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Integrative transformation of *Caenorhabditis elegans*

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A technique for introducing exogenous DNA into the chromosomes of the nematode *Caenorhabditis elegans* is presented. A cloned *C. elegans* amber suppressor tRNA gene, *sup-7*, is used as a selectable marker. The activity of this amber suppressor is selected for by injecting worms which carry an amber termination mutation in a gene (*tra-3*) whose function is required for fertility. Transient expression of *sup-7* is evidenced by the presence of fertile (rescued) animals in the generation after injection. In a fraction of cases, these fertile animals give rise to stable suppressor lines (eight have been characterized so far). Each of the stable suppressor lines carries injected DNA sequences. The suppressor activities have been mapped to chromosomal loci, indicating that the exogenous DNA has integrated into the genome. This technique has been used to introduce a chimeric gene containing a *Drosophila* heat shock promoter element fused to coding sequences from the *Escherichia coli* β-galactosidase gene. This chimeric gene functions and is heat inducible in the resulting stably transformed lines.

Key words: C. elegans/transformation/amber suppressor/selection/integration

Introduction

A short generation time and well-characterized anatomy have made *Caenorhabditis elegans* a system of choice for developmental genetics (Brenner, 1974). Mutants affecting a wide variety of essential and non-essential functions have been isolated, so that the genetic map now consists of >500 complementation groups (Edgley *et al.*, 1985). Molecular biology of *C. elegans* genes began with the cloning and characterization of a variety of abundant structural proteins from different tissues (Kramer *et al.*, 1982; Karn *et al.*, 1983; Files *et al.*, 1983; Blumenthal *et al.*, 1984). More recently, the use of a transposon (Tc*1*) has allowed the cloning of genes defined solely by genetic and phenotypic criteria (Emmons *et al.*, 1983; Eide and Anderson, 1985a; Greenwald, 1985; Moerman *et al.*, 1986).

The functional characterization of cloned genes would be greatly facilitated by the availability of techniques for assaying the function of DNAs *in vivo*. This has been most striking in the case of *Drosophila*, with an efficient DNA transformation system currently being used in analyses of many different genes (Rubin and Spradling, 1983).

Work towards a *C. elegans* transformation system was begun by Kimble *et al.* (1982), who developed a procedure for microinjecting into the germ line of adult worms and showed that tRNA extracted from an amber suppressor strain could produce transient suppression when injected into a non-suppressor strain. Subsequently, Stinchcomb *et al.* (1985) injected a variety of plasmid DNAs and assayed (by Southern or dot blots) for the presence of the injected sequences in clonal pools of animals from subsequent generations. They found that a small fraction (<0.2%) of the progeny of injected worms had taken up the injected DNA, and that the DNA had been concatemerized *in vivo* to form long tandem arrays containing several hundred copies of the injected plasmid. These long tandem arrays are extrachromosomal, but were still heritable (although frequently lost) and could be maintained in subsequent generations by blotting DNA from clonal populations. Although DNA present in the long tandem arrays can be expressed at low levels, proper regulation has not been observed [Jefferson, 1985 (reviewed in Hirsh *et al.*, 1985)].

The recent availability of cloned *C. elegans* genes permits a somewhat more straightforward approach to obtaining functional transformation: an appropriate mutant strain is used as the recipient for injection, and progeny expressing the gene are identified by phenotypic transformation. In this work, I have used the amber suppressor tRNA gene *sup-7* as a selectable marker in obtaining integrative transformation.

Results

Use of a suppressor tRNA gene as a selectable marker

It was important to start with a *C. elegans* gene whose activity could be easily selected or screened for and whose structure was well characterized at the DNA level. The only gene which currently meets these criteria is the amber suppressor *sup-7* (Waterston, 1981; Wills *et al.*, 1983; Bolten *et al.*, 1984). A number of mutations in *C. elegans* are suppressed by a single copy of *sup-7*. For this work, I have used suppression of amber mutations in the *tra-3* gene to assay for suppressor function.

The tra-3 selection scheme

C. elegans has two sexes: XX animals are self-fertile hermaphrodites while X0 animals are male. The *tra-3* product is required for the development of normal self-fertile hermaphrodites (Hodgkin and Brenner, 1977). The absence of *tra-3* product causes partial masculinization of XX animals, resulting in abnormalities both in germ line and in somatic tissue. In particular these animals (called 'pseudomales') are sterile above 20°C and thus, homozygous *tra-3/tra-3* lines cannot be propagated above 20°C. An important property of *tra-3* is maternal rescue: expression of tra-3$^+$ in the mother is sufficient for rescue of the entire progeny brood (Figure 1). The *tra-3* amber alleles provide a very sensitive assay for amber suppression: expression of suppressor tRNA in either mother or zygote results in animals that are phenotypically rescued and hence fertile above 20°C (Hodgkin, 1985).

Transient expression assay

The initial goal was to assay for transient expression of the *sup-7* gene following microinjection. Healthy hermaphrodite animals homozygous for an amber allele of *tra-3* were obtained by taking advantage of the maternal rescue effect, i.e. parents of the injected animals were *tra-3*/+ heterozygotes with linked genetic

