the *dyf-12*(nr2477) mutation in the strains obtained from the mutagenesis experiments.

4.3.3. Genetic Characterisation of *sav* mutations.

The mode of inheritance of *sav* mutants was established by observing the resistance status of the F_1 progeny from a cross between wildtype males and *sav/sav,dyf-12*(nr2477)*/dyf-12*(nr2477). If the progeny of the genotype *sav/+,dyf-12*(nr2477)/+ are resistant than the heterozygous *sav* gene fails to suppress resistance as conferred by the dominant Dyf gene. So the *sav* gene is probably recessive (*sav/+,dyf/dyf* animals were not tested). This was the case with all the suppressed strains generated in the two mutagenesis experiments.

Using the same cross, *sav* mutations were assigned autosomal linkage if both male and hermaphrodite F_1 progeny were resistant to IVM. This was the case with all but the mutation au7 in which only hermaphrodite F_1's were resistant and this gene was assigned to the X.

Mapping of eleven of the fourteen *sav* mutations to linkage groups was attempted by observing recombination in the F_1 between *sav* mutants and six *unc* mutants which map to the five autosomes of *C. elegans*. All mapping was undertaken in a homozygous *dyf-12*(nr2477) background (see methods). The raw data from the mapping experiments are presented in appendix 2,

and the summarised results in table 4.2. The only mutations which could be unambiguously assigned to a linkage group were au5(I), au14(IV) and au15(III). au4 most probably maps to either linkage group IV or I and au17 to IV or V. The ambiguous linkage group assignments in crosses using au4 and au17 may indicate that these suppressors are informational suppressors. If the *unc* marker used in these crosses is suppressible by an informational suppressor of the same type as the *sav* mutation involved, then no Unc/IVM sensitive animals would be observed in the F_3, as animals of the genotype *unc/unc*, *sav/sav*, *dyf-12/dyf-12* will be nonUnc.

au4 may be an amber suppressor on linkage group I. unc-24(e138) is amber suppressible (Hodgkin, 1985) and no *sav*-?(au4)/*sav*-?(au4),unc-24(e138)/unc-24(e138) animals were observed, despite the pressence of 10/28 *sav*-?(au4)/*sav*-?(au4);unc-22(e66)/unc-22(e66) animals when *unc-22*(e66) is used as the linkage group IV marker instead of *unc-24*(e138). Therefore it is likely that au4 suppresses *unc-24*(e138)IV and *dyf-12*(nr2477)X but not *unc-22*(e66)IV or *unc-15*(e73)I, and maps to linkage group I at a position fairly distant from *unc-15*(e73)I (one non IVM resistant Unc recombinant was observed - appendix B). Three amber suppressor loci, *sup-24*, *sup-29* and *sup-34* have been mapped to linkage group 1, au4 could be an allele of any of these. Kondo et *al.* (1990) showed that some amber suppressors in *C. elegans* fail to suppress a number of amber mutations and *sup-34* and *sup-29* were shown to be poor suppressors of *unc-24*(e138), so if au4 is an amber suppressor linked to *unc-15*, it is most likely an allele of *sup-24*.

au17 could similarly be an informational suppressor of both *unc-22*(e66)IV and *dyf-12*(nr2477)X but not *unc-24*(e138)IV or *unc-60*(e723), making its map position likely to be on linkage group V.

To reveal whether any of the *sav* mutants are informational suppressors, effects of known informational suppressor alleles on *dyf-12*(nr2477) and effects of *sav* mutations on other mutations known to be suppressible by informational suppressors could be undertaken. If any *sav* mutations are alleles of informational suppressor loci, then they are not capable of totally restoring protein function, as IVM resistance is suppressed but the Dyf phenotype is not reversed. Other informational suppressors have been observed which partially restore wild type phenotypes, for example suppression of *unc-54* by *sup-5*(e1464) only partially restores movement (Waterston and Brenner, 1978).

Linkage data for au1, au2, au3, au8, au16 and au18 are uninformative (Appendix B). Perhaps these *sav* mutations all map to positions far from the positions of the marker *unc* mutations,

so that a high level of recombination occurs. Further mapping studies using different *unc* markers will be necessary to establish linkage of these *sav* mutations.

