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Proper expression of myosin genes in transgenic nematodes

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Caenorhabditis elegans **has four genes which encode skeletal myosin heavy chain isoforms. We have re-introduced clones of two of these genes, *myo-3* and *unc-54* at low copy number into the germline of *C.elegans*. The resulting loci behave as functional copies of the genes by two genetic criteria: (i) they can result in phenotypic rescue of strains carrying inactivating *myo-3* or *unc-54* mutations, and (ii) their presence in strains with wild-type copies of the endogenous myosin loci has genetic consequences similar to duplicating the endogenous loci. The re-introduced genes function at a level close to that of the endogenous loci. Monoclonal antibodies specific for the different isoforms have been used to localize the expressed proteins. The re-introduced genes express in precisely the same cell types as the endogenous genes, and the myosin products produced assemble into filament structures as in wild-type. Unexpectedly, we have found in the course of this work that very high copy numbers of the *unc-54* gene lead to a disruption of muscle structure which may result from overexpression of the protein product.**

Key words: Caenorhabditis elegans/gene expression/myosin

Introduction

Caenorhabditis elegans is a simple roundworm which is ideally suited for the study of cellular differentiation. The newly hatched animal has only about five hundred cells. These include many of the cell types seen in higher organisms: muscle, epidermis, intestine, and a variety of neurons and support cells. All of the differentiated cell types arise from a single fertilized oocyte within the first few hours of embryogenesis. The pattern of cell divisions (the 'cell lineage') leading from an oocyte to the constellation of differentiated cells has been described in detail for *C.elegans* (Sulston *et al.*, 1983), and is virtually identical in every *C.elegans* embryo.

We are using *C.elegans* to study the signals leading to the production of muscle cells. The muscle cells are particularly appropriate for defining developmental signals, as their function, biochemical composition, and anatomy have been the subjects of extensive characterization (for review, see Waterston, 1988). There are two prominent muscle types in *C.elegans*: the pharyngeal muscles are used by the animal to eat and grind food and the body wall muscles are primarily used for locomotion. The major components of muscle cells are a set of thin and thick filaments that slide against each other, causing muscles to contract. The thick filaments consist primarily of myosin and paramyosin and the thin filaments primarily of actin. In studying gene expression in muscle cells, we have begun with the set of genes that encode the heavy chain of myosin. There are four such genes; two separate genes (*myo-3* and *unc-54*) encode different myosin isoforms expressed in body wall type muscle (mhcA and mhcB), and two (*myo-1* and *myo-2*) encode the two pharyngeal myosin isoforms (mhcD and mhcC) (Miller *et al.*, 1986). Some of the properties of the myosin genes and their encoded isoforms are summarized in Table I.

In order to analyze myosin expression, we have used gene transfer techniques, recently developed for *C.elegans*, which involve micro-injection of DNAs into *C.elegans* oocytes (Fire, 1986; A.Fire, D.Moerman, S.Harrison, D.Albertson and R.Waterston, in preparation). In this paper we demonstrate that isolated DNA segments containing either *myo-3* or *unc-54* can function properly when re-inserted into an arbitrary point in the animal's genome.

Results

Introduction of the myo-3 gene into the germline

We wished to assay for the function of cloned *myo-3* sequences by re-introducing the gene back into the germline and then testing for complementation of a putative null mutation in the *myo-3* gene. To do so we used the transformation vector pAst, which contains a selectable marker gene (the *C.elegans* amber suppressor tRNA gene *sup-7*).

Table I. Properties of the myosin heavy chain isoforms and their genes

Myosin isoform	Protein size (K)	Expressing tissue	Gene name	Chromosome	Null phenotype
mhcB	225.1	Body wall (major isoform)	*unc-54*	I (right)	uncoordinated
mhcA	225.5	Body wall (minor isoform)	*myo-3*	V (right center)	lethal
mhcC	223.0	Pharynx	*myo-2*	X (right center)	not known
mhcD	223.3	Pharynx	*myo-1*	I (center)	not known

The correspondences between genes and proteins was determined by genetic and immunomolecular methods (Epstein *et al.* 1974; Miller *et al.*, 1986). The protein molecular weights are deduced from the complete DNA sequences of the genes (Karn *et al.*, 1983; Dibb *et al.*, 1989). The tissue expression patterns are from microdissection (Epstein *et al.*, 1974) and staining with isoform-specific monoclonal antibodies (Miller *et al.*, 1983). Chromosomal locations are from genetics (Epstein *et al.*, 1974) and *in situ* hybridization (Albertson, 1985). The null phenotypes for *myo-3* and *unc-54* are from Epstein *et al.* (1974) and Waterston (1989) respectively.

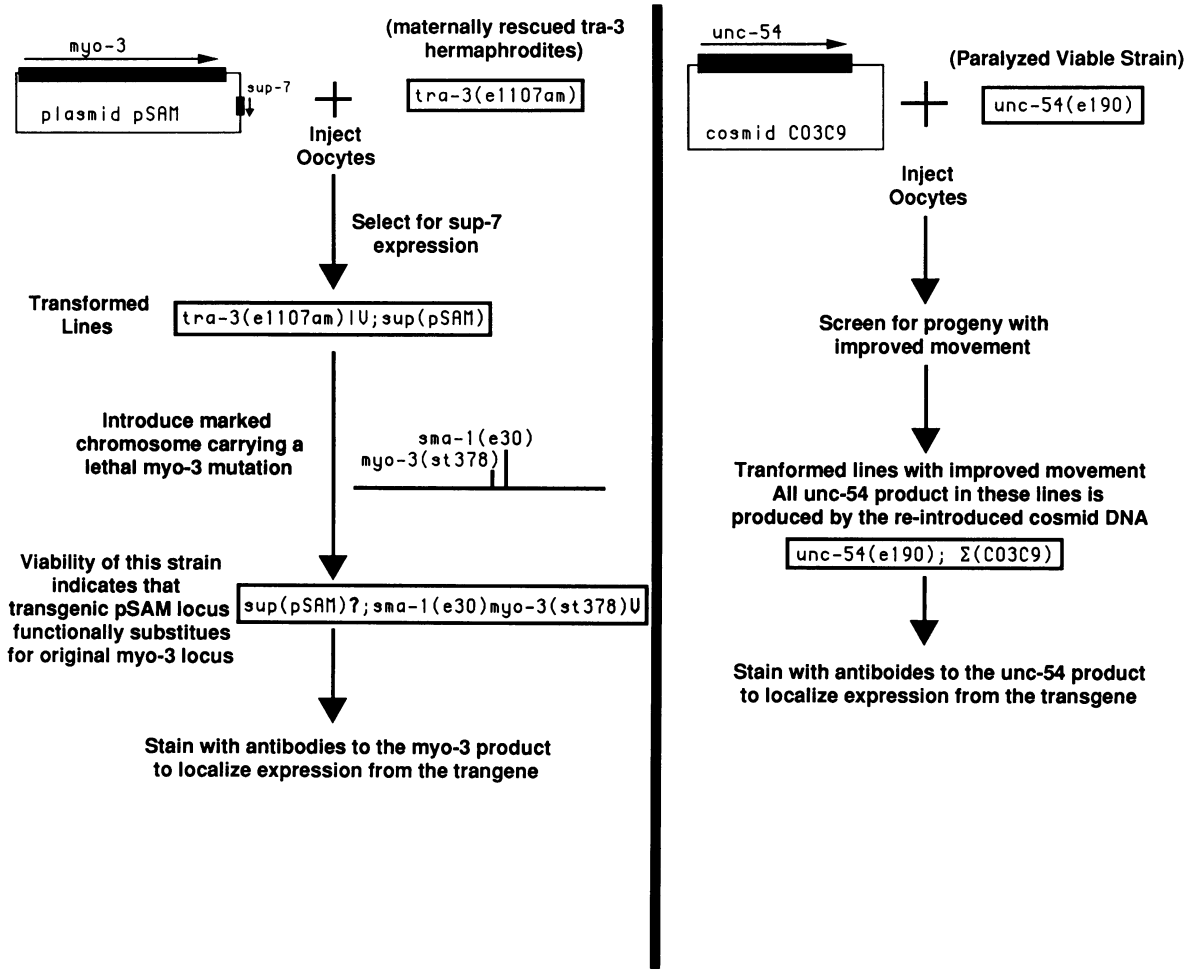

Fig. 1. Strategies for introducing the *myo-3* and *unc-54* genes into the *C.elegans* germline. The *myo-3* gene was introduced passively by co-selection for a linked marker (sup-7) while the *unc-54* gene was introduced using a direct selection for its expression. The strains and DNAs used are described in the text.

