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Efficient gene transfer in *C.elegans*: extrachromosomal maintenance and integration of transforming sequences

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We describe a dominant behavioral marker, *rol-6(su1006)*, and an efficient microinjection procedure which facilitate the recovery of *Caenorhabditis elegans* transformants. We use these tools to study the mechanism of *C.elegans* DNA transformation. By injecting mixtures of genetically marked DNA molecules, we show that large extrachromosomal arrays assemble directly from the injected molecules and that homologous recombination drives array assembly. Appropriately placed double-strand breaks stimulated homologous recombination during array formation. Our data indicate that the size of the assembled transgenic structures determines whether or not they will be maintained extrachromosomally or lost. We show that low copy number extrachromosomal transformation can be achieved by adjusting the relative concentration of DNA molecules in the injection mixture. Integration of the injected DNA, though relatively rare, was reproducibly achieved when single-stranded oligonucleotide was co-injected with the double-stranded DNA.

Key words: *C.elegans*/chromosome partitioning/DNA transformation/homologous recombination

Introduction

The nematode *Caenorhabditis elegans* has proven to be a rich genetic system with which to study a broad spectrum of biological problems (see Wood *et al.*, 1988, for review). Consequently, a growing number of *C.elegans* genes are being characterized molecularly. An essential technique for the characterization of cloned sequences is the ability to reintroduce them and to assess their function *in vivo*. Heritable extrachromosomal DNA transformation in *C.elegans* was first described by Stinchcomb *et al.* (1985a) after microinjection of DNA into the gonad cytoplasm. Although some cloned genes were expressed and regulated properly from extrachromosomal structures (for example, Way and Chalfie, 1988; Kim and Horvitz, 1990; Spence *et al.*, 1990), other cloned genes appeared to function properly only when integrated at low copy number (Fire, 1986; Fire and Waterston, 1989). Integrative transformation in *C.elegans* was achieved reproducibly only after microinjection of DNA directly into maturing oocyte nuclei (Fire, 1986). Despite the success of *C.elegans* DNA transformation, little was known about how extrachromosomal arrays are generated or what specific factors govern array formation as opposed to the chromosomal integration of transforming sequences.

Here we describe a simple and efficient microinjection procedure for *C.elegans* DNA transformation, including the use of a convenient dominant-acting genetic marker. We used these tools to study the formation, composition and heritability of transgenic extrachromosomal elements. We note similarities between *C.elegans* and other systems including mammalian cells, *Drosophila, Xenopus*, sea urchin and fish with regard to the metabolism of transforming sequences (Scangos and Ruddle, 1981; Steller and Pirrotta, 1985; Marini *et al.*, 1988; McMahon *et al.*, 1985; Stuart *et al.*, 1990). These similarities include a tendency of injected molecules to assemble into large arrays by recombination. Our data suggest that the extrachromosomal compartmentalization of highly reactive newly injected DNA may be a key event which stimulates the production of heritable transgenic structures while reducing the frequency of integration. We demonstrate that by changing the composition of the injection mixture both extrachromosomal and integrative transformation of *C.elegans* can be achieved with a single type of injection.

Results

Transformation methodology

We used a cloned mutant collagen gene, *rol-6(su1006)*, (Kramer *et al.*, 1990) as a dominant genetic marker for DNA transformation. Animals carrying the semi-dominant right roller allele, *rol-6(su1006)*, exhibit a helically twisted cuticle and body. Muscle contractions which would normally propel the animal forward or backward in a sweeping sinusoidal motion instead cause the animal to roll over about its longitudinal axis and to move in circles. We found that microinjection of the plasmid pRF4, which contains *rol-6(su1006)*, conferred this characteristic rolling behavior upon the progeny of injected wild-type animals.

We found that transformation frequency was optimal when a single high volume injection of DNA solution was made into the central core of cytoplasm in each arm of the hermaphrodite gonad (Figure 1). The ovary of the adult hermaphrodite consists of a core to cytoplasm surrounded by a sheath of germ nuclei arrested in meiosis I. As oocytes mature in the proximal region of the ovary, membranes encapsulate individual germ nuclei along with portions of the core cytoplasm. Because of the large size of the gonad cytoplasm, insertion of the needle was easily accomplished, and the distortion of the gonad due to the influx of DNA solution provided a readily visible indicator of successful

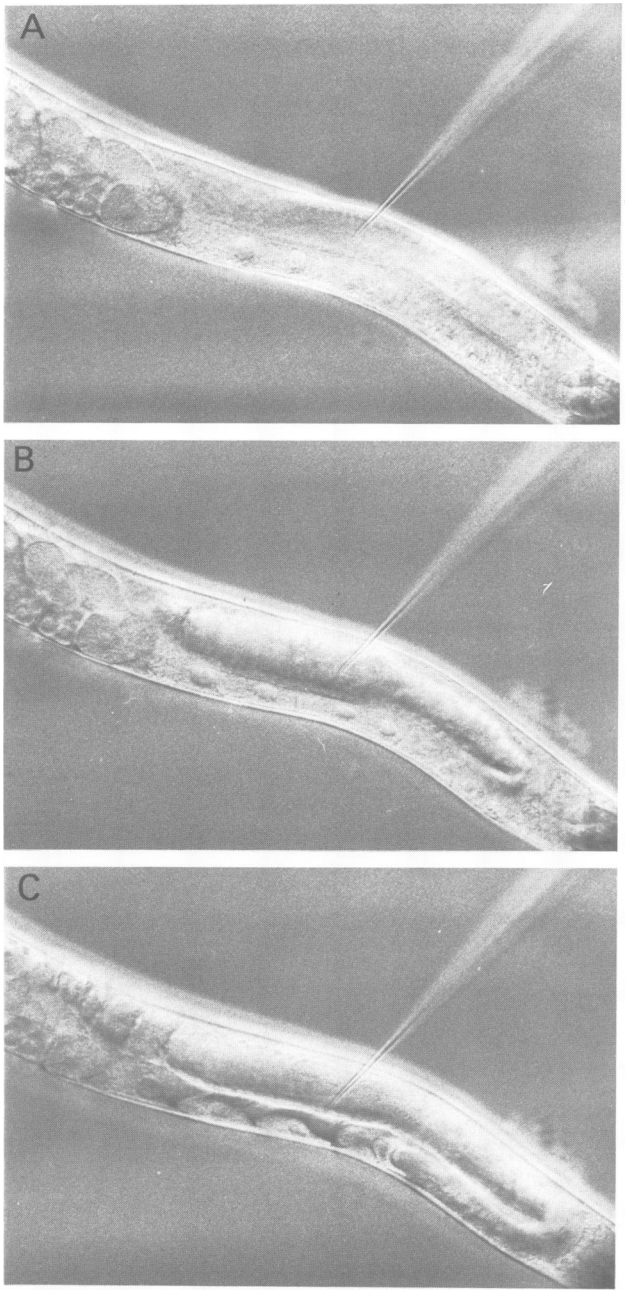

Fig. 1. Nomarski optical section through a wild-type adult hermaphrodite during injection. (**A**) The needle tip is inserted in the cytoplasm of the distal gonad. The small meiotic germline nuclei which surround the syncytium are visible as rows on each side of the central grainy core of cytoplasm. Several large oocyte nuclei are visible in the proximal region of the gonad. (**B**) When pressure was applied to the needle the gonad expanded in volume as a wave front of injected material infiltrated the cytoplasm in both directions from the site of injection. (**C**) Within 1–2 s, the wave front traveled around the bend of the gonad and past the row of oocytes. The membrane boundaries which divide the maturing oocytes become visible as they obstruct the flow of injection solution.

injection. Approximately 20–50 transformed roller progeny could be obtained from each injected animal.

Transformed animals were allowed to self-fertilize and the segregation pattern of the transformed phenotype was determined. In this study, ~6000 transformants were obtained and these fell into two major groups: first-generation

Table I. Effect of pRF4 concentration on transformation frequency

pRF4 (μg/ml)	Number injected	F1 rollers			% heritable expression lines/total F1
		Total	Per inj. animal[a]	Maximum	
12.5	11	78	7.1 ± 5.1	18	1/78 (1.3%)
25	27	313	11.6 ± 6.6	27	0/313 (0%)
50	15	398	26.5 ± 10.8	56	10/398 (2.5%)
100	20	498	24.9 ± 9.5	45	47/498 (9.4%)
200	14	400	28.6 ± 8.4	50	42/400 (10.5%)

[a]Mean ± SD.