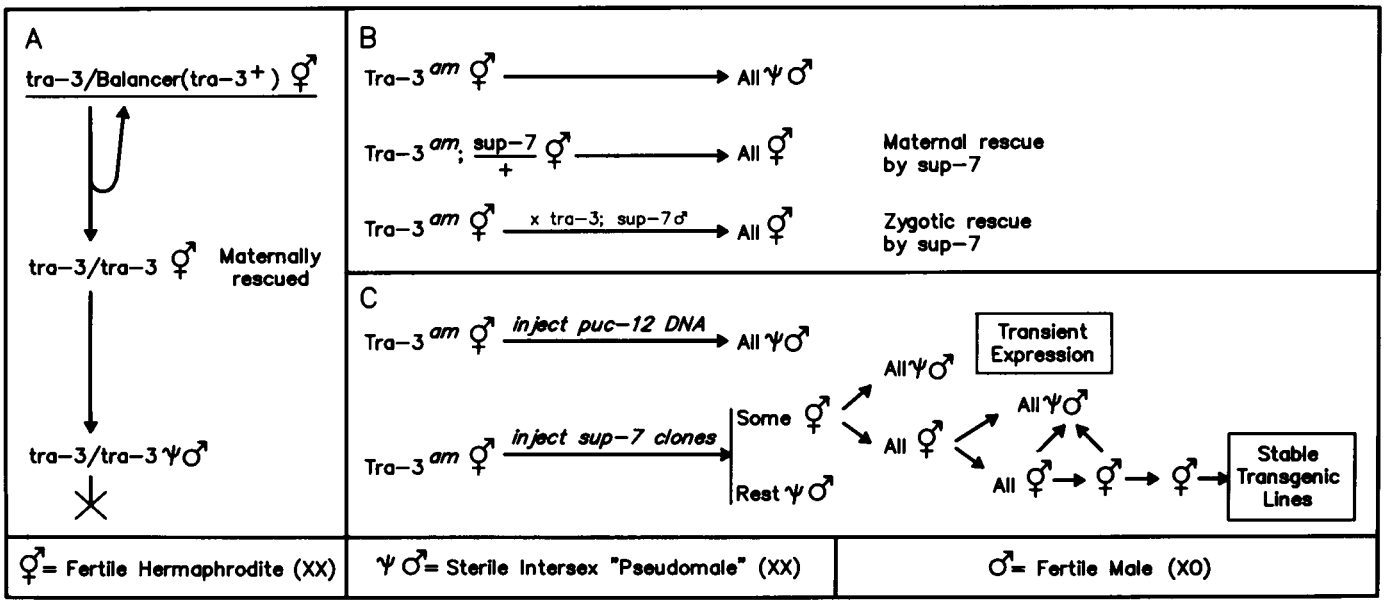

Fig. 1. (A) **Maintenance of balanced *tra-3* stocks for injection.** Maternal rescue effect allows production of homozygous *tra-3* animals that are fertile hermaphrodites (for injection). The balancer chromosome used in *nT1[unc(n754dom)let]*, a translocation between chromosomes *IV* and *V*. The translocation confers a dominant *unc* ('unco-ordinated') phenotype and carries a recessive lethal and was isolated and characterized by C.Ferguson (Ferguson and Horvitz, 1985 and personal communication). *Tra-3* homozygous hermaphrodites can be identified by their wild type movement. Occasionl (rare) breakdown of the balancer chromosome has occurred, so that stocks of the strain and putative transformants are routinely checked for *tra-3* genotype. Another balanced strain, *dyp-6(e14)sup-21(e1957)unc-58(e665)/+++;tra-3(e1107)* (kindly constructed by J.Hodgkin) was used in some of the earlier sets of injections. **(B) *tra-3* amber alleles.** *tra-3* amber alleles can be rescued by suppressor tRNA provided either maternally or zygotically (Hodgkin, 1985). For most of this work the amber allele of *tra-3* was *e1107*. **(C) Transient expression and stable transformation** with *sup-7* DNA are evidenced by the presence of hermaphrodite progeny after injection. Transient expression results in the appearance of hermaphrodites in the first (and occasionally in the second) generation after injection. Expression of suppressor activity in subsequent generations is indicative of heritability of the *sup-7* gene and in all such cases, stably transformed lines have resulted.

markers to identify *tra-3/tra-3* progeny for injection (Figure 1). The maternally rescued *tra-3/tra-3* hermaphrodites give rise to a large brood (100–300) consisting entirely of sterile pseudomales. Similar broods are seen after injection of buffer, or of vector sequences. Injection of the *sup-7*-carrying plasmids results in phenotypic rescue and fertility of some of the resulting progeny. Surprising variability in the number of rescued progeny (ranging from 1 to 150) was observed from different injections. It should be noted that expression of the tRNA gene in the injected gonad (without incorporation into the embryos) would be sufficient for rescue of large numbers of progeny (maternal rescue effect). Alternatively zygotic expression following incorporation of the DNA into the developing oocytes would also be sufficient for rescue.

Optimizing transient expression

To investigate the considerable variability in observed rescue frequencies, several equivalent sets of injections were performed in which the DNA was injected into different locations in the gonad (Figure 2). The distal arm of the gonad is a syncytium in which the ~1000 nuclei form a 'queue' (Klass *et al.*, 1976). Those most advanced in the queue (toward the turn region) are more mature and are more likely to be incorporated into the ~150 oocytes that are fertilized. Injection of the *sup-7* DNA near the turn in the distal arm results in some rescue of progeny, although usually only a single member of the progeny brood is rescued (Table I). Similar results followed injection of early oocytes. Injection into the center bulge of the distal arm (which until now has been standard for most studies) yielded little or no rescue. Surprisingly, the highest frequencies of rescue (generally between 5 and 80 rescued progeny/injection) were obtained after injection into the distal tip region of the gonad. In this region the nuclei are undergoing mitosis and the first stages of meiosis. Because they are too far back in the gonad, it is unlikely that these nuclei will ever be incorporated into oocytes. Rather, it seems likely that the nuclei in the tip region of the gonadal syncytium are capable of efficiently taking up and expressing the injected DNA, producing enough tRNA to maternally rescue many of the oocytes that are derived from that gonadal arm. Consistent with this hypothesis, none of the thousands of rescued progeny that have resulted from distal tip injections have themselves been transformed.

Stable transgenic lines

Although the transient expression should provide tools for analyzing genes active during oogenesis and early embryogenesis (e.g. maternal effect mutants), it would be most useful to have methods of producing transgenic lines carrying the DNA of interest. To obtain such transgenic lines, a large number of *tra-3*(am) hermaphrodites were injected with *sup-7*-containing DNA constructs, and the resulting descendents kept at 20°C over several generations, thereby selecting for heritability of the suppressor activity.

The large number of injections performed in these experiments was made possible by the development of a modified microinjection protocol. The initial protocol of Kimble *et al.* (1982) involved the anesthesia of animals and injection by pushing them against an immobile edge. I found that this procedure was too slow for large numbers of injections, and that there was not enough control on the position of injection for controlled injection experiments. By immobilizing non-anesthetized worms on dried agarose pads under a layer of halocarbon oil it is possible to inject large numbers, with considerable control of the injected region and no apparent harm to the injected animals. Between 60 and 150 animals can be injected in a single day.

Table I. Transient expression of sup-7 DNA

Location	Fertile injectants		Average no. of rescued progeny per positive brood	Fertility of rescued progeny (% fertile)
	Negative all ♀♂ F_1	Positive some ♂ F_1		
Early oocyte	7	7	1.1	50%
Turn region	12	6	1.2	86%
Distal 'bulge'	11	1	1	(100%)
Distal tip region	1	8	25.7	67%

These data result from two sets of controlled injections for each region (with equivalent results in each case). Both gonad arms of each animal were injected, and injected animals were considered 'fertile' only if >30 progeny were produced. The plasmid used for these experiments was pAst (*sup-7* in pUC-12). Comparable sets of injections of pUC-12 DNA into the distal tip and turn regions produced no rescued progeny.