A 15 kb *Pvu*II fragment containing the *myo-3* coding region (7.34 kb) and extensive flanking sequences (both 5′ and 3′) was inserted into the pAst vector at the *Hin*cII site. The resulting plasmid, called pSAM (Figure 1), was injected into oocytes of animals carrying an amber mutation in the *tra-3* gene [the selection procedure is described in Fire (1986)]. Three independent transformed lines were derived from these injections. It should be noted that at this stage we were solely selecting for expression of the tRNA gene *sup-7*. Each of the three transforming loci behaves as a simple Mendelian element and each locus is stable and viable in homozygous form. The three loci are designated *e2185*, *e2187* and *e2188*. Southern blots were used to confirm the presence of the injected DNA in each of the transformed lines (Figure 2). These blots revealed that the DNAs had integrated at low copy number, as had been previously observed for the *sup-7* selection (Fire, 1986). In addition, some novel fragments absent in the injected plasmid and the original chromosome are present in each line. These fragments may represent junctions between the integrating plasmid and the chromosome.

Function of the re-introduced myo-3 gene

A recessive lethal mutation (*st378*) thought to inactivate the *myo-3* structural gene had been isolated [described in the preceding paper (Waterston, 1989)]. The *st378* locus can be manipulated genetically by using the very closely linked genetic marker *sma-1*. This allowed us to introduce the three pSAM loci into a homozygous *st378/st378* genetic background. The resulting animals are viable and have normal motility. Each of the transgenic *myo-3* loci acts as a dominant suppressor of the lethality associated with *st378*. The transgenic loci thus exhibit both amber suppression activity (as expected from the *sup-7* marked used for transformation) and suppression of the putative *myo-3* mutation. The two suppressor activities segregate together in genetic crosses, as would be expected for two genes introduced on the same plasmid. As an important control, it was necessary to show that the observed suppression of *st378* is not due to the expression of the *sup-7* tRNA. Several amber suppressor loci have been tested and shown not to suppress *st378*. The suppressor loci tested include the original *sup-7* locus (Waterston, 1989) and several transgenic loci containing the *sup-7* gene without *myo-3*. Using the amber suppressor and *myo-3* activity it has been possible to map the three transgenic loci genetically. Two of the loci map on chromosome II (*e2185* and *e2187*) while the third locus (*e2188*) maps on the right arm of the X chromosome.

A number of studies selecting for reversion of mutations in the *unc-54* gene have shown that amplification of the *myo-3* gene can partially compensate for lack of mhcB protein (Riddle and Brenner, 1978; Waterston *et al.*, 1982; Otsuka, 1986; Miller and Maruyama, 1986). As an indepen-

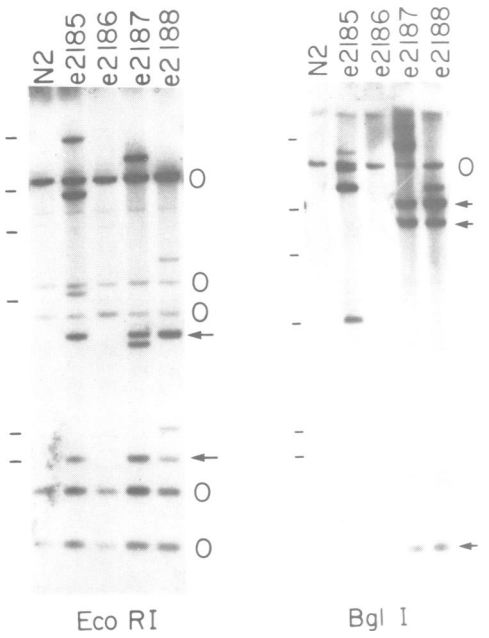

Fig. 2. Southern blots of pSAM transformed lines. DNA was extracted from wild-type animals (N2) and from populations of homozygous animals from transformed lines of the genotype *tra-3(e1107);sup(e21**)*. After cleavage with the restriction enzymes shown, the DNAs were electrophoresed on agarose, transferred to nitrocellulose (Southern, 1975) and hybridized to nick-translated pSAM. The common bands (marked with an 'O') in all lanes including N2 correspond to endogenous bands expected from the known restriction maps of the *myo-3* and *sup-7* loci (Bolten *et al.*, 1984; Dibb *et al.*, 1989, and our unpublished results). Bands with arrows are restriction fragments from the pSAM which overlap vector sequences; these are not expected in non-transformed strains. Unmarked bands present in the transformed lines presumably represent novel junctions formed during transformation. The marks on the left side of each gel represent *Hin*dIII digested lambda standards visualized by eithidium staining (marker sizes: 23130, 9416, 6557, 4361, 2322, 2027). Three of the suppressors of *tra-3(e1107am)* that were obtained following pSAM injection contained exogenous DNA; each of these loci complements the *myo-3* lethal allele *st378* (see text). A fourth suppressor of *tra-3,sup(e2186)* had no exogenous DNA; apparently *e2186* is an allele of one of the smg genes, which can mutate to suppress *tra-3* (Hodgkin, 1986; Hodgkin *et al.*, 1989). The *e2186* locus does not complement the lethality of *myo-3(st378)*.

dent test of the activity of the transgenic *myo-3* loci, we have used genetic crosses to introduce *myo-3* transgenic loci *e2187* and *e2188* into a strain with a deletion in the *unc-54* gene. In each case the transgenic *myo-3* locus leads to markedly improved movement in the *unc-54* null background. Amplification of the endogenous *myo-3* locus has also been shown to compensate for certain missense mutations in the paramyosin gene (Riddle and Brenner, 1978). All three transgenic *myo-3* loci exhibit suppression of the paramyosin missense allele *e73*.

In the genetically selected alterations in *myo-3* levels there is a correlation between the dosage of the *myo-3* gene and improved movement in the *unc-54* and paramyosin deficient backgrounds (Miller and Maruyama, 1986). From an examination of the movement of *unc-54* null animals with one or two copies of the transgenic *myo-3* locus in addition to the endogenous *myo-3*, we have constructed an activity series: *e2187* > *e2188* ≥ wild-type locus > *e2185*. A more quantitative estimate of activity was obtained by observing movement of the paramyosin defective animals with one or two copies of the transgene in the absence of endogenous *myo-3* activity. In this assay, the activity of the *e2185* locus was between 0.5 and 1.0 relative to the wild-type *myo-3* locus (defined as 1.0), *e2188* had an activity of approximately 1.0, and *e2187* activity was between 1.5 and 2.0. From the Southern blots, we estimate that *e2187* has 2−3 unrearranged copies of the transgenic *myo-3* locus, *e2188* has 1−2 copies, and *e2185* has a single copy. Table II gives a summary of activities and copy numbers for the three loci. From these data we estimate that each of the re-introduced *myo-3* copies has an activity within 2-fold of the endogenous wild-type locus.