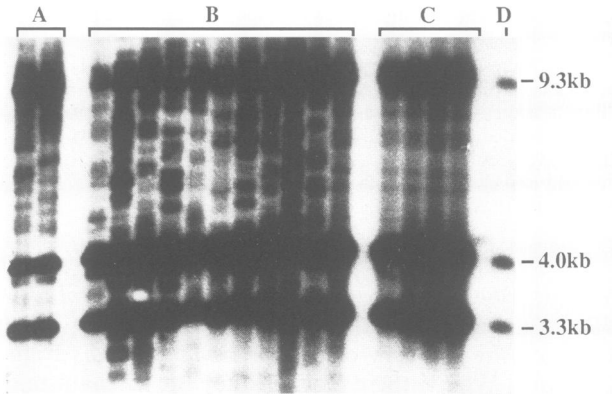

Fig. 2. Hybridization pattern of sequences within transformed populations founded by (**A**) F1 rollers from independent injected animals; (**B**) independent F1 rollers from one injected animal; and (**C**) independent F2 progeny derived from the same F1 roller. (The F1 roller parent is shown in the adjacent lane in B.) The input DNA, shown in the lane marked **D**, contained three fragments which hybridized to the probe (sizes are indicated at right). DNA prepared from transformed populations was digested with *Eco*RI, electrophoresed through a 0.7% agarose gel, transferred to Nytran, and probed with radiolabeled pRF4 DNA.

transformants that failed to pass on the transformed phenotype to their progeny (transients), and heritably transformed individuals which exhibited a non-Mendelian pattern of inheritance (extrachromosomal transformants). Integrated transformed lines were very rare except under certain injection conditions which will be described later.

DNA concentration dependence of transient expression and heritable extrachromosomal transformation

Injections were performed at pRF4 plasmid concentrations ranging from 12.5 to 200 μg/ml and the number of roller transformants obtained per injection at each concentration was compared (Table I). As the DNA concentration was increased from 12.5 to 50 μg/ml, the average number of first generation transformants increased nearly 4-fold, from seven to 27 roller progeny per injected animal (F1 expression, Table I). Above 50 μg/ml, the frequency of transformation appeared to become saturated. We found that the maximum number of transformants obtained from a single injection at each concentration (Table I, maximum) exhibited a similar concentration dependence to that observed for the average. This correspondence between the average and the maximum number of F1 transformants indicates that the groups of injections compared at each concentration were of a similar range of quality. The large standard deviation

Table II. Effect of *sup-7(st-5)* on transformation efficiency

DNA	Conc. (μg/ml)	No. injected	F1 expression[a]	Germline expression[b]
pASTRB(*sup-7*)	100	50	n.d.	0
pASTRB(*sup-7*)	10	60	n.d.	0
pASTRB(*sup-7*)	1	23	n.d.	0
pRF4+pASTRB	*rol-6/sup-7*		*F1 rollers*	
pRF4+pASTRB	100/100	136	52	0
pRF4+pASTRB	50/50	48	86	0
pRF4+pASTRB	100/10	36	221	0
pRF4+pASTRB	100/1	15	131	3r, 3rs
pRF4+pASTRB	100/0	10	163	13r
pRF4(*rol-6*)+ pPTCTS(*sup-7*)	200/2	40	250	6r, 16rs

[a]n.d. means not determined. See Materials and methods for a description of the selection for *sup-7*.
[b]'r' indicates that the transformants express the *rol-6* marker, 'rs' indicates that the transformants express both the *rol-6* marker and the *sup-7* marker.

(per injected animal, Table I) appears to reflect differences in the response of individual animals to injection.

The proportion of F1 rollers that produced heritably transformed lines was determined (% heritable transformation, Table I). The F1 rollers obtained at DNA concentrations <50 μg/ml were far less likely to transmit the roller phenotype to their progeny than were the F1 rollers obtained at higher DNA concentrations. The percent heritable transformation increased ~4-fold as the DNA concentration was doubled from 50 to 100 μg/ml. These data suggest that the assembly of heritable extrachromosomal elements depends on interactions between injected molecules. All of the heritably transformed lines obtained in this experiment exhibited non-Mendelian segregation patterns consistent with extrachromosomal transformation. At optimal DNA concentrations, an average of two to three transformed lines were established from each injected animal. This represents a considerable improvement over previous methods (Stinchcomb *et al.*, 1985a; Fire, 1986; Fire and Waterston, 1989).

Structure of extrachromosomal arrays

The injection approach which we describe yields several heritably transformed progeny from each injected animal. These transformants could result from independent transformation events or they could result from the amplification of a single transformation event within the injected gonad. To distinguish between these possibilities we looked at the structure of the transforming sequences after restriction digestion and Southern analysis (Figure 2). Transformed progeny obtained from separate injected animals always contained extrachromosomal arrays with a complex and heritable structure, including many unique rearrangements of the injected DNA (Stinchcomb *et al.*, 1985a; Figure 2A and data not shown). Similarly, we found that transformed lines obtained from a single injected animal always contained arrays as different from each other structurally, (Figure 2B, and data not shown) as did transformed lines obtained from separate injected animals (Figure 2A). When a single transformed line was split into several clones, these clones always exhibited transforming sequences with essentially identical structure (Figure 2C and data not shown). These data support the model that independent transforming elements assemble from the injected DNA and are inherited by separate progeny of the injected animal.

Composition of arrays assembled from a mixture of genetically marked molecules

When distinct DNA molecules are co-injected in *C.elegans*, they often interact with each other, assembling into a common structure during DNA transformation (Fire, 1986; Fire and Waterston, 1989; Kim and Horvitz, 1990). In order to investigate the frequency of interactions between injected molecules during array formation, we injected the *rol-6* plasmid, pRF4, together with progressively lower concentrations of a second genetically marked plasmid molecule. By measuring the frequency with which the more dilute molecule failed to be incorporated into the resulting arrays, we could derive an estimate of the minimum number of injected molecules required to assemble a single heritable structure.

The suppressor tRNA gene *sup-7(st5)* was chosen as a genetic marker for these experiments. In addition to suppressing amber alleles of a variety of genes, *sup-7(st5)* causes slowed growth and/or sterility (Waterston, 1980). Thus, arrays that incorporate more than a few copies of the suppressor gene would be expected to confer sterility or inviability on the progeny that receive them. The recipient animals in this experiment carried an amber suppressible lesion in the major DNase gene of *C.elegans*, *nuc-1(e1392)*. (*nuc-1* animals are sensitive to the drug methotrexate; we used improved viability in the presence of the drug to select or screen for low copy *sup-7* expression.)

As previously observed (Fire, 1986), viable extrachromosomal transformants were not obtained after injection of the *sup-7* plasmids alone (Table II), nor were any low copy integrated transformed lines obtained from these injections. High concentrations of the *sup-7* plasmid mixed with pRF4 in the injection solution appeared to 'poison' heritable *rol-6* transformation; F1 rollers were obtained at a lower frequency and these rollers always failed to produce healthy rolling progeny. Viable heritably transformed rolling progeny were obtained only when the *sup-7* plasmid was reduced to 1/100th of the total injected plasmid DNA (Table II). Despite the fact that the *sup-7* plasmid was 100-fold less prevalent than the *rol-6* plasmid in the injection solution, 19 of 28 transformed lines established as rollers also expressed the suppressor gene (Table II, rs). In each of the 19 cotransformed lines, the suppressor and the roller phenotypes appeared to segregate together, suggesting genetic linkage to a single extrachromosomal array.

The structure of sequences within arrays established by injecting a 100-fold dilution of *sup-7* plasmid relative to *rol-6* plasmid is shown in Figure 3. Southern blot hybridization analysis revealed that two transformed lines established and maintained under selection for *sup-7* contained a prominent band corresponding in size to the 1.3 kb suppressor fragment present in the injected DNA but absent from the worm genome (Figure 3A, lanes 3 and 4). Three of six transformed lines established as rollers ('non-selected', lanes 6, 9 and 10, in Figure 3) also contained the transgenic *sup-7* fragment. Among a total of 11 rolling transformed lines analyzed by Southern analysis (Figure 3 and data not shown), we found a 100% correlation between the presence of the *sup-7* 1.3 kb transgenic fragment and the presence of suppressor function. Based on the heritability of arrays bearing *sup-7* and on the relative abundance of the *sup-7* sequences in transformed populations, we estimated that the arrays bearing *sup-7* were maintained at < 10 copies per genome (see Figure 3 legend). Consistent with the 100:1 ratio of pRF4 to *sup-7* plasmid in the injection mixture, pRF4 DNA was present at high copy number in all of the transformed populations (Figure 3B).

In striking contrast to the very high frequency of coassembly between plasmid molecules, mixtures of plasmid and phage clones exhibited a much lower frequency of cotransformation. After injection of the *rol-6* plasmid together with an equal concentration of a non-homologous phage clone, four of 27 extrachromosomal arrays that conferred the rolling phenotype failed to contain any of the phage sequences. Some of the remaining 23 arrays analyzed contained mostly the pRF4 plasmid with relatively few copies of the phage sequences while other arrays were composed almost entirely of phage DNA with relatively few copies of the *rol-6* plasmid (data not shown). These observations suggest that shared regions of homology may be important for driving the assembly of molecules into arrays.