The proportion of sensitive Unc F_2s was often significantly higher than the expected 1/4 sensitive (protocol A) or 1/8 sensitive (protocol B) proportions for *sav* mutations unlinked to a particular *unc* mutation (a significantly lower ratio was an indication of linkage). In most of these instances, chi-square analysis showed that the high ratio of sensitive Unc F_2s was fitted ratios ($P>0.05$) of 3/4 (protocol A) or 3/8 (protocol B) sensitive progeny. One explanation for this data would be a dominant maternal effect of the *sav* mutation in certain *unc* homozygotes (see figure 4.1.). Instances where this was observed are listed below:

unc-15(e73) - au1
unc-4(e120) - au1, au5, au18
unc-32(e189) - au2, au4, au16
unc-22(e66) - au18

There were an additional two instances in which the proportion of sensitive F_2 Unc individuals was too high to be explained by a dominant maternal effect (ie the proportion of sensitive Unc worms was significantly higher than 3/4 (protocol B) or 3/8 (protocol A) (chi-squared $P<0.05$). These were seen in crosses with au16 and *unc-4*(e120) and with au18 and *unc-60*(e723). Although there is no obvious reason for these unusual results, they could be explained as chance occurrences due to small sample size. Recombination data between au16 and *unc-4* differed according to the protocol used (Appendix B.2.).

au7 was assigned X-linkage based on the sensitivity of F_1 males from the cross between N2 males and *sav/dyf-12*(nr2477) double homozygotes. The initial au7 strain also segregated Dpy progeny when outcrossed and Dpy males were observed in the F_1 from the above cross growing on NGM. The possibility that the Dpy and the Sav phenotypes were due to the same mutation was then considered. Also relevant was the observation that other *dpy* mutations suppressed the Avr phenotype of Dyf mutants (including nr2477 - see next section). Subsequent to these observations the au7 strain was outcrossed to remove *dyf-12*(nr2477), and a complementation test was carried out with \the X-linked *dpy* mutations *dpy-7*(e88), *dpy-8*(e130) and *dpy-3*(e27). *dpy-3*(e27), *dpy-7*(e88) and *dpy-8*(e130) males were mated to *sav*(au7) hermaphrodites and male outcross Dpy F_1 worms were obtained in all crosses indicating that the crosses were successful. Wildtype hermaphrodite progeny were obtained in the cross involving *dpy-8*(e130) and *dpy-3*(e27) males, indicating that these mutations complement au7 for the Dpy

phenotype. Several successful crosses between *dpy-7*(e88) males and *sup*(au7) hermaphrodites failed to yield wildtype progeny, indicating that the two *dpy* mutations au7 and e88 do not complement. au7 will be referred to as *dpy-7*(au7) for the remainder of this discussion.

To confirm the Dpy and Sav phenotypes of *dpy-7*(au7) were due to the same mutation, a number of *dpy/dyf* double homozygotes were made between *dpy-7*(au7) and Dyf mutations. Doubly mutant strains were constructed between *dpy-7*(au7) and *dyf-12*(nr272), *dyf-12*(nr2344), *dyf-12*(nr2477) and *dyf-10*(nr2389). When tested for ivermectin resistance, all four doubly mutant strains were sensitive to 5 and 10 ng/mL IVM (ie *dpy-7*(au7) suppressed IVM resistance).

Strains carrying au19 and au20 were also Dpy. To confirm that the Dpy and Sav phenotypes of these mutations were conferred by the same mutation, the *dpy* mutations were separated from *dyf-12*(nr2477) and *dpy/dyf* double homozygotes were made and tested for IVM resistance (see methods). Doubly mutant strains were constructed between au19 and *dyf-12*(nr272), *dyf-12*(nr2344), *dyf-12*(nr2477) and *dyf-10*(nr2389) and between au20 and *dyf-12*(nr2477) and *dyf-10*(nr2389). When tested for ivermectin resistance, all six doubly mutant strains were sensitive to 5 and 10 ng/mL IVM. These two *sav* mutants are referred to as *dpy*-?(au19) and *dpy*-?(au20), for the rest of this discussion.

au19 and au20 were mapped to linkage groups by observing recombination between these alleles and *unc-13*(e51)I, *unc-4*(e120)II, *unc-32*(e189)III, *unc-24*(e138)IV and *unc-60*(e723)V. The Dpy phenotypes of au19 and au20 are extremely severe and masked the Unc phenotype in double homozygotes. Recombination between these mutations and the *unc* mutants therefore was observed by progeny testing F_2 Unc worms, looking for Dpy animals in their progeny (the F_3). If Unc F_2 animals have Dpy,Unc progeny then their genotype is *unc/unc,dpy/+* and this is an indication of recombination between the *unc* and *dpy* mutations. Recombination

Table 4.2. Summary of mutant strains generated in the two ems-mutagenesis experiments screening for suppressors of the Avr phenotype of *dyf-12*(nr2477).