Re-introducing the unc-54 gene

Null mutants in the *unc-54* gene (i.e. mutants lacking mhcB protein) are viable, with a slow phenotype as larvae progressing to virtual paralysis as adults (Brenner, 1974; Epstein *et al.*, 1974). These paralyzed adults are fertile but incapable of laying eggs, so that the fertilized embryos hatch out inside the parent and eventually eat their way to freedom. To see if clones of the *unc-54* gene are active upon re-introduction into *C.elegans* we performed a direct assay for mhcB function after injection. As a source of the *unc-54* gene, two cosmids were obtained from John Sulston and Alan Coulson; both cosmids contain the 7.2 kb myosin coding domain and extensive flanking DNA. Either cosmid (or a mixture of the two) was injected into animals homozygous for a deletion in *unc-54*. A fraction of the resulting progeny are rescued in that they have improved movement as larvae and adults and exhibit normal egg laying. Most (14 of 17) of these rescued progeny result from 'F1 expression' in the sense that they gave rise to only non-rescued progeny (the F2 of the injected animals). Three of the rescued F1 animals were germline transformants in that some of their progeny were rescued. For each of the three germline transformants, a transformed line has been derived, with the rescued phenotype continually present for at least 60 generations. The corresponding genetic 'loci' are denoted *e2189*, *e2190* and *e2205*. The first two loci were derived from a mixture of the two cosmids, and the third locus from cosmid CO3C9 alone. Southern blots of DNA prepared from these strains are shown in Figure 3. All three lines have bands hybridizing to the plasmid probe which were not present in the parent strain. The copy numbers (expressed in DNA copies per haploid genome in the affected nuclei) for the lines are relatively low: 1−2 for *e2189* and *e2205* and 6−12 for *e2190*.

The three transgenic *unc-54* loci behave differently as genetic elements. The *e2189* locus is stable and viable when homozygous; the locus maps genetically to chromosome III. The *e2205* locus cannot be obtained in homozygous form; rescued animals from this line segregate approximately 1/4 dead eggs, 1/4 non-rescued progeny and 1/2 rescued progeny. This is consistent with an integration event which has created a lethal mutation or rearrangement. The *e2190* locus is similar in that no homozygote locus can be obtained. In this case, however, very few dead eggs are observed. The segregation of the *e2190* 'locus' is consistent with the segment being an extrachromosomal duplication. Large extrachromosomal fragments are visible upon staining *e2190* with DAPI; these segments apparently contain the injected sequences attached to a broken segment from a *C.elegans* chromosome (D.Albertson, personal communication).

Rescued *unc-54(0)* animals carrying *e2189* in one or two

Table II. Summary of transformed lines

Lines transformed with myo-3

Strain	Locus	Inheritance	Chromosome	Transforming DNA	Copy number	Activity (1 = Wild-type)
CB4140	*e2185*	hz	II	pSAM	1	0.5–1.0
CB4142	*e2187*	hz	II	pSAM	2–3	1.5–2.0
CB4143	*e2188*	hz	X	pSAM	1–2	~1.0
PD2	*cc2*	hz	n.d.	pSAM4	n.d.	Rescues *st378* lethality

Lines transformed with unc-54

Strain	Locus	Inheritance	Chromosome	Transforming DNA	Copy number	Activity (60 = Wild-type)
CB4144	*e2189*	hz	III	C03C9+C13G1	1	60
CB4145	*e2190*	dup (50%)		C03C9+C13G1	6–12	30
CB4173	*e2205*	let	n.d.	C03C9	1–2	25
PD26	*cc26*	hz	X	pUNK54	1–2	35
PD27	*cc27*	hz	I	pUNK54	4–7	55
PD28	*cc28*	dup (75%)		pUNK54	5–10	50
PD29	*cc29*	dup (70%)		pUNK54	2–4	40
PD30	*cc30*	dup (20%)		pUNK54	30–50	10
PD31	*cc31*	hz	n.d.	pUNK54	3–6	60
PD36	*cc36*	let	n.d.	pUNK54	1–2	45
PD37	*cc37*	hz	n.d.	pUNK54	1–2	55
PD38	*cc38*	dup (60%)		pUNK54	1–2	40

This table summarizes the transformed lines discussed in the paper. 'Strain' refers to the name of the original transformed strain; 'Locus' refers to the designation of the transgene locus (the transforming activity apparently segregates as a single locus in each case). 'Inheritance' describes the segregation of the loci. Loci referred to as 'hz' are viable and can be grown as homozygotes. Loci referred to as 'dup' contain a cytologically visible free duplication and segregate as extrachromosomal elements (the segregation frequency at each generation is given). Lines referred to as 'let' segregate dead embryos at a frequency consistent with a lethal mutation linked to the integration site. 'Chromosome' refers to the linkage group of the transforming locus as determined by genetic mapping (see Materials and methods). 'Transforming DNA' is the plasmid or cosmid originally injected (see Materials and methods). 'Copy number' is an estimate from Southern blots of the number of copies of the transforming DNA per haploid genome in rescued animals. For lines that are not viable as homozygotes, we have taken into account the fraction of the animals prepared for DNA that were not phenotypically rescued. 'Activity' for *myo-3* is an estimate of activity relative to the original *myo-3* locus, based on the genetic analysis described in the text. For *unc-54*, the activity estimate refers to the motility of the rescued animals in the transformed strain. The number given is the frequency of propagating waves (per minute) of the most vigorous young adults on a Petri plate. This is not necessarily a linear estimate of activity. Activity estimates assaying competition with the product of the dominant unc-54 allele *e1152* (see text) have also been performed with *e2189*, *e2190*, *cc26*, *cc27* and *cc28*. Loci *e2189* and *cc27* have activities within 2-fold of wild-type in this assay while *e2190* and *cc26* have activities approximately 2-fold below wild-type and *cc28* apparently has activity about 2-fold above wild-type.

doses have essentially wild-type movement and egg laying, while movement in the other two rescued strains is somewhat less than wild-type. In the case of *e2189* we have a rough estimate of the level of expression based on the movement of animals into which a dominant allele of *unc-54* (*e1152*^{d}) has been introduced genetically. Anderson and Brenner (1984) have shown that animals carrying one copy of *e1152*^{d} have distinctly different levels of mobility depending on whether 0, 1 or 2 copies of a normal wild-type *unc-54* locus are present. Based on comparisons between such strains and animals carrying one copy each of *e1152* and *e2189*, we can estimate that the activity of the *e2189* locus is within 2-fold of that of the normal resident *unc-54* gene.

Localizing the products of the re-introduced myosin genes

The restriction of the mhcA and mhcB isoforms to body wall type muscle cells had previously been shown by micro-dissection experiments (Epstein *et al.*, 1974) and by staining with isoform-specific monoclonal antibodies (Miller *et al.*, 1983). In order to test whether the re-introduced genes were expressed in the correct tissues, we stained the animals with isotype-specific monoclonal antibodies. Miller *et al.* (1983) used these antibodies to determine the spatial organization of mhcA and mhcB within the muscle filaments, showing that mhcA isoform is present at the central region of each thick filament in body wall muscle, while the mhcB isoform is present along the outer regions of the filament. We used an antibody specific to mhcA (Dm5-6) which had been directly coupled to rhodamine, and an antibody to mhcB (Dm5-8) which had been directly coupled to fluorescein. When wild-type animals were stained with a mixture of these antibodies, we observed an alternating striped pattern within each body wall muscle cell similar to that originally observed (Figure 4a). The stripes in this case are not individual filaments but rather represent the obliquely aligned central regions of sets of filaments. The observed staining by both the mhcA and mhcB antibodies was limited to body wall type muscle cells.