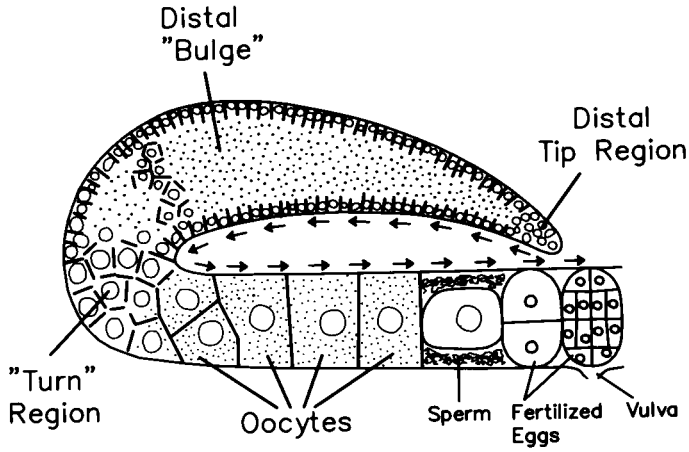

Fig. 2. The *C. elegans* adult hermaphrodite gonad (schematic diagram; an extensive microscopic analysis can be found in Klass *et al.*, 1976).

This procedure combined with the ability to select continually for suppressor tRNA expression allows stable transformation of *C. elegans*. To date eight independent transgenic lines have been characterized (see Table II). Each of these lines has continued to breed [i.e. suppress *tra-3*(am)] for 20–30 generations since establishment.

It has been difficult so far to determine precisely which factors are critical for stable transformation. The first stable lines came after several hundred unsuccessful broods (i.e. transient expression but no transgenic progeny). More recently, however, frequencies have been one transgenic line per 50–100 injected worms. One anecdotal correlation is that in the most successful sets of injections the worms were injected multiple times, aiming for nuclei around the turn in the gonad arm. That nuclei were being injected was confirmed by using the non-diffusible dye FITC-dextran. It is not known yet whether the injected nuclei survive or whether nuclear injection is necessary for stable transformation.

Although the first transformants were from injections of the *sup-7* gene cloned inside the ends of the nematode transposable element Tc*1*, it has subsequently proved unnecessary to have any Tc*1* sequences provided in *cis* or *trans*. Indeed both the frequencies of transformation and the structure of the DNA present in transformed lines so far analyzed indicate that the Tc*1* sequences have had no effect on the transformation process.

Maintenance of suppressor stocks

C. elegans is a diploid, and thus an integration event would be expected to yield a *sup/+;tra-3* animal. The suppressor should segregate as a dominant Mendelian locus in subsequent genera-

Table II. Transgenic suppressor lines

sup-7 vector[a]	Second plasmid[b]	Injection number[c]	Homozygote[d]	Level of suppression[e]	Chromosome[f]
pTC1	–	177-3	+(c.s.)	4	III
pTC1	–	232-4	–	1	?
pTC1	pM2B	273-1	–	3	I
pTC1	pM2B	274-4	+(c.s.)	4	II
pTC1	pHTC1	302–16[g]	+	2	I
pUC-12	pShZ1	326-12	–	3	?
pUC-12	pShZ1	332-2	+	2	IV
pUC-12	pShZ1	334-9	–	4	?

[a]*Sup-7* clones in two different vectors have been used: Puc-12 (Vierra and Messing, 1982) and pTC1, a vector allowing insertion within Tc*1* ends (this vector was derived from a Tc*1* element that had recently transposed into the *lin-12* locus; Greenwald, 1985).

[b]Where indicated a second plasmid was co-injected at a molar ratio of 1:1 or 3:1 (carrier to suppressor). Plasmid pShZ1 contains a *Drosophila* heat shock promoter fused to *E. coli* β-galactosidase (see Figure 5) and was constructed by S.Munro; plasmid pM2B is identical to pShZ1 except that the *Drosophila* heat shock promoter segment is replaced by a segment of the *C. elegans myo-C* gene (from I.Maruyama). pHTC1 contains a *Drosophila* heat shock promoter fused to the major open reading frame for the Tc*1* transposon.

[c]Each strain derived from a different injected parent; the strain is given the i.d. number of the parent.

[d]Homozygote: '+' indicates that a homogyzous line [*sup;tra-3*] could be derived. '–' indicates that no homozygous line was identified out of 50–100 cloned progeny. Because the homozygotes are often of low viability, it is possible that homozygous lines could yet be derived for more of the strains. For example the 274-4 homozygote strain was obtained only after the locus had been mapped. (c.s. indicates that the homozygous line is cold sensitive.)

[e]Level of suppression: arbitrary and subjective scale based on egg laying, tail morphology and cold sensitivity. High numbers indicate apparently stronger levels of suppression (all for heterozygote *sup/+;tra-3* animals).

[f]Linkage group determined as shown in Figure 3. the suppressors in strains 177-3, 274-4 and 302-16 are all closely linked to the marker used (within five map units of the 'cluster' of genes near the center of the indicated chromosome). The suppressors in strains 273-1 and 332-2 are somewhat more distantly linked to the relevant marker (~20 cM in each case). To confirm linkage *cis*-heterozygotes *dpy sup/++* have been constructed and segregation frequencies measured for all of the mapped loci except 274-4.

[g]This transformed line was obtained from a *tra-3* strain which had been crossed into a genetic background from the wild nematode strain TR403 [*C. elegans* variant isolated in the laboratory of P.Anderson; high Tc*1* copy number and active for Tc*1* transposition (personal communication)]. Transformation frequency with *sup-7* in the Tc*1* vector was no higher in this genetic background than in the standard N2 background which has no Tc*1* transposition activity (Eide and Anderson, 1985b). However, the mutator nature of the TR403 strain was evidenced in these experiments by the appearance of one other apparent transformed line in a TR403 genetic background that subsequently was shown to contain no exogenous DNA. This line is fertile as a result of a spontaneous mutation in the gene *mab-1* (J.Hodgkin, personal communication).

tions. Since the strains are all homozygous for *tra-3*, maintenance of the transgenic suppressor locus requires only the serial passage of viable animals. As a consequence of this method of passaging the strain, if multiple integration events had occurred in the orginal transformed worm there would be a strong selection for retention of only a single suppressor locus after many transfers. Stocks have routinely been frozen at an early passage but no attempt has been made to recover multiple suppressor loci that may be present in the initial transformants.

Genetic analysis of transgenic suppressor strains

Homozygous true-breeding suppressor lines have been derived from four of the eight transformants. The inability to obtain apparent true-breeding sublines from the other transformants could result from a number of different causes. (i) These suppressor loci could be more active than the original *sup-7* locus; hence two copies could cause enugh suppression to be lethal. Two lines (326-12 and 334-9) behave as very strong suppressors (with characteristic unhealthiness) even as heterozygotes; and it seems quite reasonable that the homozygotes would be inviable. (ii) The suppressor DNA could have inserted into an essential gene. (iii) The suppressor could be inherited as an extrachromosomal element. This is only a possibility for strains that have not yet been mapped: 232-4, 326-12 and 334-9. (iv) Integration could have occurred at a site where expression of the *sup-7* gene is variable, so that even in a homozygote line, some animals do not express the integrated DNA (see Levis *et al.*, 1985 for a discussion of similar phemomena in *Drosophila*).