Suppressor allele	Appearance of original strain[b]	Visible morphological phenotype	Linkage Group	IVM Resistance of original strain.
au1	Unc	None	A	<1.25 ng/mL <3.0 ng/mL[e]
au2	Unc	None	A	<5.0 ng/mL
au3	Wt	None	A	<1.25 ng/mL <3.0 ng/mL[e]
au4	Unc	None	I or IV	<5.0 ng/mL
au5	Wt	None	I	~3.0 ng/mL
dpy-7(au7)	Dpy	Dpy	X	~3.0 ng/mL
au8	Wt	None	A	<5.0 ng/mL
au14	Unc	None	IV	<5.0 ng/mL
au15[a]	Dpy	None	III	<5.0 ng/mL
au16[a]	Unc	None	A	<5.0 ng/mL
au17[a]	Unc	None	IV or V	~3.0 ng/mL
au18[a]	Dpy	None	A	<1.25 ng/mL[f]
dpy-?(au19)[a]	Dpy	Dpy[c]	III	<1.25 ng/mL[f]
dpy-?(au20)[a]	Dpy	Dpy[d]	II	<3.0 ng/mL[e]

A - autosomal. [a] - mutations generated in experiment two (initially screened for sensitivity to 3 ng/mL IVM with subsequent screens at 5 ng/mL). [b] - Unc - most of these were slow moving, with a range of severity from almost wild type to almost paralysed. Dpy strains all appeared shorter than wildtype with either normal or slightly reduced diameter. Most of these strains did not segregate distinct classes of progeny with different morphological appearance and the phenotypes are probably due to second mutations in the strains. [c] - au19 is slow growing, has reduced fertility and is shorter and thinner than wild-type animals. [d] - au20 is Dpy, slow growing and has reduced fertility. [e] - Strains with Wildtype IVM resistance. [f] - Strains supersensitive to IVM. **n.d.** - not done.

was observed between *dpy*-?(au19) and all *unc* mutations except *unc-32*(e189), indicating that *dpy*-?(au19) is on LGIII. Recombination was observed between *dpy*-?(au20) and all *unc* mutations except *unc-4*(e120), indicating that *dpy*-?(au20) is linked to LGII.

4.3.4. Effect of *dpy* mutations on the Avr phenotype of Dyf strains.

dpy-7(e88) suppresses resistance to 5 ng/mL IVM conferred by *dyf-12*(nr272) and *dyf-10*(nr2389) and resistance to 10 ng/mL conferred by *dyf-12* alleles nr2344 and nr2477. Similarly, *dpy-8*(e130) suppresses IVM resistance of *dyf-10*(nr2389) and *dyf-12*(nr2344 and nr2477) at 10 ng/mL IVM. Neither *dpy-7*(e88) or *dpy-8*(e130) alone are super-sensitive to IVM. Both strains grow on 1.25 ng/mL IVM plates but not on 3.0 ng/mL IVM plates (as for N2 - see chapter 2). Table 4.3. summarises these results.

4.3.5. Effect of *unc* mutations on the Avr phenotype of Dyf strains.

4.3.5.1. Kinesin gene mutations.

As two of the Dyf loci that have been cloned have homology to microtubule motor genes (*che-3* - dynein, Grant Pers. Comm. 1995: & *osm-3* - kinesin, (Tabish, Siddiqui et *al.*, 1995)) the possibility that Dyf genes might interact with *unc* genes which encode putative microtubule motor proteins (Hall and Hedgecock, 1991; Hedgecock and Hall, 1991; Otsuka, Jeyaprakash et *al.*, 1991; Patel, Thierry-Mieg et *al.*, 1993) was investigated. The mutations used were *unc-104*(e1265) and *unc-116*(e2310). *unc-104* encodes a kinesin-like protein and is involved in the transport of vesicles in axons; and *unc-116* has high similarity with kinesin heavy chain. Interactions between loci encoding molecular motor proteins are common in a variety of organisms (Endow and Titus, 1992).

unc-104(e1265) suppresses resistance of *dyf-12*(sa127), *mec-8*(e398), *che-12*(e1812), *osm-5*(p813) and *daf-6*(e1377). All of these Dyf mutations confer IVM resistance at 5 ng/mL in the absence of *unc-104* and *unc-104*(e1265) alone is not super sensitive to IVM (see Chapter 1). *unc-104*(e1265) does not suppress IVM resistance (5 ng/mL) of *dyf-7*(m537), *osm-6*(p811), *dyf-che-11*(e1810),

Table 4.3. - Suppression of the IVM resistance phenotype of dominant alleles of *dyf-10* and *dyf-12* by *dpy-7* and *dpy-8*.