We used the same mixture of antibodies to stain the rescued transgenic lines. For assaying *unc-54* function, the stained animals are the initial transformed lines, which carry the *e190* deletion in the endogenous chromosomal locus. When *e190* animals are stained with the antibody mixture, no staining with the anti-mhcB is observed, while strong but disorganized filamentous staining of *e190* is observed with

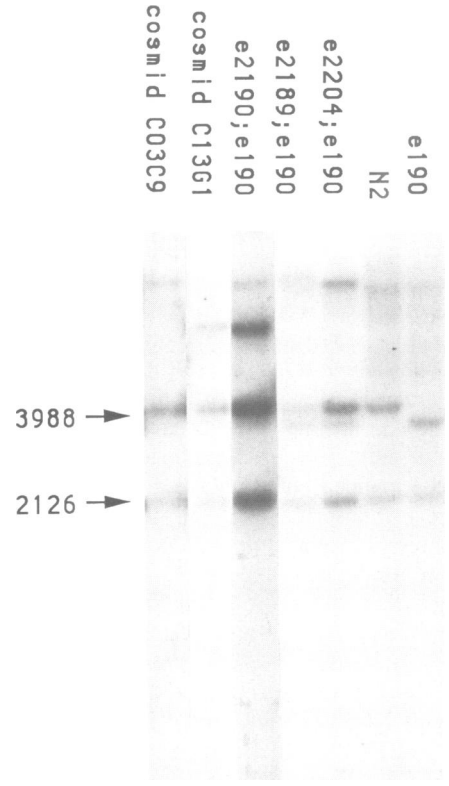

Fig. 3. Southern blot of lines transformed with *unc-54* cosmid DNA. DNAs from N2 (wild-type), and *unc-54(e190)* animals and from three transformed lines were cleaved with *Bgl*II, separated by agarose gel electrophoresis, transferred to nitrocellulose and probed with nick-translated plasmid pMRF [pMRF is pUC12 (Vierra and Messing, 1982) with nucleotides 4401 to 8955 of the *unc-54* gene inserted between *Eco*RI and *Bam*HI of the polylinker]. Comparable amounts of the five genomic DNAs were used. The two cosmid DNAs used for transformation were run as standards. The *e190* allele of *unc-54* is a 401 base deletion which lies entirely within the 3988 bp *Bgl*II fragment (Dibb *et al.*, 1985), hence all strains with *e190* have an endogenous chromosomal band migrating at 3587 bp. The cosmids each contain a wild-type copy of the gene, and hence the wild-type 3988 bp band is restored in each of the tranformed lines. The probe hybridizes also to vector sequences present in the cosmids, and these bands can also be observed in the transformed lines. Note that *e2189;e190* is viable as a homozygote, so that the DNA preparation was exclusively from animals with the transforming locus present exactly once per haploid genome. The other two DNAs were derived from mixed populations with only 50−75% rescued animals, since no homozygote line could be obtained.

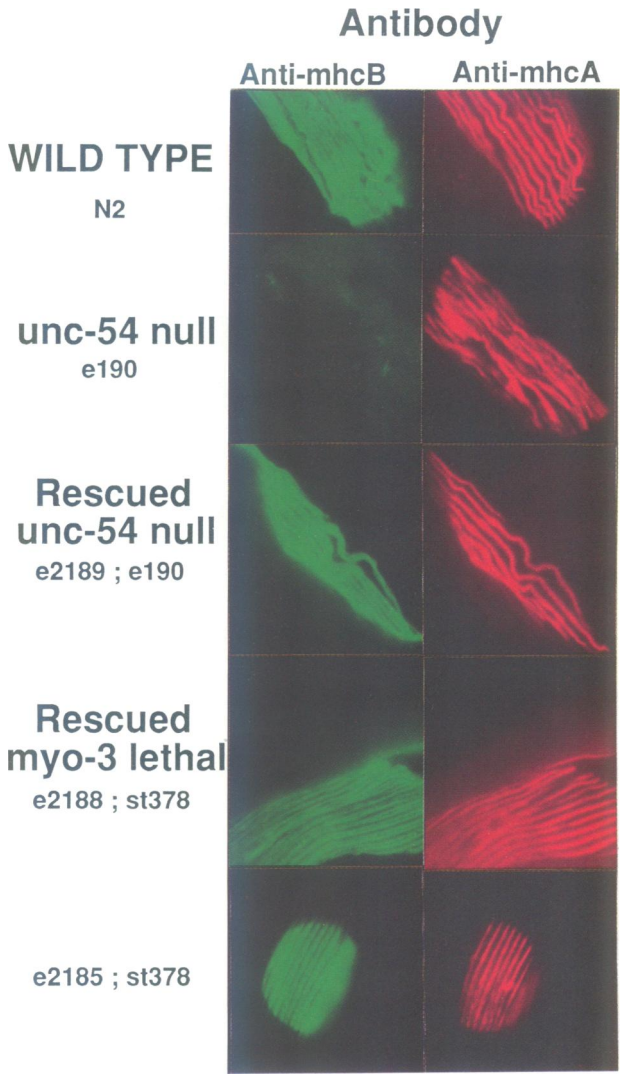

Fig. 4. Localizing expression of re-introduced myosin genes. **Top panel (a)**: wild-type (N2) and *unc-54(e190)* animals were stained with isoform-specific monoclonal antibodies (a gift of D.Miller). Fragments of muscle are shown (photographed at 1000×). Animals were simultaneously stained with two directly labeled monoclonal antibodies, 5−8 (anti-mhcB) labeled with fluorescein (left of figure) and 5−6 (anti-mhcA) labeled with rhodamine (right of figure). The stripes seen with the anti-mhcA and anti-mhcB antibodies correspond to the aligned centers and peripheral regions of muscle filaments as described by Miller *et al.* (1983). **Second panel (b)**: staining of *unc-54(e190)* animals. The *unc-54* null mutation *e190* eliminates mhcB and yields a disorganized mhcA staining pattern. **Third panel (c)**: the wild-type staining pattern is restored in muscle from animals from an *unc-54(e190)* derived transformed line carrying cosmid DNA spanning the unc-54 locus. **Bottom two panels (d and e)**: 'rescued *myo-3* null' lines carry a lethal mutation *myo-3(st378)* at the chromosomal locus encoding mhcA and copies of transgenic *myo-3* loci *sup(e2187)*II and *sup(e2188)*X. The staining pattern in these lines is similar to the wild-type pattern

the anti-mhcA antibody (Figure 4a). Each of the three transgenic *unc-54* loci restores mhcB staining in body wall muscle, with the staining pattern within the muscle regaining the wild-type pattern (Figure 4c). Staining for mhcB in these lines is found in body wall muscle and not in pharyngeal muscle or in non-muscle tissue. This is particularly evident microscopically upon the direct comparison of staining patterns with the two antibodies in wholemounts. Figure 5 shows staining of whole embryos with the mixture of antibodies.

For analyzing mhcA expression we stained lines homozygous for the *st378* mutation and for the transgemic *myo-3* locus. Since the *st378* mutation is lethal, it is not possible to examine staining in non-rescued adults homozygous for *st378*. It has been possible, however, to obtain some staining of embryos and arrested larvae homozygous for *st378*. These animals have very disorganized muscle with strong staining for mhcB. No staining with the anti-mhcA antibody was seen in embryos while only a very low level of muscle staining was seen in the arrested larvae. The rescued lines by contrast show the full wild-type pattern of staining with strong staining for mhcA and a well organized filament structure (Figure 4d, e). The staining is limited to body wall type muscle: no staining in pharyngeal muscle or non-muscle tissue is observed (Figure 5).