The original *sup-7* mutation is deleterious to the animal. *Sup-7* homozygotes can grow, but only above 20°C; these animals are inviable at 15°C (Waterston, 1981). This cold sensitivity apparently results from a higher level of suppression at lower temperatures. Of the four lines that can be grown as suppressor homozygotes, two suppress the *tra-3* mutation much less strongly than *sup-7*: at 25°C the suppressed animals seldom lay eggs and heterozygotes occasionally show partially masculinized tail morphology; both phenotypes are indicative of incomplete suppression of *tra-3* ambers (Hodgkin, 1985). The other two homozygote suppressor strains exhibit suppression comparable to *sup-7*. Not surprisingly, it is only the latter two lines that are cold sensitive. Since the heterozygote lines will grow at 15°C, the stability of the transgenic suppressors in cold-sensitive lines can be assayed by growing a homozygous population at 25°C and then selecting for growth at 15°C. From $\sim 10^4$ animals of the 177-3 strain shifted down to 15°C, no viable progeny were recovered. This suggests that the transgenic suppressor is quite stable.

To determine whether the transgenic suppressors resulted from integration into chromosomal sequences, the suppression activities can be mapped genetically. For this purpose, a set of mapping strains were constructed using '*dumpy*' genes on each of the six chromosomes as markers (Figure 3). Five of the suppressors have so far been assigned to linkage groups using this protocol (Table II). The suppression activity in line 332-2 has been further localized (using a three-factor cross) to the left arm of chromosome IV (near *unc-17*).

Structure of the integrated DNA

The inserted DNA sequences have been analyzed on Southern blots. An *Xba*I digest probed with sequences around *sup-7* serves to establish the authenticity of each transformed line. An example of such a blot is shown in Figure 4. The *sup-7* mutation creates an *Xba*I site at the anticodon (Bolten *et al.*, 1984) and hence a 0.9 kb fragment diagnostic of *sup-7*-containing DNA. The wild type allele yields a 1.3 kb *Xba*I fragment (Bolten *et al.*, 1984)

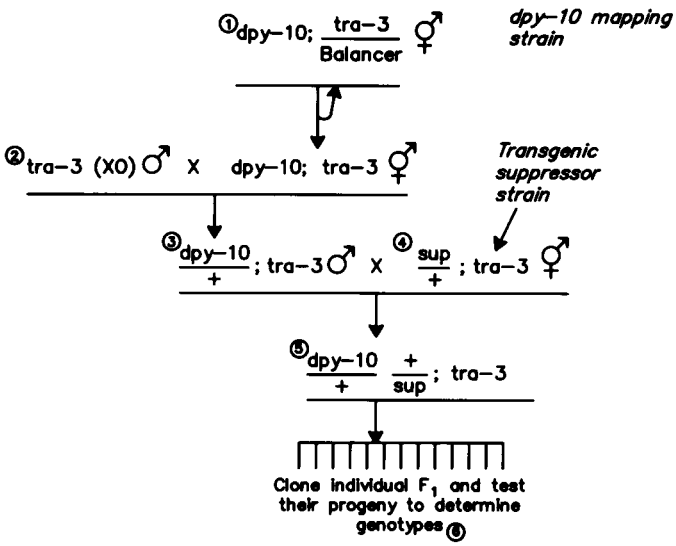

Fig. 3. Procedure for mapping new amber suppressor loci. Notes: (**1**) The mapping strains have the genotype *tra-3(e1107)/balancer;dpy*. These are analogous to the initial injection strain shown in Figure 1a in that they are self balanced and in that all well-co-ordinated hermaphrodites in each strain are homozygous for *tra-3* (the balancer is the nT1 dominant derivative described in the legend to Figure 1). These strains thus provide a source of *tra3;dpy* hermaphrodites. The dpy markers used for each chromosome are: *dpy7(e88) X, dpy-5(e61) I, dpy-10(e128) II, dpy-17(e164) III, dpy-4(e1166) IV* and *dpy-11(e224) V* (Brenner, 1974). In the latter two cases, the relevant mapping strains are actually *tra-3(e1107)dpy4(e1166)/balancer* and *tra-3(e1107);dpy-11/balancer* respectively. The mapping protocol is shown for *dpy10(e128) II*. (**2**) *Tra-3 (X0)* animals are fertile males (Hodgkin and Brenner, 1977). They are most conveniently obtained from the obligate male−female strain *tra-1(e1575dom/+;tra-3(e1107)* [XX] ♀ × *tra-3(e1107)* [XO] ♂ (Hodgkin, 1983a) [*tra-1(e1575dom)* is a dominant feminizing mutation which is epistatic to *tra-3*]. (**3**) A slight variation of this protocol is required for *X* chromosome mapping; in that case the males used in step 3 are actually *dpy-7(e88)* hemizygotes, which mate with reduced efficiency. (Note that dumpy or sup phenotypes generally decrease or eliminate male mating ability; Hodgkin, 1983b.) (**4**) Suppressor homozygotes are used here if possible. (**5**) A number of individual hermaphrodite progeny are cloned from the cross and animals of the genotype described are identified by examining progeny. (**6**) Both dumpy and non-dumpy progeny are cloned to test for the presence of the suppressor (the maternal suppression of *tra-3* forces an extra generation to assay the suppressor). If the suppressor is closely linked to the *dumpy* then most dumpy progeny should lack the suppressor and non-dumpy progeny should carry the suppressor. Both predictions have been confirmed in each case of linkage in order to rule out possible biases or gene interactions. Such biases can often distort expected ratios (particularly with *tra-3* and *sup-7*, which can both cause sterility). When possible, linked doubles [*dpy sup/+ +*] are constructed and segregation of the *cis*-homozygote examined. Sample data for strain 302-16 (suppressor on *I*): out of 42 fertile dumpy progeny of a *sup/dpy-5 I* parent, only one carried the suppressor. Of eight fertile dumpy progeny of *sup dpy5/+ +* I, all carried the suppressor.

which serves as a reference. Spontaneous *sup-7* mutations (or gene conversion mediated by the injected DNA) have never been observed; these events would be distinguished in that homozygotes would lack the 1.3-kb fragment diagnostic of the wild-type allele, and by their linkage to the *X* chromosome. The transformed strains contain between one and ten copies of the exogenous *sup-7* segment per haploid genome. Further analysis has been carried out using a variety of different restriction enzymes with both *sup-7* and plasmid vector sequences as probes (data not shown). One transformant that has been extensively analyzed (177−3) has three to four copies of the injected plasmid that appear to have been linearized randomly and joined together before integration. Two other transformants 274-4 and 302-16 appear to have tandem arrays of five to ten copies of the incom-