Homologous recombination during array formation

In order to explore more directly the frequency of homologous recombination during formation, we constructed a series of *rol-6* deletion plasmids: one 3′ deletion plasmid (p3′Δ9) and two similar sets of 5′ deletion plasmids (5′set 1 and 5′set 2, Figure 4). Each deletion inactivated the *rol-6* gene such that injection of the deletion plasmids individually failed to yield rollers (Table IV and data not shown). Expression of the roller phenotype required that 3′ and 5′ deletion plasmids be co-injected and that recombination occur within a defined region of overlapping homology inside the *rol-6* gene.

The effect of the length of homology on recombination frequency was analyzed by co-injecting p3′Δ9 together with two different 5′ deletion plasmids (5′set 2, Figure 4). When supercoiled plasmids that shared 598 bp of homology within the *rol-6* gene were co-injected, 72 heritably transformed rolling lines were obtained from 16 injected animals (Table III). This is similar to the frequency of germline transformation observed for the injection of the intact *rol-6* gene (pRF4 alone, Table III). Reducing the shared region to 295 bp reduced the frequency of rolling transformed lines by ~10-fold (Table III). Similar results were obtained with the other set of 5′ deletion plasmids (5′set 1, Figure 4, data not shown).

F1 expression of injected sequences was much more sensitive to a prerequisite for recombination than was heritable transformation. When we co-injected *rol-6* deletion plasmids which shared 598 bp of homology within the *rol-6* gene, the average number of F1 rollers obtained per injected animal declined ~4-fold relative to the number obtained for injection of the intact pRF4 plasmid (Table III, F1 expression). This suggests that when plasmids bearing the intact *rol-6(su1006)* gene are injected, a considerable portion of the non-heritable F1 expression is derived from less extensively recombined molecules.

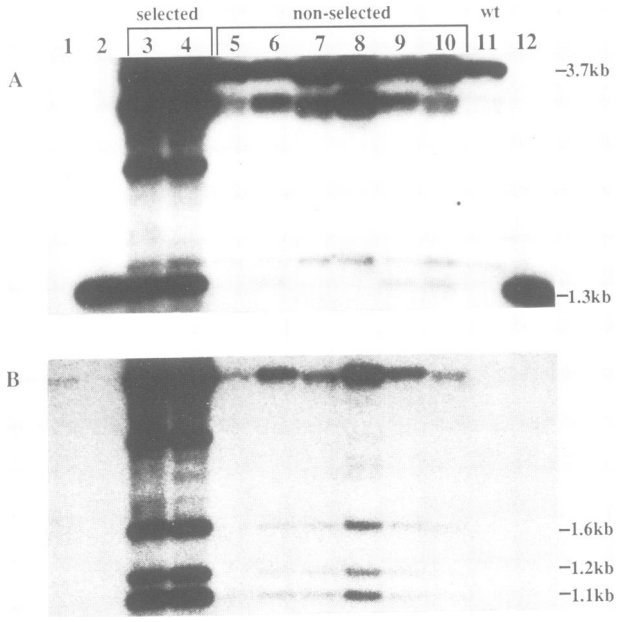

Fig. 3. Hybridization analysis of transformed lines obtained from co-injection of *rol-6* and *sup-7* plasmids at a 100:1 ratio. DNA preparations were digested with *Eco*RI and *Bam*HI and loaded as follows: lane 1, pRF4 (*rol-6*), diluted to represent the equivalent of ~10 genomic copies; lanes 2 and 12, pASTRB (*sup-7*), diluted to represent ~one genomic copy; lanes 3 and 4, DNA from populations established and maintained under selection for *sup-7* (at harvesting, the populations contained 80–98% rolling animals); lanes 5–10, DNA from populations established as rollers and maintained without further selection (at harvesting, these populations contained 5–10% rolling animals); lane 11, DNA from uninjected animals. (**A**) *sup-7* probe. The 1.3 kb pASTRB *sup-7* fragment was purified away from vector sequences through two rounds of gel electrophoresis and the isolated fragment was used to prepare a radiolabeled probe. This probe contained a small but unavoidable contamination with the pASTRB vector sequences. The contaminating vector sequences in the probe hybridize faintly to the pRF4 3.3 kb vector fragment in lane 1, and also hybridize to this 3.3 kb band in each of the lanes containing DNA from transformed populations. This hybridization is especially obvious in lanes 3 and 4 where due to selection, a much larger proportion of the populations contained the extrachromosomal arrays. In uninjected animals (lane 11), the probe hybridizes to the 3.7 kb genomic *sup-7* fragment and less intensely to three other members of the *sup-7* tRNA gene family. A 1.5 kb *sup-7* tRNA gene family member is well separated from plasmid sequences and serves as an indicator of the quantity of worm DNA loaded in each lane. Based on the proportion of transformed individuals in each population and on the relative hybridization of the probe to single copy genomic sequences, we estimate the transgenic *sup-7* copy number to be between one and 10 per transformed genome. (**B**) The same blot is shown stripped of *sup-7* probe and hybridized to a probe prepared from pRF4. In addition to plasmid hybridization at 3.3 kb in the upper portion of the gel, each of the transformed lines (lanes 3–10) shows enrichment in three *rol-6* fragments (migrating at 1.6, 1.2 and 1.1 kb). These fragments were also visible in lane 11 (DNA prepared from uninjected animals) upon longer exposure (data not shown).

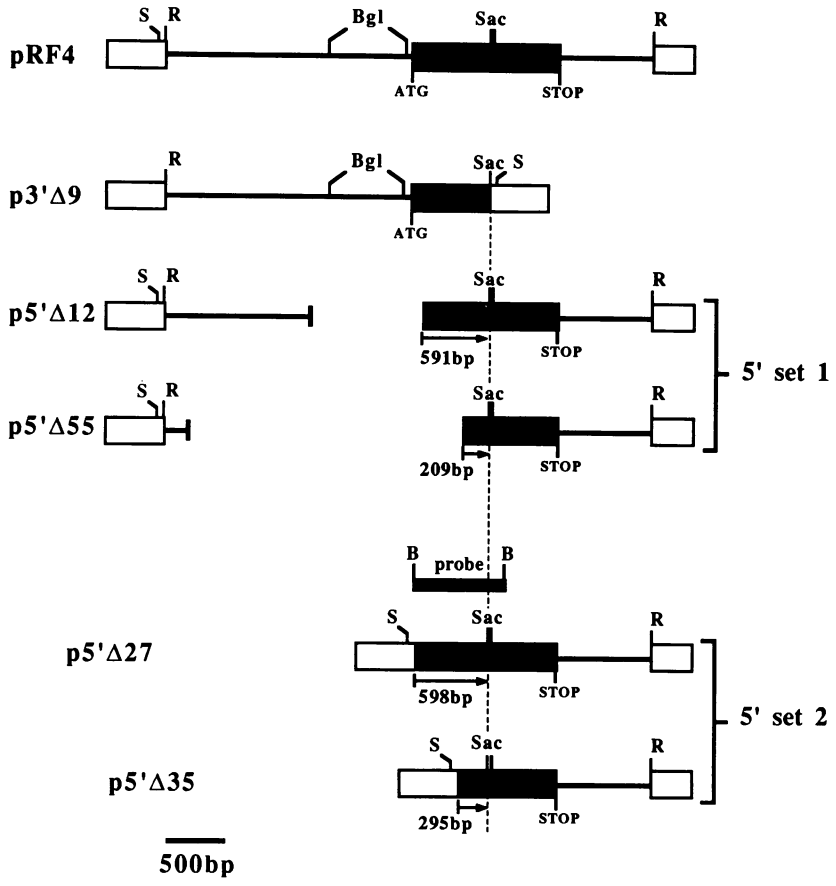

Fig. 4. Maps of the *rol-6* deletion plasmids. The intact 4 kb *rol-6* fragment is shown at the top. The junctions with vector sequences are indicated by unshaded blocks and the orientation of the plasmid is indicated by the position of the unique *Sal*I site in the Bluescribe vector. The coding region of the *rol-6* collagen gene is shown by the shaded block. The orientation of the gene is indicated by ATG (5′) and STOP (3′). The vertical hatched line indicates the *Sac*I site at the 3′ boundary of the *rol-6* sequences in plasmid p3′Δ9. The exact amount of sequence that each 5′ deletion plasmid shares with p3′Δ9 (within the *rol-6* gene) is indicated by the number (with arrow) beneath each diagram. The plasmids p5′Δ12 and p5′Δ55 (5′set 1) contain additional 5′ *rol-6* flanking sequences of 1200 and 200 bp respectively (shown aligned beneath the corresponding sequences in p3′Δ9). The gap in homology to p3′Δ9 is drawn to scale. The plasmids p5′Δ27 and p5′Δ35 (5′set 2) have the Bluescribe polylinker fused to the deletion breakpoint within the gene. Abbreviations: B, *Bam*HI; Bg, *Bgl*II; R, *Eco*RI; S, *Sal*I; Sac, *Sac*I. A 700 bp *Bam*HI fragment shown above p5′Δ27, extending from the polylinker in p5′Δ27 to a *Bam*HI site located in the *rol-6* gene, was used as a probe in subsequent experiments.