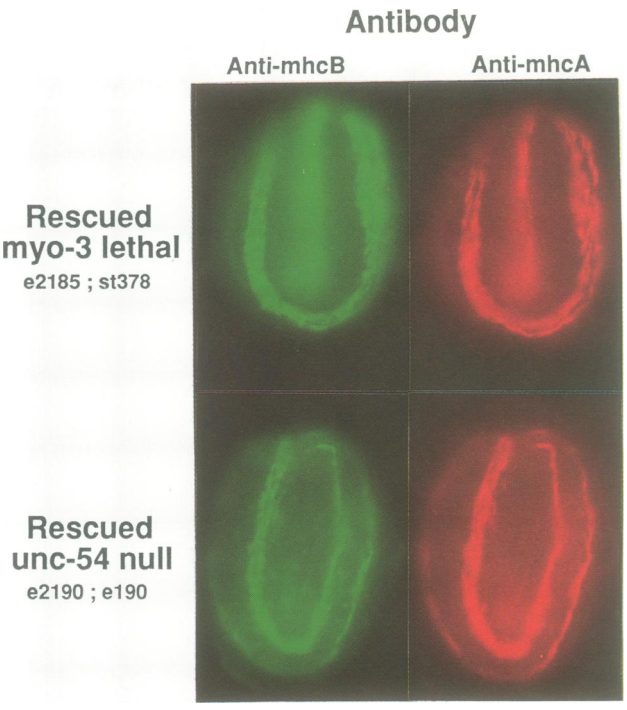

Fig. 5. Antibody staining of embryos from transgenic lines. In each case the embryo shown carries an inactivating mutation at the chromosomal locus for one of the myosin genes, with the corresponding gene re-introduced by transformation. Each photograph is an optical section of a whole stained embryo. Staining was performed as described by Albertson (1984). Anti-mhcB and anti-mhcA antibodies are illuminated on the **left** and **right** respectively (see legend to Figure 4). The two embryos are in slightly different orientation but in each case bands of staining corresponding to muscle quadrants are observed. The pattern of staining is identical to that seen in wild-type animals (not shown) stained in parallel: the two myosin isoforms are co-localized to non-pharyngeal muscle tissue in the rescued lines. No staining in pharyngeal muscles or non-muscle tissue is observed.

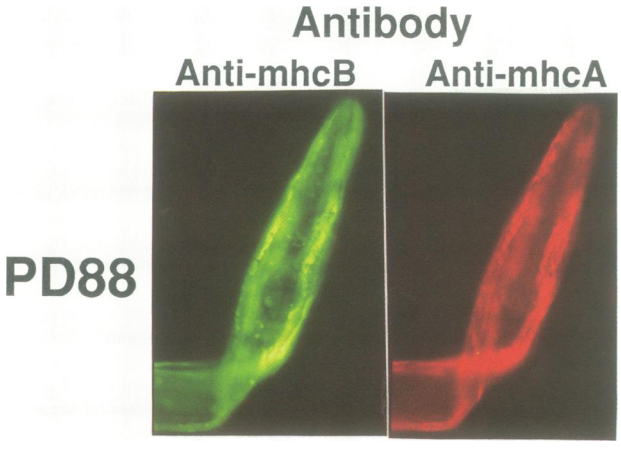

Fig. 6. Apparent overexpression of mhcB in transformed lines with high copy number tandem arrays of the *unc-54* gene. Staining was as in Figures 4 and 5. Note that intense staining with antibody to mhcB (**left**) is observed in an irregular (non-filamentous) pattern. This staining is not seen with the antibody to mhcA (**right**). The intensity of the mhcB staining is evident in a chromatic shift from green to yellow seen on the film.

Defined regions of the myosin genes are sufficient for correct expression

The segments of DNA used in the above experiments for rescuing *myo-3* and *unc-54* mutations were large segments in which only the regions around myosin genes have been sequenced (Karn *et al.*, 1983; Dibb *et al.*, 1989). It was thus a formal possibility that the observed biological activity could result from some unidentified gene present outside the sequenced regions in these plasmids. We have taken much smaller, sequenced regions of both *myo-3* and *unc-54* and repeated the assays for biological activity.

For *myo-3* we used a 10773 nt *Nae*I−*Acc*I fragment cloned into the *sup-7* vector pAst. The resulting plasmid (pSAM4) was used to make two transformed lines using the *sup-7* selection protocol. Of the two transformed lines, one was not viable as a homozygote and rescued animals exhibited properties (e.g. cold sensitivity) characteristic of overexpression of the *sup-7* gene. These properties are frequently observed with *sup-7* transformed lines; this line was not further characterized due to difficulty in genetic manipulations. The second line could be maintained as a healthy homozygous stock. The corresponding transgenic locus (*cc2*) has not been mapped but is unlinked to the original *myo-3* locus. The *cc2* insertion can suppress *st378* just as the original three transgenic *myo-3* loci do. When animals homozygous for both *cc2* and *st378* are stained with the anti-myosin antibodies, the wild-type staining pattern is observed. Thus the *Nae*I−*Acc*I fragment appears to contain all sequences necessary for *myo-3* function. No genes other than *myo-3* are evident in the sequence of this fragment.

Plasmid pUNK54 (a kind gift of Dr Ichiro Maruyama) has a 9133-nt *Pst*I−*Xba*I fragment containing the *unc-54* gene. This plasmid was injected into a large number of animals bearing a null mutation in *unc-54*. These injections resulted in rescue of the animals, both in the form of transient expression and in the production of stably transformed lines. Nine such lines were characterized. All nine transformed lines have been stained with monoclonal antibodies: each line shows staining with the anti-mhcB antibody and this staining is limited to body wall type muscle cells. For all but one of the lines (PD30, described below), the muscle cells exhibit the normal spatial distribution of the two myosins and the normal pattern of filament organization.

Effect of high copy numbers of the unc-54 gene

Given the predominance of large extrachromosomal arrays in the experiments of Stinchcomb *et al.* (1985) it was suprising that such arrays are not generally obtained after selecting for expression of mhcB. It appears that such arrays actually have a paradoxical effect: interference with muscle function. The nine mhcB rescued lines transformed with pUNK54 DNA have varying copy numbers of the injected DNA, with the highest copy numbers well below the copy numbers in the tandem arrays described by Stinchcomb *et al.* (1985). Surprisingly, the highest copy number line, PD30 (*cc30;e190*), exhibits incomplete rescue with many of the rescued animals showing a variety of movement defects as larvae and adults. When this line was stained with the mixture of isotype-specific monoclonal antibodies, large deposits of mhcB staining material were found in some cells. This intensely staining material is present only in body wall type muscle, but is apparently not present in the normal myofilament arrays. In a variety of injections into *e190* animals with mhcB encoding plasmids, two more high copy number lines similar to PD30 have been obtained. In both cases, the rescue is poor and an antibody staining pattern similar to PD30 is observed. The behavior and muscle structure in these lines suggested that high copy numbers of the *unc-54* gene could actually interfere with muscle function.

In order to test this directly, we injected the pUNK54 plasmid into wild-type animals and looked at the progeny for animals that moved poorly. Such animals were found at a relatively high frequency (four affected F1 progeny from five injected adults). Three of the four affected F1 animals gave rise to F2 progeny that also had impaired movement. In this manner three independent uncoordinated lines were selected: PD87, PD88 and PD89. The phenotypes of the affected animals in these strains vary from subtle defects in movement to complete paralysis and failure of egg laying. The defective phenotypes segregate genetically as would be expected of extrachromosomal elements. In some cases the muscle structure appears to be mosaic, with some muscles functional and others paralyzed, consistent with the possiblity of mitotic loss of the free duplication.

Strain PD88 was selected for further analysis. Southern blots reveal the presence of a large number of copies of the injected plasmid DNA, apparently present in a tandem array (200−800 copies per nucleus in the affected animals; data not shown). When paralyzed animals from PD88 were analyzed by antibody staining, many of the muscle cells showed disorganized staining with anti-mhcA and anti-mhcB antibodies. The mhcB antibody staining was particularly distinctive in that large masses of staining material were present in the muscle cells (Figure 6). These masses did not stain with anti-mhcA antisera. The masses presumably result from the presence of the long tandem arrays of the *unc-54* gene, since their presence correlates with the impaired movement of the animals: normally-moving siblings of the paralyzed animals from PD88 were stained in parallel and showed a normal (wild-type) staining pattern.

Discussion

Previous studies with DNA mediated transformation have shown that DNA can be incorporated into the *C.elegans* germline in two distinct ways. Stinchcomb *et al.* (1985) described the formation of long tandem arrays of injected plasmids, which are inherited as extrachromosomal elements. These arrays contain several hundred copies of the injected plasmid, and are segregated to progeny at frequencies varying between 5% and 95%, so that they can be maintained in a population by selection. Genes present on the long tandem arrays can be expressed (Jefferson *et al.*, 1987; Way and Chalfie, 1988), but it is not yet clear whether the long tandem arrays can generate the correct patterns and physiological levels of expression.