Genotype	Growth on IVM (ng/mL)		
	1.25	5	10
dyf-10(nr2389)	n.d.	+	+
dyf-12(nr272)	n.d.	+	+
dyf-12(nr2344)	n.d.	+	+
dyf-12(nr2477)	n.d.	+	+
dpy-6(e14)	+	-	n.d.
dpy-7(e88)	+	-	n.d.
dpy-8(e130)	+	-	n.d.
dpy-7(e88); *dyf-10*(nr2389)	n.d.	-	-
dpy-7(e88); *dyf-12*(nr272)	n.d.	-	-
dpy-7(e88); *dyf-12*(nr2344)	n.d.	+	-
dpy-7(e88); *dyf-12*(nr2477)	n.d.	+	-
dpy-8(e130); *dyf-10*(nr2389)	n.d.	+	-
dpy-8(e130); *dyf-12*(nr2344)	n.d.	+	-
dpy-8(e130); *dyf-12*(nr2477)	n.d.	+	-

+ - indicates growth at this concentration of IVM
- - indicates failure of the strain to grow at this concentration of IVM
n.d. - not done.

dyf-10(nr2389), *dyf-12*(nr2344, nr272, nr2477), *osm-3*(p802), *osm-1*(p808), *daf-10*(e1387) or *che-13*(e1805). All of these double homozygotes are Dyf.

A second kinesin gene mutation, *unc-116*(e2310), does not suppress resistance of *dyf-10*(nr2389), *osm-1*(p808), *daf-19*(m86) or *dyf-12*(nr272, nr2344, nr2477) at 5 ng/mL IVM.

4.3.5.2. Other *unc* mutations.

During the course of the work described in Chapter 3, a number of *unc/dyf* double homoygous strains were constructed. A number of these *unc/dyf* combinations were found to have reduced resistance to IVM in comparison to the Dyf mutant alone. Some of these examples of suppression are listed in table 4.4. *unc-60*(e723) suppresses IVM resistance to concentrations above 10 ng/mL IVM for three dominant IVM resistant alleles (table 4.4.). IVM resistance at 5 ng/mL is unaffected by *unc-60*(e723) when it is combined with *che-3*(nr5), *daf-10*(e1387), *dyf-6*(mn364), *dyf-7*(m537) or *dyf-12*(sa127), but testing at higher IVM concentrations might reveal some suppression of IVM resistance. In addition, at 5 ng/mL *unc-52*(e444) suppresses IVM resistance of *daf-10*(e1387) and *dyf-6*(mn364) but not *che-3*(nr5), *dyf-7*(m537) or *dyf-12*(sa127) and *unc-54*(e190) suppresses IVM resistance of *dyf-6*(mn364) and *dyf-12*(sa127) but not *dyf-7*(m537).

Additionally, there was no suppression of IVM resistance by *mah-2*(cn110) (a ts *unc* mutation) of *dyf-10*(nr2389) at 5 or 10 ng/mL IVM or by *unc-9*(ec27) of *dyf-12*(nr272, nr2344 and nr2477) at the same drug concentrations. No suppression was observed at 5 ng/mL of IVM resistance for the following *unc/dyf* combinations: *unc-3*(e151)/*dyf-10*(nr2389), *unc-31*(e169)/*dyf-12*(nr272), *unc-31*(e169)/*dyf-12*(nr2344), *unc-31*(e169)/*dyf-12*(nr2477), *unc-31*(e169)/*dyf-10*(nr2389), *unc-4*(e120)/*dyf-12*(nr2477), *unc-15*(e73)/*dyf-12*(nr2477), *unc-32*(e189)/*dyf-12*(nr2477), *unc-24*(e138)/*dyf-12*(nr2477) or *unc-22*(e66)/*dyf-12*(nr2477). Further testing of these double mutant strains at higher IVM concentrations might reveal more suppressor interactions between unc and IVM resistant mutations.

Table 4.4. - Suppression of the IVM resistance phenotype of Dyf alleles by *unc* mutations.

Genotype	Growth on IVM (ng/mL)			
	5	10	15	20
dyf-10(nr2389)	+	+	+	+
dyf-12(nr272)	+	+	+	-
dyf-12(nr2344)	+	+	-	-
dyf-12(nr2477)	+	+	+	-
unc-18(e81); *dyf-10*(nr2389)	+	-	n.d.	n.d.
unc-18(e81)*dyf-12*(nr272)	+	-	n.d.	n.d.
unc-60(e723); *dyf-10*(nr2389)	+	+	-	-
unc-60(e723); *dyf-12*(nr272)	+	+	-	-
unc-60(e723); *dyf-12*(nr2344)	+	+	-	-
unc-60(e723); *dyf-12*(nr2477)	+	+	-	-

+ - indicates growth at this concentration of IVM., **n.d.** - not done.,
- - indicates failure of the strain to grow at this concentration of IVM

4.4. DISCUSSION

4.4.1. *sav* mutations are common.