Table III. Effect of homology length on recombination frequency

DNA[a]	(Homology)	No. injected	Totals		Per injected animal	
			F1 rollers	lines	F1 rollers	lines(roll)
pRF4 (alone)		4	198	22	49.5 ± 13.8	5.5 ± 1.6
p5′Δ27+p3′Δ9	(598 bp)	16	205	72	12.8 ± 3.2	4.5 ± 0.8
p5′Δ35+p3′Δ9	(295 bp)	15	45	8	3.0 ± 1.5	0.5 ± 0.5
p5′Δ27 (alone)		16	0	0	0	0
p3′Δ9 (alone)		30	0	0	0	0

[a]Each plasmid was injected at 100 μg/ml.

To assess the structure of the products of recombination between the 3′ and 5′ deletion plasmids, DNA was prepared from several transformed lines and subjected to Southern analysis. Probes and restriction enzymes were chosen so as to allow us to distinguish between the injected molecules, the restored *rol-6(su1006)* gene, and the genomic copies of the wild-type *rol-6* gene. DNA prepared from the transformed lines contained a prominent band migrating at 692 bp, indicative of a recombination event between the injected molecules that restored the *rol-6(su1006)* gene (Figure 5, lanes 7–14). The abundance of the restored gene fragment relative to the unrecombined parental fragments (Figure 5, high molecular weight bands in lanes 7–13) suggests that recombination occurred frequently within the shared 591 bp interval during the production of these extrachromosomal arrays. In contrast, when the injected plasmids shared only 209 bp within the *rol-6* gene, the resulting arrays contained a much greater proportion of parental sequences (Figure 5, intense hybridization in the upper portion of lane 14). Fragments corresponding to the genomic copy of the *rol-6* gene were much less abundant and were visible only upon a much longer exposure (data

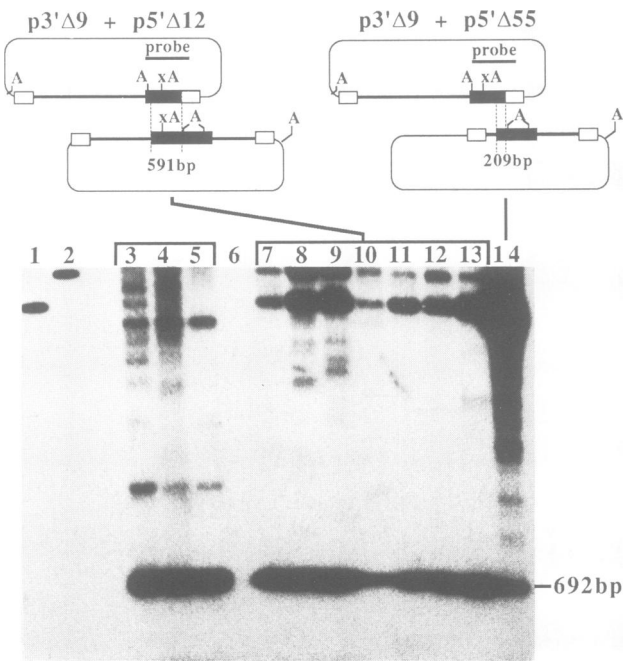

Fig. 5. Analysis of homologous recombination products within extrachromosomal arrays. The diagram illustrates the deletion plasmids aligned with respect to their shared homology within the *rol-6* gene. The exact size of the shared region is indicated by the number beneath the hatched lines. The *Aat*II sites used to analyze the recombination products are indicated by 'A' in the diagram, 'xA' indicates an *Aat*II site present in the genomic wild-type *rol-6* gene but absent in *rol-6(su1006)* (other notations are the same as in Figure 4). A 692 bp *Aat*II fragment is diagnostic for restoration of the *rol-6(su1006)* coding region. The probe, shown oriented with respect to the deletion plasmids, was described in Figure 4. DNA preparations were digested with *Aat*II and loaded as follows: lane 1, p3′Δ9; lane 2, p5′Δ12; lanes 3–5, populations transformed with intact *rol-6* (pRF4); lane 6, uninjected animals; lanes 7–13, populations transformed with p3′Δ9 p5′Δ12; lane 14, population transformed with p3′Δ9 and p5′Δ55. An equal amount of DNA from uninjected animals was loaded in lane 6. The genomic *rol-6(+)* fragments (migrating at the bottom of the gel in lanes 3–14) were not visible in this exposure. The reciprocal recombination products were not resolved from the parental fragments in the *Aat*II digest.

not shown). Since the frequency of roller transformants depended on the length of shared homology between the co-injected *rol-6* deletion plasmids (Table IV), and since the resulting transformed lines exhibited the restriction pattern of a recombinant *rol-6* gene (Figure 5), we conclude that homologous recombination was the mechanism by which the *rol-6* gene function was restored.

Double strand breaks stimulate homologous recombination

In other systems including yeast and mammalian cells (Orr-Weaver *et al.*, 1981; Kucherlapati *et al.*, 1984; Lin *et al.*, 1984; Wake *et al.*, 1985), free ends adjacent to a region of homology stimulate homologous recombination. We used the *rol-6* deletion plasmids to explore the effect of free ends on homologous recombination during array formation in *C.elegans*. The plasmids p5′Δ35 and p3′Δ9, which shared 295 bp of homology within the *rol-6* gene, were chosen for this analysis. When co-injected as supercoils, these plasmids yielded a low frequency of rollers (Table III). Digestion of either or both plasmids with the restriction endonuclease *Sal*I, which opens the plasmids adjacent to the region of *rol-6* homology (see Figure 6, diagram), caused an increase of 6- to 10-fold in the frequency of heritably transformed rolling lines (Table IV). Digestion with *Eco*RI, which opens the plasmids distal to the region of *rol-6* homology (Figure 6, diagram), did not increase the frequency of transformation (Table IV). All of the transformed lines obtained in these experiments appeared to exhibit extrachromosomal inheritance of the roller phenotype. As a control for the quality of the injections, a second intact marker gene (an actin-4–β-galactosidase fusion gene kindly provided by Jocelyn Shaw) was included in each injection mixture (Table IV, βGal).

We found that linear DNA molecules consistently gave an ~2-fold lower transformation frequency under conditions optimized for transformation with supercoiled plasmid. For example when the pRF4 plasmid containing the intact *rol-6(su1006)* gene was pretreated with *Sal*I which open the plasmid in polylinker sequences situated well away from the gene, we observed an ~2-fold drop in transformation

Table IV. Effect of double strand breaks on frequency of homologous recombination

DNA mixture[a]	Injected[b]	Per injected animal		
		[F1(roll) + F1(βGal)][c]	F1(roll)	Lines(roll)
pRF4 uncut	4	69.3 ± 9.5	53.5 ± 6.3	14.3 ± 6.2
pRF4 *Sal*I	4	45.3 ± 10.5	25.0 ± 6.8	8.5 ± 3.0
5′Δ35 uncut + 3′Δ9 uncut	10	45.0 ± 7.9	5.6 ± 0.9	0.5 ± 0.8
5′Δ35*Sal*I + 3′Δ9*Sal*I	10	43.4 ± 6.6	22.9 ± 3.4	6.3 ± 1.8
5′Δ35*Sal*I + 3′Δ9 uncut	10	37.3 ± 10.6	15.8 ± 3.1	4.4 ± 1.5
5′Δ35 uncut + 3′Δ9*Sal*I	9	34.4 ± 11.0	8.9 ± 2.8	2.8 ± 1.0
5′Δ35*Eco*RI + 3′Δ9*Eco*RI	10	33.1 ± 8.3	1.9 ± 0.9	0.3 ± 0.5
5′Δ35*Sal*I (alone)	20	n.d.	0	0
3′Δ35*Sal*I (alone)	20	n.d.	0	0

[a]In addition to 100 μg/ml of those plasmids indicated, 50 μg/ml of the plasmid pJS303 bearing a β-galactosidase fusion gene was included in order to monitor the quality of each injection.
[b]The injected animals were *rol-6(n1270e187)*. Injection of the putative null mutant animals yields higher numbers of transformed lines and facilitates the analysis.
[c]The value [F1(roll) + F1(βGal)] was determined for each injected animal. First all of the F1 rollers were cloned and then the remaining F1 animals were fixed and stained for β-galactosidase expression. Since ~90% of F1 rollers also express the β-galactosidase gene (data not shown) the total [F1(roll) + F1(βGal)] should be roughly equal for injections of equal quality, regardless of how many rollers were obtained. From this value we conclude that the injections compared were of similar quality (e.g. showed similar total F1 expression).
[d]Means are given ± SD.

efficiency (Table IV, compare pRF4 uncut with pRF4 *SalI*). The frequency of transformation observed after co-injection of p5′Δ35 and p3′Δ9, each pretreated with *SalI* is not significantly different from the frequency observed after injection of the intact *rol-6* plasmid as a *SalI* linear (Table IV).