A technique for integrative transformation of *C.elegans* has also been described (Fire, 1986). This technique uses a suppressor tRNA gene, *sup-7*, as a selectable marker. Selection for expression of the amber tRNA gene (by injecting into a recipient strain carrying an amber mutation) yields transformed lines with a low copy number (1−10) of the injected DNA. In general the injected DNA has integrated into a chromosome. Thus the tRNA selection favors low copy number integration. This probably reflects a selection against long tandem arrays containing *sup-7*: high levels of amber tRNA expression can be deleterious to the animal (Waterston and Brenner, 1978; Waterston, 1981). The integration of injected DNA into the chromosome is generally a non-homologous event. Although homologous recombination between injected plasmids frequently occurs during transformation events, homologous recombination with corresponding chromosomal sequences is extremely rare (A.Fire and S.Harrison, unpublished). The *sup-7* selection scheme has been used to introduce a fusion between a Drosophila heat shock promoter and *Escherichia coli* β-galactosidase. The integrated fusion gave heat shock induced expression of β-galactosidase, indicating that some regulation of the integrated DNA could occur (Fire, 1986). Similar transformation procedures have recently been used by Spieth *et al.* (1988) in their studies of vitellogenin synthesis.

In this work we have used the *sup-7* selection scheme to introduce a cloned copy of the *myo-3* gene. The cloned *myo-3* DNA was introduced into three independent chromosomal insertions, none of which is on the same chromosome as the original *myo-3* gene. We then tested for the ability of the newly introduced *myo-3* loci to complement a lethal mutation (*st378*) which was a strong candidate for a mutation in the mhcA structural gene (Waterston, 1989). In testing the resulting re-introduced loci for suppression of the mutation *st378* we were working with a number of unknowns. (i) It was not yet conclusively proven that *st378* was indeed a mutation affecting the mhcA structural gene. (ii) It was not known whether the cloned copy of *myo-3* was a complete functional copy with all necessary signals. (iii) It was not known whether *myo-3* (or any developmentally regulated *C.elegans* gene) could function properly when re-inserted into a different location in the genome.

The ability of each of the three transgenic *myo-3* loci to complement the *st378* allele strongly argues that the cloned *myo-3* DNA was both functional and active in different chromosomal environments, and provides strong direct evidence that the *st378* mutation indeed affects the mhcA structural gene.

Our measurements of activity and copy number for the re-introduced genes gave levels of activity which are generally within 2-fold of the endogenous gene. Since no initial selection was made for expression of mhcA, and the animal can tolerate significant increases in mhcA expression without any evident adverse effects (Waterston *et al.*, 1982), we can conclude that the large *myo-3* segment used for this analysis is likely to contain all the critical signals determining the level of expression in body wall muscle.

The *unc-54* rescue was performed using a significantly different protocol from the *myo-3* experiments (see Figure 1). With *myo-3*, we introduced the gene into the chromosome by selecting for the function of the *sup-7* gene, i.e. without selecting at all for myosin function. The transformed lines were then assayed to test the function of *myo-3* for each of the different independent insertions. The conclusion from these experiments is that the *myo-3* gene can function normally in virtually any environment (four of four in these studies) permissive for integration and *sup-7* activity. For *unc-54* we selected directly for function of the myosin gene in creating the transformed lines. Thus is it quite possible that some insertions were not selected because no scorable rescue occurred. It is notable, however, that the frequencies of transformed lines obtained using *unc-54* selection and *sup-7* expression have been similar for these studies (12/105 and 5/75). Thus it is likely that the integrative transformed lines that we select after *unc-54* injection represent a substantial fraction of all germline integration events that actually occur following the injections.

The *unc-54* gene can apparently transform to give both

very high copy number tandem arrays and lower copy number lines, the latter mostly integrated. Gene expression can occur from either type of structure, but some feature of the high copy number arrays actually interferes with muscle function. Our best hypothesis to explain this interference is that the *unc-54* gene present at very high copy number produces a large excess of the normal mhcB protein and that this excess protein interferes with muscle function. This is consistent with the observed excess of mhcB-staining blobs in muscle cells carrying the high copy number arrays. Several other explanations of the muscle disruption by large *unc-54* arrays are possible: the presence of *unc-54* DNA at high copy number might interfere with normal muscle gene expression or the *unc-54* DNA present in the long arrays might encode an aberrant protein (perhaps from a rearranged copy or copies) that interferes with muscle function.

Regardless of its mechanism, the disruption of muscle structure by high copy number arrays of *unc-54* provides a dominant selection for introducing and maintaining DNA. Since this scheme does not require any mutation in the recipient animal, it should be applicable in other nematodes which have not been extensively characterized genetically. We have used this to make a single transformed line of the related nematode species *C.briggsiae* (data not shown).

Tissue specific expression

We have used isoform-specific monoclonal antibodies (Miller *et al.*, 1983) to probe the tissue specificity of myosin expression in the transformed lines. In all cases the products of the re-introduced myosin genes are seen only in muscle cells, and never in non-muscle tissue. Within the different classes of muscle it is clear that the products of the re-introduced genes are found in the body wall type muscle cells, and not in the pharyngeal muscles which normally express mhcC and mhcD isoforms.

Would the antibody staining protocol detect myosin in non-muscle tisuse if it were inappropriately expressed there but did not assemble? We do not have examples of muscle myosins expressed in non-muscle tissue, but the high abundance of myosin in muscle cells argues that comparable levels could be detected in non-muscle tissue in virtually any state. The preservation and staining of myosin does not require its assembly into muscle filaments. This was shown by staining a translation termination mutant, *unc-54* (*e1300*), which produces a truncated mhcB protein which fails to assemble into filaments; animals homozygous for *e1300* stain well with anti-mhcB antibodies but the staining is throughout the muscle cell bodies and not organized into filaments (J.Priess, A.Fire and D.Miller, unpublished). Likewise in wild-type young embryos before muscle assembly is complete, unassembled myosin is visible in the muscle cell bodies (R.Waterston, 1989; R.Francis and J.Curry, J.Priess, personal communications).

The restriction of myosin expression to body wall muscle could occur by a variety of mechanisms including transcriptional and translational regulation as well as regulation of the stability or processing of the myosin proteins and/or messages. Our recent studies using myosin·β-galactosidase gene fusions (A.Fire and S.Harrison, in preparation) have shown that transcriptional regulation plays a large part in the specificity of expression.

Materials and methods

Source of DNA

All *myo-3* sequences were derived from the clone λsu3a, kindly provided by Nick Dibb and Ichiro Maruyama. This clone originally derived from the *sup-3*$^+$ strain CB1405 [in which the *myo-3* locus is duplicated on one homolog (Miller and Maruyama, 1986). This was the same clone that was used for determining the *myo-3* DNA sequence. Plasmid pSAM contains a *Pvu*II fragment containing the entire sequenced segment of *myo-3* inserted by blunt end ligation into the unique *Hin*cII site of plasmid pAST (Fire, 1986); plasmid pSAM4 is identical except that the myosin segment used is the *Nae*I−*Acc*I fragment spanning *myo-3*, with DNA polymerase used to blunt end the *Acc*I site. For both pSAM and pSAM4, the *myo-3* and *sup-7* genes are oriented in parallel. Cosmid clones C13G1 and C03C9 carrying the *unc-54* gene and extensive flanking regions were obtained from J.Sulston and A.Coulson. The plasmid pUNK54 was obtained from Ichi Maruyama. All of the *unc-54* clones were derived from wild-type *C.elegans* (N2).

Plasmids were prepared by the alkaline lysis protocol of Birnboim and Doly (1979), and further purified LiCl precipitation treatment with RNase and protease K, extractions with phenol−$CHCl_3$ and $CHCl_3$, and precipitation with ethanol.