The ems mediated mutagenesis experiments conducted to isolate *sav* mutants yielded one mutant per 250 mutagenised genomes, indicating that there are many loci that can be mutated to impart a Sav phenotype. The results presented above indicate that there are at least 6 loci defined by the mutations isolated in the mutagenesis screen for suppressors of *dyf-12*(nr2477). There are also 5 *unc* loci with a Sav phenotype implicated by this work and a sixth, *unc-116* has been observed to suppress IVM resistance of a number of alleles of *che-3* (Grant, Pers Comm., 1995). There are also at least two additional *dpy* loci with a Sav phenotype, *dpy-8* (above results) and *dpy-5* (Johnson, Pers. Comm., 1995). Synaptotagmin is a protein involved in proper synapse function and two synaptotagmin mutants *snt-1*(md290) and *snt-1*(md325) have also been observed to have a Sav phenotype (Grant, Pers. Comm., 1995). Some of the *sav* mutants isolated as part of this work could be nonUnc mutations at any of these loci, and others could be informational suppressors which partially restore wild type protein function (a complete restoration of function would be expected to reverse the Dyf phenotype as well as reverting IVM resistance to wild type levels). An additional class of suppressor may be mutations which impart super sensitivity to IVM. Additive effects between supersensitive mutations and resistant mutations might be expected to result in a double homozygote with reduced sensitivity to IVM in comparison to the Avr parent. Supersensitive mutants included alleles *mab-5*, *unc-86*, *che-7*, *eat-1*, *eat-2*, *eat-3*, *eat-6*, *eat-7* and *eat-8* (see Chapter 2), however none of these have been combined in double homozygotes with Dyf mutations to test this possibility. Further mutagenesis experiments and analysis of *dyf/unc* and *dyf/dpy* double homozygotes will be needed to establish the total number of loci which can be mutated to impart a Sav phenotype. Other candidate genes for suppression of IVM resistance by Dyf genes should include those in which mutations are super sensitive to IVM.

4.4.2. *sav* mutants suppress IVM resistance of Dyf mutations but not the associated Dyf phenotype.

In none of the instances of suppression of IVM resistance described above was the Dyf phenotype suppressed along with the

IVM resistance phenotype. IVM resistance is also apparent in nonDyf (*dyf*/+) heterozygotes carrying dominant alleles (Chapter 3) and in nonDyf alleles at Dyf loci (Johnson, Pers. Comm., 1995). This indicates that the two phenotypes are separable even though all loci associated with a low level resistance phenotype are characterised by Dyf alleles (see also chapter 2 and Chapter 3).

It is expected that mutations which suppress IVM resistance and dye-filling defective phenotypes are possible. Some informational suppressors of point mutations in Dyf genes, for example, could act to suppress both phenotypes by totally restoring normal gene function, however partial reversion of phenotypes by informational suppressors is also possible (Waterston and Brenner, 1978; Hodgkin, 1985) and informational suppressors which restore wild type gene function to levels which reverse IVM resistance but do not reverse the dye filling defect might be possible. Ambiguous map data for the *sav* mutations au4 and au17 may indicate that one of these is an informational suppressor of nr2477. To further investigate this possibility it would be necessary to test suppression of nr2477 by known informational suppressor mutations (e.g. amber suppressors *sup-5*, *sup-7*, *sup-21*, *sup-24*, *sup-28*, *sup-29*, *sup-33*, *sup-34* (Kondo, Makovec et *al.*, 1990)) and to test the alleles au4 and au17 for suppression of known mutations suppressible by informational suppressors (e.g. amber suppressible mutations *unc-13*(e450), *unc-51*(e369), *unc-24*(e138), *unc-15*(e1214), *unc-52*(e669), *dpy-20*(e2017), *lin-1*(e1777) (Kondo, Makovec et *al.*, 1990)). Eventually, sequence information for nr2477 may indicate whether the mutation could be suppressible by aberrant tRNA molecules. Also neomorphic mutations which allow a mutant protein to substitute for another missing component might completely reverse amphid defects, as with *sup-11* suppression of *unc-93* (Greenwald and Horvitz, 1982). As no suppressors reversing both Dyf and IVM resistance were observed in the mutation experiment, these are probably rarer than the Sav suppressors isolated.