DNA was recovered from transformed lines and Southern analysis was performed in order to assess the structure of the transformation products. Probes and restriction enzymes were chosen such that the hybridizing fragments predicted from the restored gene and from the reciprocal recombination product could be visualized on a single Southern blot. Transformed lines obtained from injection of *SalI* linearized plasmids contained a prominent 793 bp band corresponding in size to the restored *rol-6* gene (Figure 6, lanes 4−7). This fragment was less abundant in extrachromosomal arrays that assembled from the supercoiled or *Eco*RI cut deletion plasmids (Figure 6, lanes 8−15). Conversely, a 310 bp band corresponding in size to the predicted reciprocal recombination product was not detectable within extrachromosomal arrays that assembled from the *SalI* cut deletion plasmids, but was present in several of the transformed lines obtained from the uncut and *Eco*RI cut plasmids (Figure 6, lanes 8, 10, 11 and 13−15). Thus, pretreatment with *SalI* appears to stimulate a recombination event in which the sequences proximal to the free ends are not conserved. This observation is consistent with the stimulative effects of double-strand breaks in other systems (Szostak *et al.*, 1983).

Integrative transformation

The high frequency of recombination between injected molecules encouraged us to look for integration of the transforming sequences into worm chromosomes. We looked for integrated transformed lines in two different ways. First, we cloned four or more self progeny of transformed individuals and then looked for 100% transmission of the transformed phenotype among the progeny of a single individual. Over 400 transformed lines were screened in this way and only one transformed line exhibited 100% transmission and in subsequent analysis exhibited a Mendelian segregation pattern and genetic linkage to a worm chromosome. In a second approach to finding integrated transformed lines, we attempted to use the *sup-7* gene (which is toxic at high copy number) to eliminate the high background of extrachromosomal transformation and to help select for low copy number integration events. Injection of *sup-7* into oocyte nuclei has been used to generate integrated transformed lines (Fire, 1986; Spieth *et al.*, 1988; Fire and Waterston, 1989). However, using the *sup-7* marker with the cytoplasmic injection method, we failed to obtain an integrated line from >400 injected animals (see Table I). These observations suggest that sequences injected into gonad cytoplasm may encounter some obstacle to interaction with the chromosomes.

In an effort to overwhelm or to bypass obstacles to integration we injected large molar quantities of a single-stranded 50 base oligonucleotide bearing the dominant *rol-6(su1006)* mutation. We then looked for gene conversion between the oligonucleotide and the genomic copy of the *rol-6* gene (Moerschell *et al.*, 1988). In the course of these experiments we also explored the effect of the oligonucleotide on the transformation efficiency of the *rol-6* plasmid. Very high concentrations of oligonucleotide co-injected with pRF4 reduced the fertility of the injected animals. However, roller transformants were still obtained. Surprisingly, several of the resulting transformed lines yielded broods of self progeny

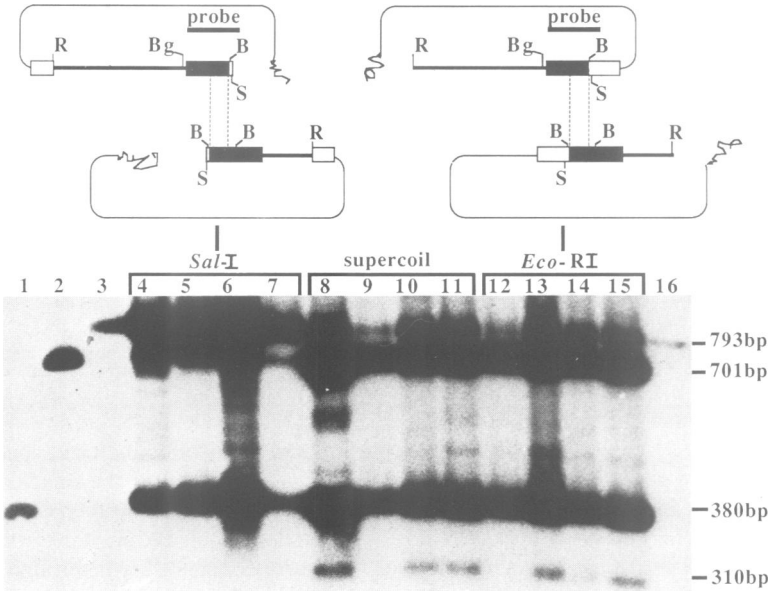

Fig. 6. Effect of double-strand breaks on recombination. The diagram illustrates the deletion plasmids p3′Δ9 (top) and p5′Δ35 (bottom) aligned with respect to the 295 bp shared region (indicated by hatched lines). The sites used to linearize the deletion plasmids are shown, as are the sites used to digest the DNA recovered from the transformed lines (notation as in Figure 4). The probe prepared from p5′Δ27 (see Figure 4) is shown oriented with respect to the deletion plasmids. Recombination that restored *rol-6* function should produce a 793 bp *Bam*HI−*Bgl*II fragment, while the reciprocal recombination reaction should produce a 310 bp *Bam*HI fragment. DNA preparations were digested with *Bgl*II and *Bam*HI, and were loaded as follows: lane 1, p5′Δ35; lane 2, p3′Δ9; lane 3, pRF4; lanes 4−7, DNA recovered from populations transformed with *SalI* cut deletion plasmids; lanes 8−11, DNA recovered from populations transformed with uncut deletion plasmids; lanes 12−15, DNA recovered from populations transformed with *Eco*RI cut deletion plasmids; lane 16, uninjected animals.

Table V. Effect of oligonucleotides on the integration of plasmid DNA

DNA (mg/ml)		Animals	F1	Lines (roll)	
pRF4	oligo-type[a]	No. type[b]	rollers	arrays	integrated
0.08	0.7 A	63 wt	188	3	3
0.08	0.7 A	21 null	227	10	2
0.02[c]	1.0 A	47 wt	156	24	2
0.02[d]	1.0 A	44 wt	135	14	1
0.16	0.7 A	50 wt	650	23	1
0.16	1.0 B	200 wt	1000	53	2
0.16	1.0 B	60 null	400	20	1
0.08	0.5 C	30 null	800	57	0

[a]Oligonucleotide type: A = 50mer homologous to *rol-6*, see Materials and methods B = 50mer non-homologous, C = random hexamers.
[b]Animal type: wt = N2, null = *rol-6(n1270e187)* putative null allele.
[c]Injected DNA was a mixture of 20 μg/ml of pRF4 and 60 μg/ml of a second plasmid.
[d]Injected DNA was a mixture of 20 μg/ml of pRF4 and 20 μg/ml of a second plasmid. The second plasmid was co-integrated with the *rol-6* plasmid.

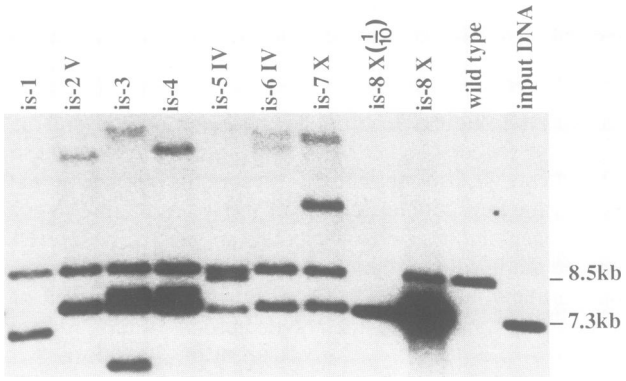

Fig. 7. Southern analysis of integrated transformed lines. DNA preparations were digested with *SalI* and loaded as indicated. Probe was prepared from a fragment of the *rol-6* gene isolated from p5′Δ27 (Figure 4). The *rol-6* plasmid, pRF4 (input DNA), contains a single *SalI* site and migrates at 7.3 kb. The genomic copy of the *rol-6* gene resides on an 8.5 kb *SalI* fragment visible in wild-type DNA. The genetic linkage to a worm chromosome (I–V and X), if determined, is indicated by the appropriate Roman numeral. Some integrated *rol-6(su1006)* lines differ in the severity of their roller phenotype, suggesting position effects on *rol-6* expression. For example, is-2 and is-4 exhibited no phenotype in the presence of a single copy of *rol-6(+)*, whereas other integrated lines did exhibit a phenotype.

containing 100% rolling animals (Table V). These apparently integrated transformed lines were obtained at a variable frequency, ranging from approximately one in 20 to one in 100 injected animals (Table V). Homology between the oligonucleotide and the plasmid was not necessary (oligonucleotide type B, Table V). Increasing the plasmid concentration from 80 to 160 μg/ml caused an increase in the number of extrachromosomal transformed lines, but not in the proportion of injected animals which gave rise to stable transformed lines (Table V). We did not obtain an integrated line from co-injection of *rol-6* plasmid along with random six-base oligonucleotides in one experiment (oligonucleotide C, Table V).