Microinjection

Oocyte injections were performed as described by Fire, 1986. Injections of paralyzed mutants such as the *unc-54* null mutant *e190* can be performed by essentially the identical procedure used with phenotypically normal animals. Because the paralyzed animals are thin, they are placed more closely on the agarose pad for injection, and a shorter time is needed for drying. Because *e190* animals fail to lay their eggs, adult animals with as few fertilized eggs as possible (generally 0−2) are injected. The hatching of embryos inside their mother prevents the subsequent production of viable eggs by the maternal gonad. Therefore it is advantageous to arrest each of the fertilized enzymes in the injected mother. This can readily be done by poking each fertilized embyro once with the microinjection needle. The fertilized embyros will not fully develop after such treatment.

Screening progeny of injected animals

The selection of transformed animals using the amber suppressor gene *sup-7* as a selectable marker on the injected plasmid has been described (Fire, 1986). In the initial description it was noted that *de novo* mutations in several genes can lead to suppression of the *tra-3* (*e1107*^{am}) mutation [see Hodgkin (1986) for a more complete description of *tra-3* reversion experiments]. One non-transformed revertant strain has been isolated in the course of this work (*e2186;e1107*, Figure 2). No exogenous DNA is detected in the strain; instead there appears to be a mutation in one of the smg genes (Hodgkin *et al.*, 1989). This stresses the importance of confirming each transformed line, either by hybridization to the exogenous DNA or by demonstrating expression of an independently assayable genetic marker on the introduced plasmid. As noted previously the vast majority (>80%) of fertile lines derived after injecting *sup-7* plasmids into a *tra-3*^{am} background are indeed bona-fide transformed lines.

Scoring for phenotypic rescue in an *unc-54* null background is done on each of several consecutive days. The non-rescued animals are delayed at hatching and have severely impaired movement as early larvae progressing to paralysis as adults. Because the extent of movement impairment at a given stage is virtually invariant, even slight improvements can be reproducibly scored. The rescued animals varied in the extent of rescue. All animals scored as rescued were picked onto individual plates and observed for several days to confirm that their movement was indeed improved. For some rescued animals, motility worsened progressively as adults, while in other cases, rescue was evident in the original rescued animal even after several weeks. A fraction of the rescued animals lay eggs, indicating rescue in the vulval muscles. As a control, several plasmids containing only part of the *unc-54* coding region or with deletions in the coding region have been injected. None of these plasmids gives any phenotypic rescue.

Suppression of *unc-54* null mutations due to amplification of the *myo-3* gene had been described, and thus it was possible that this selection scheme could yield spontaneous amplification mutants in the *myo-3* gene. To date no such amplification mutations have been obtained, indicating that the spontaneous amplification frequency for the endogenous gene is much lower than our frequency of transformation.

Antibody staining

Animals were stained by a modification of published procedures (Albertson, 1984). Flow Laboratories 8 well multi-test slides (number 60-408-05) were

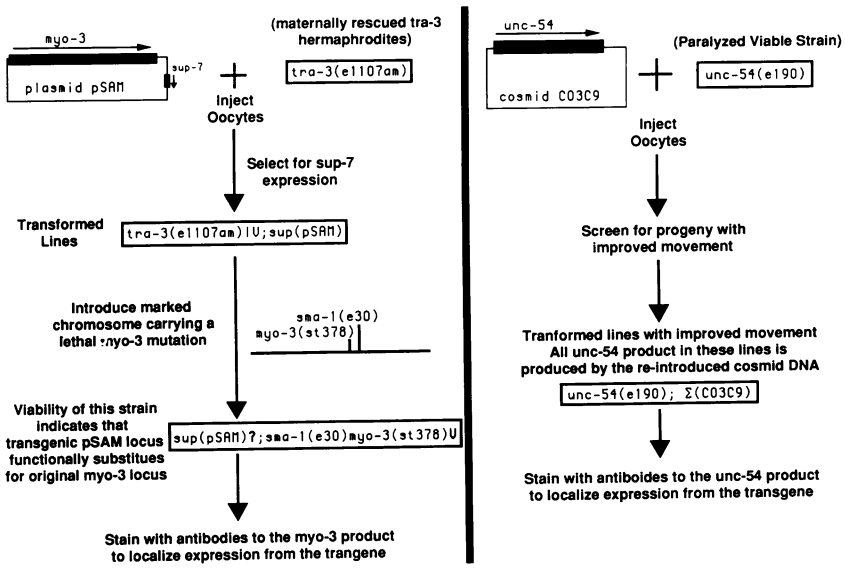

Fig. 7. Crosses for analyzing transgenic *myo-3* loci. The crosses shown were performed with *e2185*, *e2187* and *e2188*. For each locus, amber suppressor positive animals in generation '3' also gave rise to a high fraction of viable Sma progeny. For *e2187* and *e2185* animals without the amber suppressor activity were also recoverd in generation '3' (this is the genotype shown at left); no complementation of *myo-3(st378)* lethality was observed in the progeny of these animals (i.e. the only viable Sma progeny were rare recombinants in the *myo-3–sma-1* interval). The X linkage of the *e2188* locus precludes the appearance of the class of progeny on the left in generation 3, so that tight linkage of the *sup-7* and *myo-3* activities on the X chromosome could not be established in this cross. A reciprocal cross to that shown in the figure (in which the *sma-1(e30)myo-3(st378)* chromosome is introduced in males mated with the original transformed lines] has been performed with all four transgenic *myo-3* lines (not shown). This cross also demonstrated the ability of the transgenic loci to complement the *myo-3* lethal, and showed linkage between the amber suppressor and *myo-3* complementing activities for each of the four *myo-3* transformed strains.

overlayed with 20 μl of 0.05% polylysine 300 000 (from Sigma) per well, and left at room temperature for 10 min, after which the fluid was wiped off and the slides heated to 50°C (polylysine coating was done on the day of use). Animals were washed off Petri plates in buffer EN (0.1 M NaCl, 10 mM EDTA pH 7.5), rinsed in 4% sucrose 1 mM EDTA and pipetted (~3 μl per well) onto wells 1 and 8 only (leaving the other wells empty facilitates squashing and eliminates contamination between wells). A 22 × 40 mm coverslip was then overlayed and the animals squashed by applying gentle pressure. The slides were placed immediately after squashing onto an aluminium block cooled in dry ice, maintaining gentle pressure over the samples. After freezing, the coverslips were flipped off with a razor blade and immediately plunged into −20°C methanol. After 4 min, the slides were transferred to −20°C acetone for 4 min, and then washed for 1 min each at room temperature in 75%, 50%, 25% acetone and Tween-TBS (Priess and Thomson, 1987). The slides were then placed in Tween-TBS with 1% BSA for 30 min, excess liquid removed and 10 μl of antibody staining solution per well were added. After 12 h at room temperature the slides were washed for a total of 2 h in several changes of Tween TBS and for 30 min in Tween TBS with 1% BSA. After a final wash in Tween TBS, the slides were mounted in 80% glycerol 5% *n*-propyl gallate and observed in a Zeiss standard fluorescence microscope.

For these experiments, the antibody staining solution contained monoclonal antibodies which were a kind gift of David Miller. Antibody Dm5-6 (specific for mhcA) was labelled with rhodamine by Miller *et al.*. Antibody Dm5-8 was directly labelled with FITC as ascites fluid, dialyzed, and subsequently purified by chromatography on Sepharose. The differential staining of *e190* and wild-type animals by the labelled Dm5-8 confirms that the isotype specificity has been retained (Figure 3). The staining mixture contained antibodies 5–6 and 5–8 at approximately 1/5000 and 1/2500 dilutions from ascites fluid in Tween TBS + 1% BSA + 1 μg/ml DAPI.