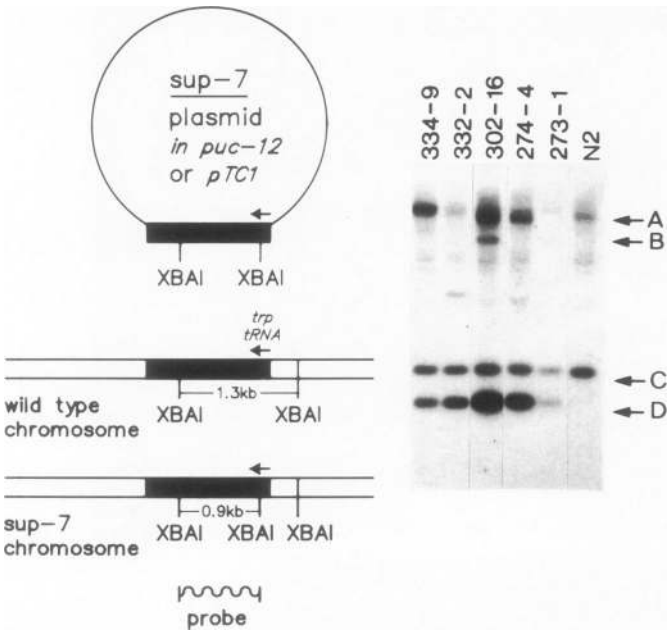

Fig. 4. Presence of exogenous DNA in transgenic suppressor lines. DNA from each line was cleaved with *Xba*I, electrophoresed in agarose (1.2%), transferred to nitrocellulose (Southern, 1975) and probed with a ^{32}P-labeled fragment from the *sup-7* locus. Note that one of the two *Xba*I sites in the *sup-7* segment is methylated (and hence fully resistant to cleavage) when grown in Dam$^+$ *E. coli*. This cleavage site is restored after replication in *C. elegans*. The positions of the initial *sup-7* plasmids (Dam$^+$) cleaved with *Xba*I are noted: **(A)** *sup-7* in pUC-12 (pAst); **(B)** *sup-7* in pTC1. Also noted are the 1.3 kb *Xba*I fragment derived from the wild type chromosome **(C; lane N2)** and the 0.9 kb fragment present in *sup-7* **(D**; lane not shown). [The high mol. wt. fragments appearing in lines 334-9, 302-16 and 274-4 (which do not co-migrate with the two plasmid markers) are presumably due either to partial digestion or to breaks or rearrangements that have occurred within the 0.9 kb *Xba*I fragment in individual inserted copies.]

Table III. Heat inducible β-galactosidase expression in transgenic lines

	β-galactosidase activity (enzyme units/g protein)	
	20°C	Heat shock 34°C 4 h
N2	<25	<25
e1107/balancer	<25	<25
332-2	<25	1490
334-9	<25	300

Table entries show soluble β-galactosidase activities in each strain. Worms were grown on bacterial strain P90C (deleted for the β-galactosidase gene; Miller *et al.*, 1977), harvested in M9 buffer (Brenner, 1974) plus 1 mM dithiothreitol, and homogenized by very sharp agitation for 1 min in the presence of an equal volume of acid-washed sand. After centrifugation at 12 000 *g* for 30 sec to remove sand and debris, protein concentrations were assayed by the method of Bradford (1976) and 55 μg of each sample added to 1 ml of assay mix: 1 mM DTT, 5 mM $MgCl_2$, 200 μg/ml chloramphenicol, 0.1 M Na-Phosphate, 0.1 M Tris-Cl (final pH 8.3), with 0.2 mg/ml ONP-galactoside. ONPG hydrolysis was measured spectrophotometrically, and enzyme assays were linear over a period of at least 24 h at 30°C. One unit is defined as the amount of enzyme required to hydrolyze 1 nmol of ONPG/min under the conditions described. A purified preparation of β-galactosidase from *E. coli* (from Sigma) was used as a standard. The specific activity of this preparation was 1.4×10^8 units/g under these conditions. Activities of control N2 and *tra-3(e1107)/balancer* animals and non-heat-shocked transformed lines 332-2 and 334-9 as well as of other transformed lines not carrying the HSP-β*GAL* segment (latter data not shown) were below the limit of detection of this assay (25 units/g). Similar results have been obtained using sonication to disrupt the animals, and using a fluorogenic substrate 4-methylumbelliferyl-β-D-galactosidase.