sav suppressors of Dyf mutants should be useful in elucidating the mechanisms of resistance to IVM. Mosaic analysis for example (Herman, 1984; Herman, 1987) could be used to discover the cell types expressing both *dyf* and *sav* genes. Predictions of functional interactions between *sav* and Dyf mutations may be aided by molecular characterisation; and nine of the *sav* loci mentioned in the above discussion (*dpy-7*, *dpy-5*, *unc-18*, *unc-52*, *unc-54*, *unc-104*, *unc-116*, *snt-1* and *snt-2*) have been cloned as have nine of the 30 low-level IVM resistance loci. Additionally, the cell types important to the Dyf phenotype but unimportant for IVM resistance could be identified using either

mosaic analysis or expression of recombinant constructs of Dyf genes.

4.4.3. nonUnc nonDpy *sav* mutations may interact with *unc* mutations.

During genetic mapping experiments, the normally recessive *sav* mutants au1, au2, au3, au4, au5, au16, and au20 appeared to have a dominant maternal effect, increasing the proportion of sensitive animals (*sav*/+, *unc*/*unc*) in the presence of certain *unc* mutations (see appendix B). This effect is both *sav* and *unc* locus specific, as no one *unc* mutation interacted with all *sav* genes. No effect on dominance of *sav* mutants was observed with *unc-24*(e138), however there were interactions between *unc-4*(e120) and au1, au5 and au18, *unc-15*(e73) and au1, *unc-32*(e189) and au2, au4, au16 and au18, *unc-22*(e66) and au18 and between *unc-60*(e723) and au3 (Appendix B). This observation may indicate that suppression of IVM resistance can be enhanced by nonSav mutations.

Dominant effects of normally recessive mutations in certain genetic backgrounds have been described previously in *C. elegans* (Kusch and Edgar, 1986). No maternal effects of *sav* mutations were observed in simple outcrossing experiments, or in any experiments other than the genetic mapping. Further investigation will be needed to confirm or disprove any hypothesis involving the interaction of *unc* and *sav* mutations, however these results may indicate that the action of IVM in *C elegans* can be modified by a cascade of gene products within one or more complex biochemical pathways.

Another explanation for these results might be that some of the *sav* mutations are nonUnc alleles of the *unc* marker mutations used. For example, *unc-60*(e723) slightly suppresses IVM resistance in *dyf-12*(nr2477), a nonUnc allele of *unc-60* which suppresses resistance to 5 ng/mL might be possible. If a Sav mutation was an unusual allele of *unc-60*, 3/4 of the Unc F_2 progeny (protocol A) would be nonAvr if the Sav and Unc *unc-60* alleles failed to complement for both the Sav and Unc phenotypes.

4.4.4. *dpy* mutations can suppress IVM resistance.

A number of *dpy* genes are implicated in suppression of IVM resistance. These include the collagen gene *dpy-7* (Johnstone, Shaffi et *al.*, 1992), as well as *dpy-8*, *dpy-5* and two unidentified *dpy* genes on LGII (au20) and LGIII (au19). An explanation for the suppression of IVM resistance by *dpy* genes may be related to the temperature sensitivity of IVM resistance in

Dyf strains (see Chapter 2.). Levy, Yang and Kramer, (1993) proposed a mechanism for the suppression of *glp-1* and *mup-1* by the collagen genes *dpy-2*, *dpy-10* and *sqt-1*. They proposed was that mutant collagen proteins could accumulate in an abnormal unfolded state, inducing a stress response similar to heat shock. As the alleles of both *glp-1* and *mup-1* that are suppressible by mutant collagen genes are temperature sensitive, induction of a stress response similar to heat shock in strains homozygous for these mutations could clearly result in suppression. IVM resistance in Dyf strains is also temperature sensitive (Chapter 2,3) and therefore the suppressor action of *dpy* genes may be mediated by a similar mechanism to that proposed by (Levy, Yang et *al.*, 1993). At least one of the *dpy* genes involved in suppression of IVM resistance (*dpy-5*) is not a collagen gene (Baillie, Pers. Comm., 1994). However it is possible that *dpy-5* is important in collagen localisation or processing and that mutants in this gene could result in the accumulation of incorrectly processed collagen.