Southern analysis was performed to assess the copy number of the transforming sequences. Hybridization with a DNA probe prepared from the *rol-6* gene revealed that each stable transformed line contained the genomic copy (8.5 kb in Figure 7) and one or more additional transgenic copies of *rol-6*. The transformed line 'is-1', appeared to contain one novel hybridizing band present at approximately one copy per genome. The other stable lines contained two or more novel bands, and in each case one of the new bands co-migrated with the injected plasmids at 7.3 kb, consistent with insertion of tandem copies of the plasmid. The other novel hybridizing fragments may represent either internal rearrangements within the tandem copies of pRF4 or junction fragments between pRF4 sequences and the genomic site of insertion. All of the transformed lines except for line 'is-8' appeared to contain fewer than five copies of the pRF4 plasmid. Transformed line is-8 appeared to contain between 10 and 20 tandem copies of the *rol-6* sequence (a 10-fold dilution of is-8 was loaded for comparison [is-8(1/10) in Figure 7]. None of the stable transformed lines showed a shift in the size of the 8.5 kb genomic fragment predicted for a homologous insertion of pRF4 into the *rol-6* locus on chromosome II. Five of the stable lines were genetically mapped with respect to known chromosomal genetic marker genes and in each case the roller phenotype displayed linkage to a single worm chromosome (Figure 8, Roman numerals).

Discussion

Here we describe a convenient dominant reporter gene, *rol-6(su1006)*, and a simplified microinjection procedure for *C.elegans* DNA transformation. We have used these tools to study DNA transformation in *C.elegans*. Our findings allow us to reconstruct the probable fate of DNA sequences injected into the *C.elegans* gonad cytoplasm. The newly injected sequences are initially highly reactive and undergo frequent homologous recombination reactions. At appropriate DNA concentrations, these reactions drive the assembly of the injected sequences into large contiguous arrays. Arrays which attain a sufficient size become heritable as extrachromosomal elements. Smaller transgenic molecules can be expressed among the progeny of injected animals but are not transmitted in the germline. Interactions between the chromosomes and the exogenous sequences are rare, suggesting that the injected sequences may be sequestered away from the chromosomes during the highly reactive period of array assembly. After array assembly the extrachromosomal transforming sequences assume a much less recombinogenic form and are propagated indefinitely with little further rearrangement.

Array formation

The high frequency of cotransformation between the *rol-6* and *sup-7* plasmids indicates that arrays assemble directly from a large pool of the injected plasmid molecules. The fact that high concentrations of the toxic *sup-7* sequences completely abolished heritable *rol-6* transformation suggests that arrays cannot form through the replication of one or a few founder molecules or by selectively incorporating only one type of plasmid molecule. Rather, the high frequency of cotransformation between *rol-6* and *sup-7* plasmids suggests that arrays must be large, and that they assemble directly from the injected DNA.

Shared regions of homology appear to be important in driving the co-assembly of sequences into arrays. Injected molecules that shared homology were incorporated into arrays much more efficiently than were completely non-

homologous molecules. By co-injecting plasmids bearing non-overlapping deletions in the *rol-6* gene, we showed that homologous recombination occurred frequently during array formation. Similar recombination between homologous DNA molecules after co-injection into *C.elegans* has been observed previously (A.Fire, personal communication). We found that double-strand breaks stimulated homologous recombination during array assembly in a fashion consistent with the effect observed in yeast and mammalian cells (Orr-Weaver *et al.*, 1981; Kuchlerlapati *et al.*, 1984; Lin *et al.*, 1984; Wake *et al.*, 1985).

Extrachromosomal arrays contain numerous rearrangements of the injected DNA. The relatively low frequency of coassembly which we observed between non-homologous molecules suggest that the majority of these rearrangements must occur within or between homologous molecules. For example, the joints between linear molecules as well as many of the rearrangements preserved within arrays could be formed after molecules are brought into proximity by homologous pairing. The synapsis-mediated fusion of identical linear molecules into an inverted dimer plasmid has been demonstrated to occur in yeast (Kunes *et al.*, 1990). Rearrangements of the injected sequences could also occur if broken molecules are efficiently ligated during array formation. Such ligation activity may occur intramolecularly or may occur preferentially between homologous molecules. A better understanding of the mechanisms which result in rearrangements of the injected molecules and in the joining of non-homologous molecules during DNA transformation in *C.elegans* will require further study.

Chromosomal integration

We have shown that DNA molecules injected into the *C.elegans* gonad cytoplasm undergo a transient period of reactivity resulting in the formation of large heritable extrachromosomal elements. We and others (Stinchcomb *et al.*, 1985a) have observed that, once established, these extrachromosomal structures undergo very little further rearrangement. A transient period of reactivity has also been observed during DNA transformation in other systems. For example, by performing successive injections into individual mammalian cell nuclei, Folger *et al.* (1985) showed that newly injected sequences remained highly reactive for only approximately the first 60 min after injection. If more than 60 min elapsed between successive injections, the plasmid molecules failed to interact and were always found integrated at separate chromosomal sites.

When exogenous DNA is microinjected directly into nuclei it is exposed to chromosomes throughout the reactive period. After cytoplasmic injection, however, DNA sequences may experience substantial modification prior to exposure to the chromosomes. This may be particularly true of DNA delivered into oocyte cytoplasm where stockpiles of DNA packaging and modifying proteins are thought to be kept in store for the rapid biosynthetic activities of early embryogenesis (reviewed for *Xenopus* oocytes by Laskey *et al.*, 1979). For example, in *Xenopus* oocyte cytoplasm, foreign DNA sequences are rapidly condensed into chromatin and are assembled into structures resembling typical eukaryotic nuclei (Forbes *et al.*, 1983). If similar activities exist in the *C.elegans* gonad cytoplasm, then the reactive period of array formation may be over long before the injected sequences are exposed to the chromosomal nuclear environment. This could explain the strong correlation observed between nuclear injection and integration in previous work on *C.elegans* transformation (Fire, 1986).

In this study, using a cytoplasmic injection technique, we reproducibly obtained integrated lines only after co-injection of plasmid DNA together with an excess of single-stranded oligonucleotide (Table V). The effect of the oligonucleotide on integration appeared not to be sequence specific, since no homology between the plasmid and oligonucleotide was required. Perhaps the oligonucleotide competes for factors present within the gonad cytoplasm which would normally convert the double-stranded DNA into a less reactive form. If the injected DNA persists in a reactive form until after fertilization, then it may be exposed to chromosomes during the cell divisions of early development. Whatever the reason, our results indicate a significant positive effect of oligonucleotide on the frequency of interaction between injected sequences and the chromosomes. In fact, the frequency of integration which we report is comparable with the frequency of integration observed after nuclear injection of DNA in *C.elegans* (A.Fire, personal communication).

Presently we do not know anything about the fate of the oligonucleotide. For example we do not know if the oligonucleotide can be incorporated into arrays or into the chromosomes. Furthermore, we do not know how parameters such as oligonucleotide sequence, length and strandedness affect the frequency of integration. Among the progeny of 500 animals receiving injections of the *rol-6(su1006)* oligonucleotide, we identified a single possible gene conversion event between the oligonucleotide and the wild-type *rol-6* genomic locus (data not shown). Further study, utilizing a selection for the desired gene conversion event or the co-injection of the oligonucleotide and the target sequence will determine the utility of oligonucleotides as vectors for gene conversion of *C.elegans*.

All of the plasmid integration events observed in this study appeared to be non-homologous; however, a few examples of homologous integration have been observed in *C.elegans* (Sheryl Broverman and Andy Fire, personal communication). With improved selections for recovering integrated transformed lines, it may be possible to use the cytoplasmic injection method described here to target the insertion of double-stranded DNA molecules into homologous chromosomal sites. The high frequency of homologous recombination between co-injected DNA molecules and the stimulative effect of double-strand breaks that we have observed during *C.elegans* transformation are reminiscent of gene transfer in mammalian cells (Folger *et al.*, 1982; Kucherlapati *et al.*, 1984; Lin *et al.*, 1984). Strategies like those employed for the targeted insertion of sequences in mammalian cells (Thomas *et al.*, 1986) may prove useful for obtaining homologous integration in *C.elegans*.