Genetics

Testing loci for complementation of myo-3(st378). In order to test whether a given re-introduced locus complements the putative *myo-3* null mutation *st378* we first constructed a balanced strain carrying *st378* with a tightly linked marker *sma1*. The resulting strain is designated PD4003 = *DnT1*(IV,V)/*tra-3*(*e1107*^{am})IV *sma-1(e30)myo-3(st378)*V. *DnT1* is a reciprocal translocation between chromosomes IV and V, which acts as a balancer for most of chromosomes IV and V; *DnT1* also carries a dominant mutation conferring an uncoordinated phenotype and a recessive lethal mutation (Ferguson and Horvitz, 1985). Since *st378* is also a lethal, the strain PD4003 behaves as a perfectly balanced strain. The cross illustrated in Figure 7 can then be used to test whether a given transforming locus can complement *st378*. Note that between steps 3 and 4 of the cross it is conceivable that the *myo-3* and *sma-1* loci could become separated by recombination. The rarity of such recombination events is due to the close linkage of the markers. Recombination can easily be distinguished from bona fide complementation by two criteria.

First, the frequency of Sma animals in generation 4 in the protocol is characteristic: in the case of bona fide rescue, 3/4 of the *e30* bearing embryos carry the *st378* rescuing activity so that 20% of total viable progeny have the Sma phenotype. In the absence of reducing activity the frequency of Sma progeny is less than 1%, consistent with a distance of about 0.25 map units between the genes.

Second, the Sma animals can be checked to test whether they still carry the *st378* mutation, by outcrossing with wild-type animals. The Sma animals which are rare recombinants invariably contain a chromosome with the *e30* locus with no linked *st378* mutation, while the true rescued animals still carry *st378* on both homologs.

For each of the four transgenic *myo-3+sup-7* loci characterized, a homozygous line carrying *tra-3(e1107)*, *sma-1(e30)*, *myo-3(st378)*, and the transforming locus was obtained and outcrossed to confirm the presence of the two suppressed markers [*myo-3(st378)* and *tra-3(e1107)*].

In an independent set of experiments, the complementation activities of each of the transgenic *myo-3* loci derived from pSAM were confirmed using a different balanced strain in which the linked marker *sqt-3* V is used to follow *myo-3(st378)* in place of the balancer chromosome *DnT1* (data not shown).

Co-segregation of myo-3 and unc-54 activities for pSAM derived lines. Examination of the self progeny of the animals in step 3 of the cross described in Figure 7 can be used to determine the presence of the *myo-3* complementing activity (evidenced by a high frequency of viable Sma progeny) and *sup-7* suppressor activity [if this is absent in animals homozygous for *tra-3(e1107)* then all progeny will be pseudomales]. There is complete correlation between these two activities, indicating that two genes present in the same plasmid retained their original linkage after transformation.

Mapping of transgene loci. The mapping of transgenic *myo-3* loci *e2185*, *e2187* and *e2188* was performed by standard genetic means. Doubles between each of these suppressor loci and markers on chromosomes I, III, IV and V occur at the frequencies expected for non-linkage. [Markers used were *dpy-5(e61)*I, *unc-101(m1)*I, *dpy-17(e164)*III, *dyp-20(e1282)*IV and

*sma-1(e30)*V]. Crosses with *tra-2(e1095)*II indicate that *e2185* and *e2187* are linked to chromosome II: approximately 100 *tra-2* chromosomes derived from *tra-2*/sup heterozygotes were examined in each case. For *e2185*, two recombinant (sup *tra-2*) chromosomes were found, while none were found for *e2187*. This places both markers near the central genetic cluster on chromosome II.

Linkage to the X chromosome was tested directly in a cross assaying for transmission of the trait from males to both male and hermaphrodite progeny. In these crosses, *e2187* and *e2185* males passed their suppressor traits to both male and hermaphrodite progeny, while *e2188* males passed the trait to hermaphrodite cross progeny and not to male cross progeny. Two and three factor crosses with *e2188* have positioned the locus on the right arm of the X chromosome: *e2188* behaves as very distantly linked to *unc-1(e719)*X, and 10 ± 4 map units from *unc-3(e151)*X (by two factor cross). In a three factor cross, segregation of *lon-2(e678)e2188/unc-3(e151)* heterozygotes was examined. Eight of eight recombination events in the *e2188–unc-3* interval also break between *lon-2* and *unc-3*. This places *e2188* to the left of *unc-3* on the genetic map (Edgley and Riddle, 1988) at coordinate +9(±4).

Mapping of transgenic copies of *unc-54* was performed using a set of strains which are homozygous for an *unc-54* null mutation [$e1092^{ochre}$; *Dibb et al.* (1985)] and contain other genetic markers on several chromosomes. Males from the rescued strains were crossed with the marker strains, cross progeny hermaphrodites were identified by their improved movement and selfed, and linkage determined by scoring phenotypes of the resulting progeny. Linkage to the X chromosome is particularly evident in this procedure in that the male cross progeny of the cross would invariably be paralyzed. In some cases a second strategy has been used to determine linkage: males from a rescued *unc-54* null strain are crossed with a tester strain (e.g. a dumpy strain with a wild-type *unc-54* locus), and the cross progeny hermaphrodites are selfed. The absence of non-dumpy *Unc-54* progeny in the resulting broods indicates linkage between the transgenic $unc\text{-}54^{+}$ locus and the dpy marker. For the latter cross, the numerical prediction is U/(total progeny) = $p*(2-p)/16$, where U is the number of Unc progeny which are not Dpy and p is the recombination frequency between the transgenic *unc-54* locus and the dumpy marker. In mapping *e2189* with respect to *dpy-17(e164)*III we obtained zero Uncs of approximately 800 progeny, while in a similar cross with *vab-7(e1562)*III, we obtained 4 Unc (non Vab) animals of approximately 800 progeny. This data (and a three factor cross with *dpy-18* and *vab-7*) places *e2189* to the left of *vab-7* on the genetic map, at a position of −2.6 ± 2 on chromosome III.

Other strain constructions. Strains carrying transgenic *myo-3* loci with mutations in the endogenous *unc-54* locus were constructed by crossing sup;*myo-3(st378)sma-1(e30)* hermaphrodites with *unc-54(e1092)*/+ males. Cross progeny hermaphrodites were selfed and broods containing uncoordinated animals examined closely for the presence of animals whose movement was intermediate between that of *unc-54* nulls and wild-type. These animals were selfed and found to be homozygous for *unc-54(e1092)* and to contain at least one copy of the transgenic *myo-3* locus. The activities described in the text were obtained by comparing the movement of homozygous *unc-54(e1092)* animals with 0–2 doses of the wild-type chromosomal *myo-3* locus and 0–2 doses of each of the suppressor loci. For example, we assigned the activity of each of the suppressor loci to be at least half that of the wild-type *myo-3* locus by analyzing animals whose only body wall myosin comes from having two copies of a suppressor locus [in a *unc-54(e1092);myo-3(st378)* homozygous background]. These animals have a phenotype similar to the *unc-54* null phenotype; in particular, they are less severely paralyzed than *unc-54(e1092);sma-1(e30)myo-3(st378)*/+ animals whose only functional body wall myosin comes from a single wild-type *myo-3* locus. Strains carrying the paramyosin missense mutation *unc-15(e73)* with 0–2 doses of the endogenous and transgenic *myo-3* loci were constructed and tested in a manner analogous to that described above.

Interactions between transgenic copies of *unc-54* and the dominant *unc-54* allele $e1152^{d}$ (Anderson and Brenner, 1984) were analyzed by crossing males from the rescued transgenic lines with *unc-54*($e1152^{d}$) hermaphrodites and comparing the phenotypes of cross progeny with those of *unc-54*($e1152^{d}$)/+ heterozygotes as well as animals carrying a free duplication with the wild-type *unc-54* locus [i.e. *unc-54*($e1152^{d}$)/+/+ and *unc-54*($e1152^{d}$)/*unc-54*($e1152^{d}$)/+].

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