An alternative explanation for Dpy-mediated suppression of Avr might be that Dpy mutations can alter the accesibility of various nematode tissues to IVM. Some *dpy* genes alter collagens which are found in extracellular cuticle (Edgar, Cox et *al.*, 1982; Johnstone, 1994; Kramer, 1994) and changes in the cuticle might affect the ability of IVM to penetrate it. Although the cuticle of *A. suum* is permeable to IVM, various chemical treatments increase the rate of entry of the drug through isolated patches of cuticle (Ho, Geary et *al.*, 1990). It is possible therefore that the kinetics of IVM entry are changed by *dpy* mutations so that the drug concentration in target tissues is increased. The drug concentrations which kill Dyf worms might therefore be reduced in Dpy,Dyf worms. One problem with this model is that Dpy worms are not super sensitive to IVM (Chapter 2), as would be expected if increased drug entry was apparent in these worms. One possibility might be that drug kinetics are unaffected by cuticle structure at low drug concentrations because the cuticle is not saturated by the drug, and that cuticle structure only affects drug entry at higher concentrations.

4.4.5. *unc* mutations suppressing IVM resistance of Dyf mutants may be useful in elucidating the mechanism of resistance to IVM.

4.4.5.1. Kinesin-like *unc* mutations.

Mutations in the *unc-104* kinesin-like gene of *C. elegans* result in defects in the transport of synaptic vesicles in axons (Hall and Hedgecock, 1991; Hedgecock and Hall, 1991). Suppression of IVM resistance by *unc-104* therefore suggests that normal axonal (and synaptic) function is necessary for IVM resistance. Recent evidence that synaptotagmin mutants are also capable of suppressing IVM resistance (Grant, Pers. Comm., 1995) also suggests that synaptic function must be normal for IVM resistance by Dyf mutants.

Dyf mutants are commonly dauer-formation defective (see Chapter 1 and (Albert, Brown et *al.*, 1981)), however if the amphid neurons ADF, ASG, ASI and ASJ are killed by laser ablation, the resultant phenotype is constitutive dauer larvae formation (Bargmann and Horvitz, 1991), also if all amphid cilia are missing due to a mutation *daf-19*(m86), dauer larvae are formed constitutively (Perkins, Hedgecock et *al.*, 1986). The Dyf mutants which have been analysed however, have less severe amphid ultrastructural abnormalities than *daf-19* and are Daf-d. The discrepancy between killing amphid neurons and isolating them from the environment can be best explained by assuming that external stimuli inhibit, rather than stimulate, amphid neurons (Bargmann and Horvitz, 1991). These observations are consistent with there being a constitutively transmitted signal from amphid neurons in Dyf worms and support the following hypothesis that explains suppression of IVM resistance by kinesin and synaptotagmin mutants.

Dyf mutations prevent exposure of chemosensory amphid cilia to the external environment (Perkins, Hedgecock et *al.*, 1986; Starich, Herman et *al.*, 1995). If in the absence of environmental stimulation, the amphid neurons constitutively transmit a signal important for IVM resistance, then mutations preventing the transmission of that signal can result in suppression of IVM resistance. The nature and function of this signal is not known but it may be similar to the constitutive signal that inhibits dauer formation (Bargmann and Horvitz, 1991), it is unlikely however that the signals are identical, as *daf-19*(m86) is Daf-c (Perkins, Hedgecock et *al.*, 1986) and Avr (Chapter 2.).

The failure of *unc-104*(e1265) and *unc-116*(e2310) to suppress IVM resistance in all Dyf mutants can be explained by

considering that e1265 and e2310 are mild loss of function alleles (Hall and Hedgecock, 1991; Patel, Thierry-Mieg et *al.*, 1993) and may fail to completely block a constitutive signal produced by mutant amphid neurons in some Dyf strains. In combination with weakly Dyf strains, in which amphid neurons may still receive some inhibitory input from the external environment, weak alleles of *unc-104* and *unc-116* may be able to block amphid neuron signalling. This is consistent with the above observations in which the weakly Dyf alleles *che-12*(e1812) and *mec-8*(e398) (Perkins, Hedgecock et *al.*, 1986) were suppressed by *unc-104*(e1265) whereas the strongly Dyf alleles *osm-1*(p808), *osm-3*(p802) and *osm-6*(p811) were not.