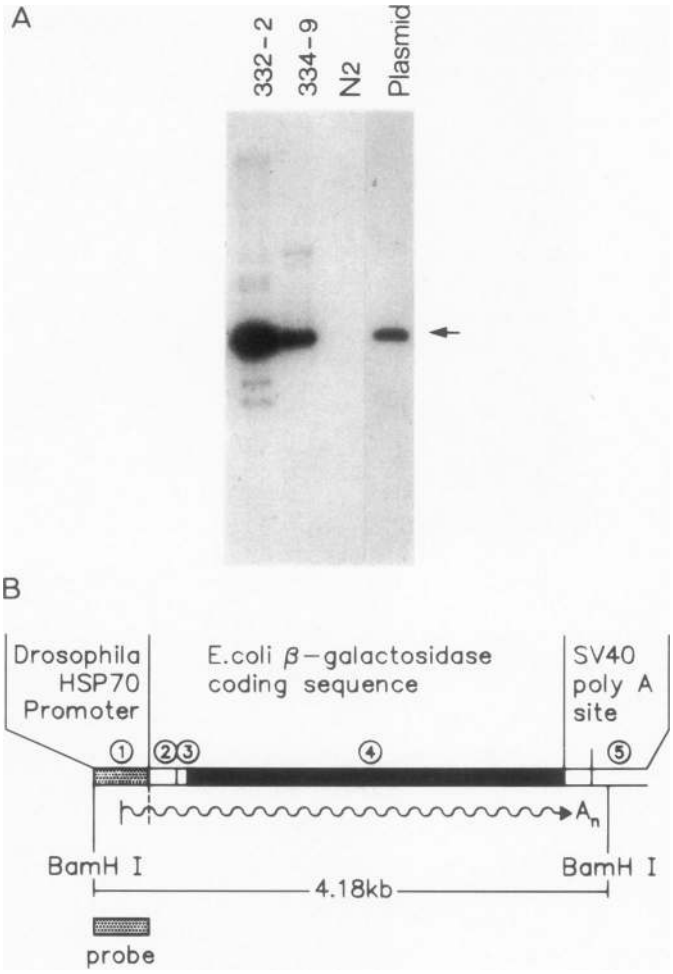

Fig. 5. Co-transformation using the *sup-7* selection. **(A)** The transgenic lines 332-2 and 334-9 are independent suppressor lines derived after co-injection of the *sup-7*-containing plasmid pAst with a second plasmid pShZ1. Strain 'N2' is the wild type parental strain. DNAs from the indicated strains were cleaved with *Bam*HI, electrophoresed, transferred to nitrocellulose, and hybridized with the probe shown in Figure 5B. Comparison with a known amount of pShZ1 DNA in the 'plasmid' marker lane suggests that 5−20 copies of pShZ1 are present in each line. Note that rearrangements or breaks have occurred within the 4.18 kb *Bam*HI fragment in some of the copies, but that in most copies the fragment appears to be intact. **(B)** Map of plasmid pShZ1 showing fragment used as probe. The probe sequences are derived from the *Drosophila* heat shock promoter element and do not hybridize to any *C. elegans* sequences. The pShZ1 plasmid was derived by inserting a fragment of pCH110 (Hall *et al.*, 1983) into a *Drosophila melanogaster* heat shock vector (from S.Munro). The final plasmid contains sequences from pBR322, the SV40 origin and polyadenylation (5) regions, a herpes tk polyadenylation site, *E. coli* β-galactosidase (4), and sequences from *E. coli trp* tRNA synthetase (3), *E. coli* guanine phosphoribosyl transferase (2), adenovirus (11 bp), and two different *D. melanogaster* heat shock loci (1). This plasmid has been shown to function in mammalian (COS) cells (S.Munro, personal communication) and in *Drosophila* tissue culture cells (G.Riddihough, personal communication). It is not known whether any of these segments contain tissue-specific regulatory elements that might cause the localized expression in *C. elegans*.

ing plasmid which could have been created by homologous recombination between several plasmsid molecules (or by a rolling circle replication mechanism). There is no correlation between the strengths of suppression and the copy number in the different suppressor strains. Thus expression appears to be at least partly dependent on the local and/or global chromosomal environments of the re-introduced *sup-7* genes.

A number of novel restriction fragments not present in the

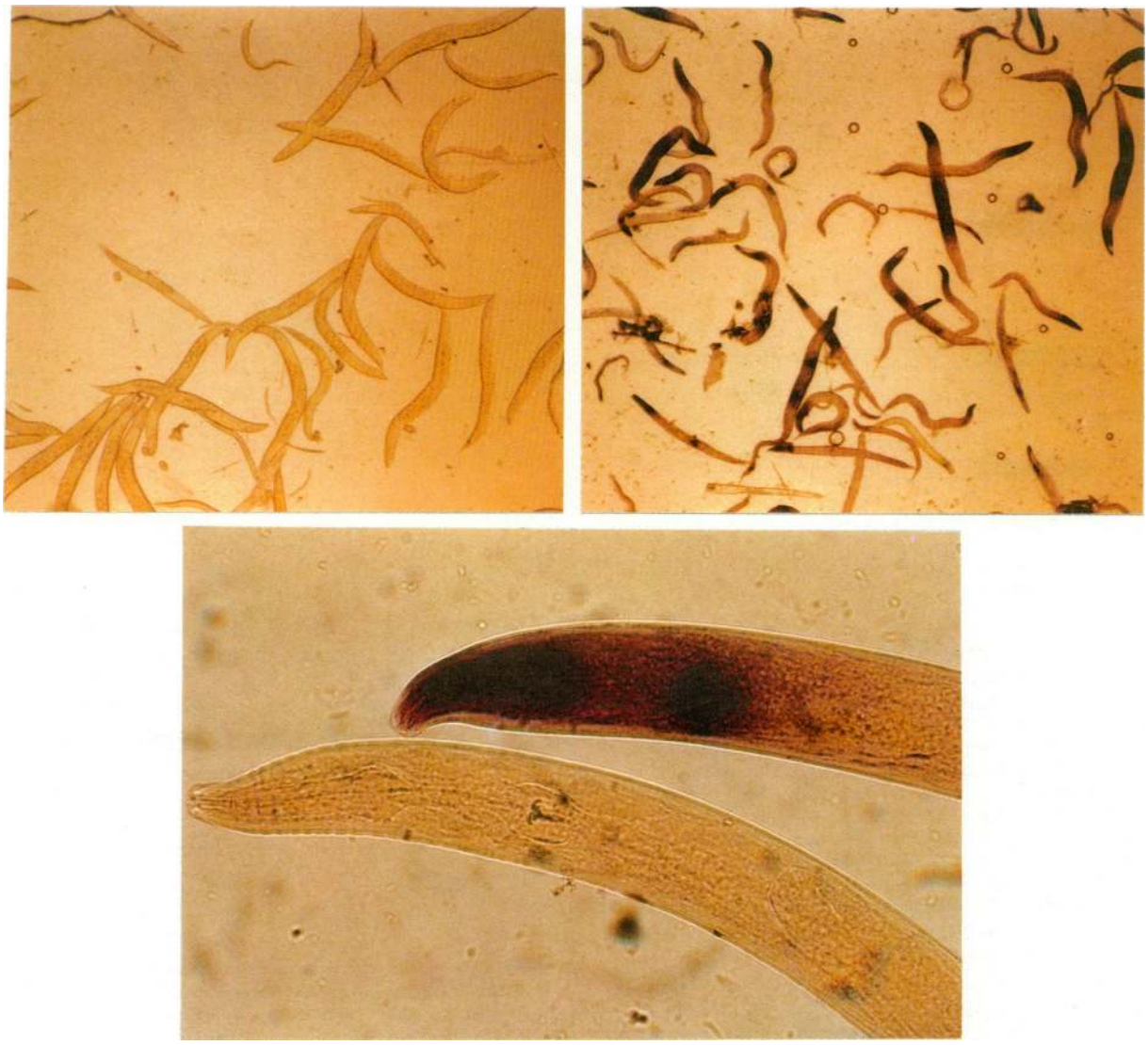

Fig. 6. Histochemical demonstration of β-galactosidase activity in transgenic lines. Whole worms were fixed and stained as follows. Worms grown on *E. coli* strain P90C were washed in water and placed in a 50 μl drop of water on a glass multi-test slide. Slides were dried for 5–10 min in a desiccator jar under vacuum (2 mb), dipped in acetone, and re-dried at room temperature in open air. The worms were rehydrated by adding 100 μl of staining solution [0.2 M sodium phosphate pH 7.5, 1.0 mM dithiothreitol was saturated at 65°C with 6-bromo-2-naphthyl D-galactoside (available from Sigma) and SDS was added to a concentration of 0.004%]. After 1 h at room temperature 20 μl of a fresh 0.125% solution of fast blue B diazonium salt was added (higher concentrations were required for some commercial preparations of the diazonium salt). The substrate is cleaved by β-galactosidase to yield a local insoluble precipitate of 6-bromo-2-naphthol, which reacts with the diazotized fast blue to form a turquoise dye (Rutenberg *et al.*, 1957). **Left panel:** 332-2 animals without heat shock. Similar patterns (i.e. no staining) are obtained in 334-9 without heat shock, in the parental strain with or without heat shock, and in independent sup-7 transformed lines not carrying a β-galactosidase fusion. With long exposures to substrate, all lines stain very weakly in regions around sperm (presumably endogenous enzyme activity). **Right panel**: 332-2 animals stained after heat shock (3 h at 34°C). Note pharyngeal staining of all stages. The staining inside of adults is apparently of early embryos inside the adult gonad. A similar staining pattern is seen with strain 334-9. **Lower panel**: stained and unstained animals (both are pseudomales from an outcross of 332-2) showing pharyngeal staining at high resolution.