Heritability of transgenic structures

The germline transmission of extrachromosomal elements in *C.elegans* appears to require that arrays attain a minimum size. The high frequency of co-transformation we observed between the *rol-6* plasmid and the 100-fold more dilute *sup-7* plasmid indicates that a large number of plasmid molecules must assemble to form a single array. We used the observed frequency for zero incorporation of the *sup-7* plasmid into arrays to make a statistical estimate of the number of plasmid molecules required to form a single heritable extrachromo-

somal element (see Materials and methods). Depending on assumptions regarding the mechanism of array assembly we estimate that arrays are composed of 110–165 plasmid molecules. Hybridization analysis of extrachromosomal lines bearing the *sup-7* plasmid suggests that <10 copies are present per transformed genome. Furthermore, the relative abundance of the *rol-6* plasmid in these arrays is consistent with an array size of >100 plasmid molecules (Figure 3). Stinchcomb *et al.* (1985a) estimated that transformed cells contained 70–300 plasmid copies. Our data support this estimate and further indicate that these plasmid copies are contained in one or a few arrays per transformed cell.

There is evidence from other systems that replication of exogenous DNA is more efficient for relatively large molecules. For example, larger DNA molecules appeared to be preferentially sequestered and replicated within nucleus-like structures that formed in isolated *Xenopus* oocyte cytoplasm (Newport, 1987). Biochemical analysis revealed that plasmid sequences injected into *Xenopus* or sea urchin egg cytoplasm were replicated only after they were assembled into high molecular weight concatenates (Marini *et al.*, 1988; McMahon *et al.*, 1985). A bias for replication of large arrays may explain the limitation of array size on heritability of extrachromosomal elements in *C.elegans*. Large size may also be required for the mitotic and meiotic partitioning of extrachromosomal arrays. In the yeast *Saccharomyces cerevisiae*, increasing the length of artificial chromosomes increased their mitotic stability (Murray *et al.*, 1986). Chromosome-mediated gene transfer in mammalian cells results in both large and small transgenic fragments. The large, cytologically detectable fragments were lost at a much lower frequency than were the smaller, cytologically undetectable fragments (Klobutcher *et al.*, 1980). In *C.elegans*, chromosomal fragments generated by radiation damage ('free duplications') exhibit a range of heritability, with larger duplications more stable than smaller ones (Herman, 1984). We and others (Stinchcomb *et al.*, 1985a; Way and Chalfie, 1988; Andy Fire, personal communication) have found that individual extrachromosomal arrays exhibit characteristic high or low levels of heritability. Perhaps these differences in heritability simply reflect differences in array size.

We do not know if endogenous worm sequences are added to arrays during transformation. If the heritability of extrachromosomal arrays depends solely on the addition of specialized sequences such as telomeres and replication origins, then our data suggest that they are only added to relatively larger molecules. Injection of yeast centromere- and telomere-containing sequences, and worm sequences which confer plasmid partitioning activity in *S.cerevisiae* (Stinchcomb *et al.*, 1985b), but no effect on transformation frequency or on the stability of the resulting arrays (data not shown).

Expression of transgenic sequences

For some transgenes, relatively normal phenotypic expression is possible over a broad range of copy number. For example, extrachromosomal arrays bearing as few as ~10 copies to >100 copies of the *rol-6* plasmid exhibited very similar rolling behavior. By contrast, expression of *sup-7(st5)* was tolerated only at a much lower gene dose, ~1–10 copies per extrachromosomal array.

Integrated copies of a particular transgene exhibited a sensitivity to gene dose very similar to that observed for extrachromosomal expression. For example, as seen for extrachromosomal transformation, a broad range in copy number of the *rol-6(su1006)* plasmid was found in integrated transformed lines (Figure 7, and data not shown). Similarly, as was observed for extrachromosomal transformation, only a low copy number of *sup-7(st5)* was tolerated in integrated transformed lines (Fire, 1986). Thus, the expression of a transgene from extrachromosomal arrays may be generally comparable with expression after chromosomal integration.

Conclusion

These findings have clear practical applications for *C.elegans* transformation. Transformed lines can be recovered easily. The composition of extrachromosomal arrays can be controlled, allowing one to produce transformed lines expressing a low or high dose of a gene of interest. The high frequency of homologous recombination which occurs during array formation allows contiguous sequences (from a chromosome walk for example) to be introduced with confidence that a gene partially contained on overlapping molecules will be restored during array formation. The *rol-6* marker and the injection methods which we describe have already been applied by others to recover *C.elegans* transformants (Aroian *et al.*, 1990; Han and Sternberg, 1990; Krause *et al.*, 1990; Aamodt *et al.*, 1991; Ahringer and Kimble, 1991).

The ease with which *C.elegans* DNA transformation can now be accomplished coupled with the rich genetics and the nearly complete physical map now available in *C.elegans* (Coulson *et al.*, 1986, 1988) make this well characterized nematode an ideal organism in which to apply molecular techniques to the questions of gene function and regulation. We believe that it will also be possible to use these resources to study the cellular processes which govern chromosome structure, replication and partitioning.

Materials and methods

Strains and culture

Caenorhabditis elegans strains were handled and maintained as described by Brenner (1974). Worm strains and alleles were from the Cambridge collection (Brenner, 1974) unless otherwise noted. Strains used for injection were; N2 *var* Bristol; *rol-6 (n1270e187)* a putative null allele of *rol-6* (wild-type in behavior, though slightly dumpy in appearance, Park and Horvitz, 1986); *nuc-1(e1392)*, an amber allele of the major DNase of *C.elegans*. Strains used for genetic mapping included MT465 [*dpy-5 (e61)I; bli-2 (e768)II; unc-32 (e189)III*] and MT464 [*unc-5 (e53)IV; dpy-11 (e224)V; lon-2 (e678)X*] (provided by H.R.Horvitz).

DNA for injection

Plasmid and cosmid DNA was prepared for injection using a single round of CsCl gradient purification (Maniatis *et al.*, 1982). pRF4 carries a 4 kb *Eco*RI fragment of *C.elegans* genomic DNA containing the *rol-6(su1006)* collagen gene (Kramer *et al.*, 1990) in the Bluescribe vector (Stratagene Inc.). pJS303, provided by Jocelyn Shaw, is a pBR322 derivative which contains the 5′ and 3′ sequences of the *C.elegans actin-4* gene fused to the β-galactosidase gene from *Escherichia coli*. pASTRB is a derivative of pAST (Fire, 1986) and contains the 1.3 kb *Eco*RI–*Hin*cII fragment of *C.elegans* genomic DNA including the *sup-7(st5)* gene (Bolten *et al.*, 1986) inserted in the *Eco*RI–*Sma*I site of pUC18 (Yanisch-Perron *et al.*, 1985). pPTCTS is a derivative of pASTRB which, in addition to *sup-7*, also contains the 4 kb *rol-6(su1006) Eco*RI fragment.

Phage DNA was prepared using a CsCl gradient to isolate phage particles followed by phenol extraction and ethanol precipitation (Maniatis *et al.*, 1982). Single-stranded oligonucleotides were phenol extracted twice and ethanol precipitated. The 50mer homologous to *rol-6* (oligonucleotide A) had the following sequence: 5′-GGAACCGCTTCCAACCGTGTGAG-ATGTCAACAATATGGAGGATATGGAGC-3′.

The buffer used for most of the injections was described by Fire (1986).

Alternatively, DNA was injected in 10 mM Tris pH 7.5 and 1 mM EDTA (TE), with no apparent loss in transformation efficiency. DNA diluted in TE could be stored indefinitely and seemed to flow through the needle more easily than did other solutions.

Syncytial injection technique

Needles were pulled on a Kopf model 720 Vertical Pipette Puller (David Kopf Instruments). Glass blanks (World Precision Instruments Inc. item no. 1B100F-4) were pulled symmetrically and both halves were used. To ensure adequate and reproducible flow, needles were routinely treated with a hydrofluoric acid solution (HF). The HF was obtained from EM Science as a 48% solution. The packaging allows a small quantity of the acid solution to be recovered from the cap after opening. Once a small droplet of HF solution (15 μl) was transferred onto a plastic Petri plate, it did not pose an inhalation hazard and could safely be handled outside the hood. However, extreme caution should be exercised when handling HF. To perform the HF treatment, a needle is loaded into its carrier (Leitz microtool collar) and pressurized to 50 lb/square inch. The tip of the needle is immersed in a 15 μl drop of the 48% HF solution on a plastic Petri dish (this is most easily accomplished if the needle is hand held by its carrier while the tip and drop are observed under a dissecting microscope). After ~1 s, when the needle starts to bubble, it is immediately transferred to an adjacent drop of water to rinse the tip. The size of the tip opening can be controlled by varying the length of time in the HF, and can be monitored by the rate of bubbling in the HF and/or water, and by inspection at high magnification. Approximately 40 needles can be etched in 20 min. The HF droplet should be diluted carefully with excess water and discarded. For a less desirable, yet serviceable alternative to etching, needle tips can be broken against particles of dust embedded in the agar pad.