The data presented here, however, also show that the strongly Dyf mutations *daf-6*(e1377) and *osm-5*(p813) (Perkins, Hedgecock et *al.*, 1986) were *unc-104*(e1265) suppressible and other data (Grant, Pers. Comm., 1995) show that several strongly Dyf alleles of *che-3* are *unc-104*(e1265) and *unc-116*(e2310) suppressible. There is a discrepancy between the above proposal and results showing suppression of IVM resistance in strong Dyf mutant strains by unc-104 and unc-116. One possibility may be that the Dyf mutations involved inhibit neuron signalling as well as altering cilia morphology, thus a weak impairment of neuron signalling via *unc-104*(e1265) or *unc-116*(e2310) may be additive with weak interuptions to signaling mediated by *che-3* or *osm-5* alleles to block constitutive signals in Dyf,Unc strains. If this were the case, then the severity of the Dyf mutation may not be related to the strength of constitutive signals produced from amphid neurons in Dyf worms. A final model for suppression of IVM resistance of Dyf mutations by weak kinesin mutations must await further characterisation of the functions of the Dyf genes involved. More severe alleles of *unc-104* or *unc-116* might suppress IVM resistance more efficiently than the alleles used in this study, however the viability of more severe alleles is reduced (Hall and Hedgecock, 1991; Patel, Thierry-Mieg et *al.*, 1993), and this might mask suppression effects. Another approach might be to create mutant strains carrying *unc-104*(e1265), *unc-116*(e2310) and Dyf mutations, in the expectation that the combined action of both Unc mutations might suppress IVM resistance more efficiently then either alone.

4.4.5.2. *unc* mutations involved in muscle structure.

The mutations *unc-52*(e444) *unc-54*(e190) and *unc-60*(e723) were shown to be suppressors of IVM resistance conferred by some Dyf genes. Each of these mutations affect muscle structure (Wood, 1988). The action of *unc-60*(e723) on resistance of two alleles of *dyf-12*(nr272 and nr2477) and on *dyf-10*(nr2389) is of special interest. There seems to be a limit on resistance at some point between 10 and 15 ng/mL IVM in strains homozygous for *unc-60*(e723). Assembly of a collection of double mutant strains homozygous for *unc-60*(e723) and Dyf mutations that confer resistance to concentrations of IVM above 15 ng/mL, might reveal an important limiting effect of *unc-60*(e723). The deduced protein sequence of *unc-60* is homologous to actin-binding proteins that have been implicated in actin filament assembly in vertebrates (McKim, Matheson et *al.*, 1994).

A mutation in the *unc-52* gene suppresses IVM resistance conferred by *dyf-6* and *daf-10* but not *dyf-7*, *dyf-12* or *che-3*. The protein product of the *unc-52* gene has homology to perlecan, which is a protein found in basement membranes and which may function in muscle tissue to anchor thick and thin filaments to the extracellular matrix (Rogalski, Gilchrist et *al.*, 1995).

The principal site of action of IVM has been proposed to be neuronal or muscular in both nematodes and arthropods (Scott and Duce, 1985; Martin and Pennington, 1989; Martin, Kusel et *al.*, 1992), and in *C. elegans* and *H. contortus* the pharynx appears to be extremely sensitive to the drug (Avery and Horvitz, 1990; Geary, Sims et *al.*, 1993), and may be more accessible to IVM than other tissues of the nematode (see section). It is possible that mutations which weaken the pharynx muscle might increase the sensitivity of the pharynx to IVM, and so decrease IVM resistance in Unc;Dyf worms through some additive action. If mutations such as *unc-52*(e444) and *unc-60*(e723) increase sensitivity of pharyngeal muscle to the actions of IVM, then these mutants might also be super sensitive to IVM.

The observation that *unc-54*(e190) suppresses IVM resistance in *dyf-12*(sa127) and *dyf-6*(mn364) however conflicts with this idea as *unc-54* is not essential for pharyngeal muscle function (Waterston, 1988). Perhaps the interaction with *unc-54*(e190) reflects interactions on some pleiotropic phenotype that is not directly related to IVM resistance. Some Dyf mutations exhibit a wide range of pleiotropic phenotypes and in *mec-8* mutants (Lundquist, Shaw et *al.*, 1993) this includes affects on body wall muscle.

unc-52;mec-8 double mutants also exhibit a synthetic lethal phenotype (Lundquist, Shaw et *al.*, 1993). Perhaps the combined actions of some Dyf mutations with muscle Unc mutations and IVM produce a similar synthetic lethality. This type of interaction would be expected to be locus (and perhaps allele) specific, and interactions between muscle Unc mutations and Dyf mutations observed so far have been locus specific. Unlike observations in section 4.4.5.1. using kinesin Uncs, the small number of muscle-Unc;Dyf allelic combinations observed here is not large enough to make predictions on the specificity of these interactions.

These preliminary results indicate that analysis of mutations affecting muscle structure and their interactions with Dyf mutations may yield relevant information contributing to our understanding of IVM resistance in *C. elegans.* Also interactions between muscle structure mutants and Dyf mutants might reveal which Dyf mutants have pleiotropic effects in muscle cells. Observations of interactions between Dyf mutants and mutations affecting only non-pharyngeal muscle might be especially illuminating.