original plasmid have been observed: some of these have been identified as novel joints between plasmid sequences and some are presumably junctions with chromosomal DNA. The short tRNA gene (70 bp) is an unlikely target for rearrangements. Unfortunately this is not so with most genes of interest. It is encouraging, despite the breaks and novel joints that are observed, that in most cases large segments (>7 kb) of the original plasmids have been delivered apparently intact.

Co-transformation with mixed DNAs

Some of the transformed strains came from experiments in which a *sup-7*-containing plasmid had been co-injected with a second unselected plasmid. In five such strains so far analyzed, sequences from the second plasmid are also present in the transformed line (Figure 5 and data not shown). Similar co-transformation phenomena in mammalian systems (Wigler *et al.*, 1979) have proven useful for introducing various genes of interest.

A Drosophila heat shock promoter element functions in C. elegans

The unselected plasmid that was co-injected with *sup-7* in constructing strains 332-2 and 334-9 was a fusion construct containing a *Drosophila* heat shock promoter element (HSP) upstream of coding sequences for *E. coli* β-galactosidase (βGAL). The presence of the 4.18 kb *Bam*HI fragment in Figure 5 suggested that the HSP–βGAL fusion segment was intact. The ability of this segment to function in response to heat shock in *C. elegans* was tested using both soluble enzyme assays (Table III) and histochemical staining (Figure 6). The temperature and time

dependence of the *C. elegans* heat shock response have been described by Snutch and Baillie (1983); for this work a heat shock of 3 h at 34°C was chosen.

The soluble enzyme assays indicated a dramatic stimulation in β-galactosidase activity after heat shock in both 332-2 and 334-9. No such stimulation was observed in the parental lines or in *sup-7* transformed lines not carrying the HSP−βGAL fusion.

Whole worms were fixed and stained for β-galactosidase activity as described in Figure 6. No staining was observed in the parental line (with or without heat shock) or in 332-2 or 334-9 growing at 20°C. Staining of heat-shocked animals of either strain 332-2 or 334-9 was very intense, and showed an unexpected localization, with most of the staining observed in embryos and in the pharynx. The staining pattern was similar in the two independent lines and in several outcrossed lines derived from each. This localization is unlikely to represent simple permeability differences, since cut and/or squashed animals give similar patterns of staining. Additionally, injection of pure enzyme into the gonad, gut, or pharynx of wild type animals followed by fixation and staining resulted in specific staining in each case of the injected tissue. The restricted localization of β-galactosidase activity in induced animals could result either from properties of the *C. elegans* heat shock response, from differential stabilities of β-galactosidase protein and message or from possible fortuitous expression signals in the HSP−βGAL fusion plasmid that was used.

The histochemical staining assay has been used to study the segregation of the heat-inducible β-galactosidase activity. To do so, heterozygous (*sup/+;tra-3*) suppressor lines were selfed and individual progeny picked. Presence of the suppressor could be determined by *tra-3* suppression and the presence of the heat-inducible β-galactosidase assayed by staining the whole progeny brood. In this way it was shown that both suppressor loci are closely linked (<3 map units) to loci encoding heat-inducible β-galactosidase. Strain 334-9 contains no additional HSP−βGAL loci, but strain 332-2 contained at least one extra HSP−βGal locus unlinked to the suppressor. The latter strain was repeatedly outcrossed with *tra-3(e1107)* males, yielding a strain with a single locus on chromosome IV (4 map units from *unc-17*) which encodes both the suppressor function and heat-inducible β-galactosidase activity. Thus in both cases at least one copy of the unselected plasmid has apparently integrated at the same locus as the suppressor tRNA.

For strain 332-2, rough estimates of the induced β-galactosidase as a fraction of total protein were obtained from soluble enzyme assays and from histochemistry (by comparison with injections of pure enzyme). These estimates are of the order of 0.001 and 0.01% respectively (the difference may reflect losses in solubilizing the enzyme).

Discussion

The use of a suppressor tRNA as a marker allows exogenous DNA to be introduced into the *C. elegans* germ line at a low copy number. Most if not all of the resulting strains have the exogenous DNA integrated into one of the chromosomes. This technique produces some disruption of the introduced DNA, but large segments of the transforming plasmids (at least 7 kb) apparently can be maintained intact. The expression per copy number of the re-introduced *sup-7* gene appears to vary over a range of 10- to 20-fold. Similar variations upon re-introduction of genes has been observed in a number of other organisms.

The total working time required for production of each transformed line is ~2 days, including the time spent for injections, maintaining stocks and reagents, and screening progeny. Only ~30% of this time is involved with injections.

Surprisingly, none of the transgenic lines carried the type of tandem arrays described by Stinchcomb *et al.* (1985). It seems likely that such arrays would provide lethal doses of suppressor tRNA and are thus selected against. Alternatively, it is conceivable that such long tandem arrays are formed with *sup-7*, but that expression from this structure is insufficient to rescue *tra-3*. In either case it is hoped that the chromosomal environment provided by the *sup-7* selection will allow the proper expression and regulation of introduced genes. The proper regulation of an integrated heat shock promoter element is very encouraging in this respect.

In addition to the production of transgenic lines, injection of *sup-7*-containing plasmids was shown to lead to transient expression in the injected gonad. If the injection is targeted to the 'distal tip' region of the gonad, then the transient expression becomes very efficient and reproducible, with almost every animal showing expression of the injected DNA. The ability to readily assay transient expression of genes in the maternal gonad may in some cases avoid the considerable tedium of producing transgenic lines. For example, this procedure may be useful in identifying 'maternal effect' genes whose expression in the maternal gonad is required for proper development of progeny. A variety of such genes have recently been identified (e.g. Kemphues *et al.*, 1986).

Recently, the transformation protocol described in this paper has been used to introduce several different DNA constructs carrying the *sup-7* marker (M.Shen, personal communication: J.Cane, S.Bektesh, A.F. and T.Blumenthal, unpublished observations), and a total of 21 independent suppressor lines have now been derived.