Needles were loaded for injection by inserting a drawn-out 100 μl capillary pipette (freshly pulled to avoid dust contamination) down the wide bore of the needle, and depositing the DNA solution just behind the tip, as described by Fire (1986). Needles were loaded into a Leitz microtool collar and were pressurized through 1/16 inch polyethylene tubing attached to a bottled nitrogen source. A pressure regulator, and a Swagelok one-way toggle value (B-1GM4-S4) on the nitrogen tank were used to apply a controlled pressure to the system and to obtain the desired rate of flow. A three-way Swagelok ball valve (part no. B-41XS2) was used to turn flow on and off during each injection. Agarose injection pads for immobilizing animals were prepared as described by Fire (1986). Prior to injection, a pad was spread with halocarbon oil (series 700, Halocarbon Products Corp.) A worm pick was touched to the oil surface on the injection pad and the oil drop was used to pick up worms for injection. Large healthy adult hermaphrodites which had produced < 100 eggs were taken directly from the seeded culture plates and deposited in the layer of halocarbon oil above the agarose pad. The worms (usually 10 at a time) were aligned in a row across the short axis of the pad with the ventral side facing away from the needle and with enough space between worms to allow the needle access to each animal. If the worms did not stick well to the pads it was sometimes necessary to either dry them by leaving the plate in the incubator with the lid off for a few hours prior to injection or to use slightly thicker agarose pads.

The microscope used for injection was a Ziess Axiovert 35 equipped with differential interference contrast optics and a free sliding oil cushion stage. The microscope was mounted on a Micro-g, vibration isolation table (Technical Manufacturing Corp.). Injections were performed at ~450× magnification using a Ziess (PLAN-NEOFLUAR, 40×,0.75 pol) air objective. A Leitz micromanipulator was used to hold and position the needle (but not to insert the needle, see below). Injections were performed by first focusing on the grainy textured cytoplasm in the center of the distal gonad arm. The needle tip was then brought into the same focal plane and the stage was moved (by hand) pushing the worm into the needle tip. A gentle tap to the back of the micromanipulator was usually required to cause the needle to penetrate the cuticle and gonad. Nitrogen pressure was then applied to the needle, and maintained until the entire gonad was filled with DNA solution (1−2 s, as shown in Figure 1A−C), or until the 'wave front' of injected material stopped moving. The procedure was repeated for the second gonad distal arm. Misaligned animals were injected by inserting the needle through the body and into the distal gonad. Based on the flow rate of the needle outside the gonad we estimate that between 30 and 50 pl of solution are typically delivered into each gonad arm. Adequate flow and retention of DNA solution in the gonad appears to depend upon resilient membranes making a seal around the needle at the point of insertion. Therefore, animals were not allowed to become desiccated while on the injection pad. To avoid leakage of the injected solution, animals were injected only once in each gonad arm.

Immediately after injection, a drawn out capillary was used to apply a small drop of sterile M9 salts to the worms and to transfer them to a seeded culture plate. Typically, the recovered animals were active immediately or shortly after removal from the injection pad. A new batch of animals was then applied to a dry portion of the injection pad and the procedure was repeated (as many as five times per 22 × 50 mm pad). Injected animals were cultured in groups of one to five per plate with a single transfer to a fresh plate after 24 h. Typically, about half of the transformed progeny arise on each plate.

Methotrexate selection for sup-7(st5) in nuc-1(e1392am) animals

Wild-type and *nuc-1* animals were assayed for viability over a range of methotrexate concentrations. Methotrexate blocks *de novo* purine synthesis. Wild-type animals are insensitive to the drug, presumably because they are able to scavenge nucleotide from their food. *nuc-1(e1392am)* animals have an amber mutation in the major DNase of the worm and are sensitive to methotrexate. A concentrated stock (10 mg/ml) of methotrexate (+amethopterin, Sigma Chemical Co.) was prepared in 0.2 M NaOH. Dilutions were prepared in M9 buffer and mixed with concentrated *E.coli* (OP50) and 100 μl of the mixture was seeded onto 53 mm NGM agar plates. Alternatively, 100 μl of the appropriate drug solution was deposited directly onto the bacterial lawn of a standard pre-seeded plate. (We found that selection worked best when contamination with other bacterial and fungal colonies was avoided.) *nuc-1(e1392)* adults placed on plates containing between 20 and 100 μg/ml methotrexate produced approximately normal numbers of progeny, but these progeny were uncoordinated, developmentally arrested, and/or infertile. Wild-type animals were essentially unaffected by this range of methotrexate concentrations.

Transformed lines identified using the pRF4 marker in co-injection experiments with *sup-7(st5)* plasmids were scored as methotrexate-resistant if they produced fertile, healthy, coordinated progeny when transferred to methotrexate plates. A broad range of sensitivity was observed. Transformed lines were also obtained by directly selecting for methotrexate resistance. Injected animals were placed in groups of one to five on plates containing 50 μg/ml methotrexate. Provided the plates were seeded thickly with *E.coli*, transiently rescued F1 animals matured and produced arrested and sterile progeny on the original plate without exhausting the food supply. Germline transformants were then identified in the F2 generation with no intermediate cloning steps required. The presence of the *rol-6* marker facilitated the identification of transformed animals in the F2.

Statistical analysis of array formation

The probability of forming an array which fails to contain molecule B, P_0(B), after injection of a mixture containing two types of molecule (A and B) should equal the probability of incorporating A, *P*A, at each of the '*x*' steps where interactions between A and B are possible, or $P_0B = PA^x$. To solve for '*x*', values for P_0B and *P*A must be determined. If molecule B is genetically or physically marked, then the P_0B can be measured empirically. The probability of incorporating molecule A, *P*A, depends on the mechanism of array formation. For example, in the simplest case (and one for which our data provide a test), injected molecules associate with equal probability anywhere along their length (i.e. *P*A equals the relative concentration of molecule A in the injection mixture). Two instances where we have measured P_0 allow a test of this hypothesis. In the first experiment, co-injection of the *rol-6* and *sup-7* plasmids, yielded a P_0 for *sup-7* plasmid incorporation of 9/28 or 0.33 (Table II) and the relative concentration of *rol-6* plasmid in the injection solution was 0.99. Substituting these values into the equation above ($0.33 = 0.99^x$), gives $x = \sim 110$ assembly steps. In the second experiment, co-injection of plasmid and phage sequences yielded a P_0 for incorporation of phage DNA of 4/27 or 0.15, and the relative concentration of *rol-6* plasmid in the injection solution was 0.5. Solving the equation, $0.15 = 0.5^x$, gives $x = 3$ assembly steps. These calculations suggest that plasmid molecules interact much more frequently than do plasmid and phage molecules during array formation. This conclusion is clearly inconsistent with a mechanism wherein sequences coassemble with an equal probability per unit length.

An alternative model consistent with all of the data presented here, is that molecules assemble primarily through regions of shared homology. Accordingly the probability of incorporating molecule A, *P*A, should reflect not its relative concentration but rather its fraction of the total homologous sequence in the injection solution. In this case, the data from the *rol-6* and *sup-7* co-injection experiments would indicate an even larger number of assembly steps than was calculated assuming an equal probability of assembly per unit length. The two plasmids share homology over ~1/3 of the 7 kb *rol-6* plasmid or ~1/300th of the total injected homology. Solving the equation, $0.33 = 0.9933^x$, gives $x = 200$.

Molecular biology

Caenorhabditis elegans DNA was isolated as described by Sulston and Hodgkin (Wood, 1988). Standard techniques for modifying, transferring

and hybridizing DNA were used unless otherwise indicated (Maniatis *et al.*, 1982). Radioactive probes were prepared using a random primer labeling kit (Boehringer Mannheim Biochemicals).

The *rol-6* deletion plasmids were constructed as follows. The 2.4 kb *Sac*I–*Eco*RI fragment from pRF4 was inserted into the polylinker of Bluescribe (Stratagene Inc.) to obtain the plasmid p3′Δ9. The plasmid pRF4 was digested with *Bgl*II followed by dilution and ligation to obtain the vector pFR4ΔBG lacking the 5′ 600 bp *Bgl*II fragment. *Bal*31 exonuclease was used to extend the 5′ deletion in pRF4ΔBG, followed by dilution and ligation to produce p5′Δ12 and p5′Δ55. The plasmids p5′Δ27 and p5′Δ35 were obtained by digesting with *Sma*I prior to dilution and ligation so as to juxtapose the vector polylinker and the deletion endpoint. Sequencing was performed to determine the deletion breakpoints within the *rol-6* gene (Sanger *et al.*, 1977).

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