Materials and methods

DNA

Bacterial plasmid DNAs were purified by alkaline lysis (Birnboim, 1983) followed by equilibrium centrifugation in cesium chloride−ethidium bromide. For injection, DNA was precipitated in 0.1 M KAc pH 7.4 with two volumes of ethanol, washed with ethanol, resuspended at a concentration of 5 mg/ml in 10 mM KPO_4 pH 7.5 and stored at −20°C. Injection solutions were 2 mg/ml DNA, 0.125% Lucifer Yellow CH (Stewart, 1978), 2% polyethylene glycol 6000, 20 mM potassium phosphate, 3 mM potassium citrate (adjusted to pH 7.5 with KOH). Injection solutions were centrifuged for 10 min at 12 000 *g* in an Eppendorf microcentrifuge to remove particulate matter just prior to filling needles.

The *sup-7*-carrying plasmid 'pAst' was constructed by inserting the 1.3 kb *Eco*RI−*Sal*I fragment from pRW91 (Bolten *et al.*, 1984) into pUC-12 (Vierra and Messing, 1982).

Worms

Handling of worm stocks was as described by Brenner (1974). Preparation of nematode DNA was as described (Emmons and Yesner, 1984) except that worms were grown on bacterial strain P90C (Miller *et al.*, 1977) which is deleted for the *lac* operon. This eliminates any hybridization to *lac* sequences contained (Vierra and Messing, 1982) in Puc- or M13mp-derived probes.

The microinjection protocol, which requires relatively little manual dexterity, relies on the ability to immobilize live worms on a dried pad of agarose under an oil layer. I have used voltalef 3X or 3S oil for this purpose. (Occasional batches of 3S oil do not allow adhesion; these batches were discarded.) The worms lose water to the dry agarose pad and thus become somewhat desiccated. With agarose pads that are too thick, the worms rapidly dry out and die. Pads that are too thin fail to sufficiently immobilize the worm for injection. The optimal pad thickness depends on the size of the worms to be injected. Pads are formed on 32-mm diameter glass coverslips (#1½): a drop of molten 2% agaorse is placed on a microscope slide and the coverslip is laid on top with gentle pressure. The slide is removed, with the agarose pad adhering to the coverslip. The pads are then dried (100°C for 15 min) and become permanently affixed to the coverslips.

It has been found experimentally that both the ease of injection and viability of injected worms can be increased by: (i) keeping the worms cool during the time they are immobilized (15−17°C) and (ii) transferring the worms to a par-

tially desiccated agar plate without bacteria before transfer onto the oil-covered immobillizing pad. The latter transfer is performed with a sharpened platinum pick and care is taken not to stress the worm once it has made contact with the agarose surface of the coverslip (otherwise the cuticle can be stretched, which can decrease viability). The picked worm is allowed to touch the agar surface at a single point and the pick is gently withdrawn, allowing the worm to find its own orientation on the agar surface. Once the worms have been mounted, they remain viable on the agar surface for 15–30 min. This is sufficient time to allow injection of 5–20 worms/pad. It is convenient to mount the worms along a single line to keep track of which worms have been injected.

Clarke GC120F15 needles were drawn using a two-stroke electrode puller. Tips were initially < 1 μm, but were often broken during the course of injection and could be readily used with bore sizes as large as 3 μm. The needles were filled by placing 0.1–1.0 μl of solution inside the needle just before the tip using a capillary tube that had been drawn to a long, narrow bore by hand. Unusable broken or clogged needles were discarded immediately, with each needle generally being used for 3–40 worms. To facilitate changing of needles, a simple air pressure system is used: a small jack is glued to the base of a ringstand and used to exert a constant pressure on a plastic syringe (50 ml) which is held upright on the stand with a clamp. A disposable three-way plastic valve is used to allow rapid release of pressure. The needles were held using a Clarke electrode holder (#EH2R) and connected to the pressure system using silicon tubing. This system allowed pressures of up to 200 p.s.i. to be maintained if necessary. Much lower pressures were generally sufficient, and pressure could generally be adjusted so that liquid would only flow out of the needle when inside a worm (i.e flow into the oil is prevented by surface tension).

The microscope used for injection is a Zeiss IM which is equipped for simultaneous differential interference contrast and fluorescence with a Plan-40 or Plan-Neofluor-40 lens. The injections are monitored by following the fluorescent Lucifer Yellow using filters for fluorescein or formaldehyde-induced fluorescence. A glide stage with centered rotation was critical for rapid positioning of worms for injection. A C.Zeiss/Jena micromanipulator was used for injections, with injections close to the horizontal (< 5°C). In general, greater viability was obtained by pressing the needle firmly against the cuticle before injection and then hitting the micromanipulator or the microscope to drive the needle into the desired location. In this way 10–20 injections could be made into each worm without significantly decreasing viability. Injection volumes were not carefully measured, but probably varied between 1 and 10 pl/injection.

The worms dry out to varying extents during the course of injection. By using an isotonic recovery buffer [0.1% salmon sperm DNA (for viscosity), 4% glucose, 2.4 mM KCl, 66 mM NaCl, 3 mM $MgCl_2$, 3 mM $CaCl_2$, 3 mM Hepes pH 7.2] it is possible to recover even extremely desiccated worms. When all worms on a pad are injected, two drops of sterile recovery buffer are added, the animals immediately become hydrated and detach from the agarose substrate. The coverslip is transferred from the microscope stage to a humidified chamber (a Petri plate with agar is convenient for this). After several hours, M9 buffer (Brenner, 1974) is added to reduce osmotic strength. After the injection process, the worms are quite sensitive to osmotic shock; therefore the M9 buffer is added dropwise (20 drops) over the course of about 1 h. The worms are then transferred to individual Petri plates with nutrient agar and bacteria for food and maintained at 20°C.

For the *tra-3* selection it is important to follow the progeny of single worms since occasional breakdown of balancer chromosome is observed. Breakdown of the balancer is evidenced by single broods with 100% hermaphrodites, but would not be as evident if progeny of injected worms were kept as a mass population. Four to five days after injection, hermaphrodite progeny are picked from each plate and cloned.

It was conceivable that a background level of *tra-3* suppression due to spontaneous mutations would be observed. Hodgkin (1985, 1986) has used chemical mutagenesis to carry out extensive reversion analyses of both amber and non-amber alleles of *tra-3*: in addition to their supression by amber suppressors, *tra-3* amber alleles can be suppressed by intragenic reversion at the *tra-3* locus, or by compensatory mutations in several other genes affecting the sex determination pathway. Spontaneous mutations are rare in the standard 'Bristol' (N2) strain from which the standard injection strains were derived (Greenwald and Horvitz, 1980; Eide and Anderson, 1985b) and no such mutants have been observed in these studies. Nevertheless, it remains standard procedure to confirm each newly derived 'transformant' by demonstrating the presence of exogenous *sup-7* DNA sequences on Southern blots.

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