Creation of Low-Copy Integrated Transgenic Lines in *Caenorhabditis elegans*

Vida Praitis, Elizabeth Casey, David Collar and Judith Austin

Department of Molecular Genetics and Cell Biology, University of Chicago, Chicago, Illinois 60637

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ABSTRACT

In *Caenorhabditis elegans*, transgenic lines are typically created by injecting DNA into the hermaphrodite germline to form multicopy extrachromosomal DNA arrays. This technique is a reliable means of expressing transgenes in *C. elegans*, but its use has limitations. Because extrachromosomal arrays are semistable, only a fraction of the animals in a transgenic extrachromosomal array line are transformed. In addition, because extrachromosomal arrays can contain hundreds of copies of the transforming DNA, transgenes may be overexpressed, misexpressed, or silenced. We have developed an alternative method for *C. elegans* transformation, using microparticle bombardment, that produces single- and low-copy chromosomal insertions. Using this method, we find that it is possible to create integrated transgenic lines that reproducibly express GFP reporter constructs without the variations in expression level and pattern frequently exhibited by extrachromosomal array lines. In addition, we find that low-copy integrated lines can also be used to express transgenes in the *C. elegans* germline, where conventional extrachromosomal arrays typically fail to express due to germline silencing.

THE development of techniques that allow exogenous DNA to be introduced into an organism has transformed many diverse areas of experimental biology. Transgenic DNA constructs have been used to rescue mutant genes, express reporter genes, and test the relationship of gene structure and function *in vivo*. In many cases, transgenic DNA is maintained within an organism extrachromosomally in the form of a plasmid or a multicopy array. Alternatively, transgenic DNA can be integrated into the organism's genomic DNA either by random insertion or homologous recombination.

In *Caenorhabditis elegans*, transgene DNA injected into the syncytial cytoplasm of the hermaphrodite germline undergoes intermolecular ligation and recombination to form multicopy extrachromosomal arrays. Association of an extrachromosomal array with a germline nucleus results in formation of a transgenic embryo. Transmission of extrachromosomal arrays from one generation to the next is dependent on array size and can range from 10 to 90% (Mello and Fire 1995); it has been estimated that these extrachromosomal arrays contain at least 80–300 copies of the injected plasmids (Stinchcomb *et al.* 1985; Fire and Waterston 1989; Mello *et al.* 1991; MacMorris *et al.* 1994).

Although extrachromosomal arrays created by germline injection have been used successfully to study patterns of gene expression and to identify genes by phenotypic rescue, they have disadvantages that limit their usefulness. Due to the high number of copies of the transgene in an extrachromosomal array, total transgene expression can be elevated relative to that of the corresponding endogenous gene (Fire and Waterston 1989); in addition, expression pattern can vary from animal to animal due to mosaic loss of the extrachromosomal array (Stinchcomb *et al.* 1985). Further complicating matters, the presence of tandemly repeated sequences in an array can trigger gene-silencing mechanisms (Okkema *et al.* 1993; MacMorris *et al.* 1994; Kelly *et al.* 1997; Hsieh *et al.* 1999). Transgene silencing is a particular problem in the *C. elegans* germline, where high-copy-number extrachromosomal arrays are rapidly silenced after a few generations (Kelly *et al.* 1997; Kelly and Fire 1998; Seydoux and Strome 1999), limiting the ability of researchers to study germline development and function.

One way to avoid problems associated with high-copy extrachromosomal arrays would be to create transgenic lines by direct insertion of transgenes into chromosomes. Unfortunately, the ease with which extrachromosomal arrays are formed and maintained in *C. elegans* has made it difficult to identify less frequent events such as chromosomal insertion of transforming DNA. One solution to this problem has been to inhibit array formation by including a "poison sequence." For example, transgenes containing the *C. elegans* suppressor tRNA gene *sup-7* are unable to form high-copy extrachromosomal arrays after injection of transgene DNA into either germline cytoplasm or oocyte nuclei because the *sup-7* gene is toxic when present in high copy number (Fire 1986; Mello *et al.* 1991). Injection of *sup-7*-containing plasmids directly into oocyte nuclei, however, can be used to create low-copy integrated lines with 1–10 copies of the transforming construct inserted into a chromo-

Corresponding author: Judith Austin, Department of Molecular Genetics and Cell Biology, University of Chicago, 920 E. 58th St. Chicago, IL 60637 E-mail: jaustin@midway.uchicago.edu

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some (Fire 1986; Spieth *et al.* 1988; Fire and Waterston 1989). Low-copy integrated lines have also been obtained by germline injection of high concentrations of oligonucleotides along with the transforming plasmid (Mello *et al.* 1991). For both of these methods, however, the low frequency of integrated lines obtained, relative to the number of germline injections, has prevented their general use. In a different approach, γ-irradiation has been used to integrate extrachromosomal arrays into *C. elegans* chromosomes (Mello and Fire 1995), but the high-copy-number DNA in these integrated arrays can still produce elevated levels of transgene expression and gene silencing (Krause *et al.* 1994; Hsieh *et al.* 1999).

The difficulty of making low-copy-number integrated lines in *C. elegans*, coupled with the mosaicism and silencing observed in extrachromosomal array lines, has restricted the analysis of gene function in *C. elegans*. Experiments first carried out by A. Rushforth and P. Anderson (personal communication), and more recently by Wilm *et al.* (1999) and Jackstadt *et al.* (1999), have shown that microparticle bombardment can be used to create extrachromosomal arrays in *C. elegans*. In this article, we describe the use of microparticle bombardment to create low-copy integrative transformants in *C. elegans*. We find that integrated lines created using this approach typically contain only a few copies of the transforming DNA and can be used to express transgenes in both the *C. elegans* germline and soma.

MATERIALS AND METHODS

Strains: We used the following strains: LGI, *dpy-5(e61)*, *dpy-14(e188)*; LGII, *dpy-10 (e128)*; LGIII, *unc-119(ed3)*, *dpy-18(e364)*, *dpy-1(e1)*, *dpy-17(e164)*; LGIV, *dpy-4(e1166)*, *dpy-13(e184)*; LGV, *dpy-11(e224)*, *sma-1(ru3)*; LGX, *dpy-6(e14)*. AZ188 [*unc-119(ed3)III*; *azEx1*(pDP#MM016b; pAZ75)] contains an extrachromosomal array created by germline coinjection of pDP#MM016b and pAZ75 [pAZ75 contains 11 kb of *sma-1* genomic DNA cloned into BS(SK+)]. pOK100.03 contains a multimerized *myo-2 C* subelement enhancer oligo fused to a *myo-2* minimal promoter and green fluorescent protein (GFP; Thatcher *et al.* 1999). OK0023(*cuEx16*) contains an extrachromosomal array of pOK100.03 and pRF4 *rol-6(su1006)*; OK0039(*cuIs2)IV* contains an integrated array created by γ-irradiation of OK0023; both strains were kindly provided by P. Okkema and A. Fernandez. DP132*(edIs6)IV* carries an integrated array of the UNC-119::GFP fusion pDP#MMUGF12. SS599 [bnEx2(pJH4.52 *pie-1*::GFP::H2B, pRF4 *rol-6(su1006)*; N2 genomic); S. Strome, J. Powers, M. Dunn, K. Reese, G. Seydoux and W. Saxton, personal communication] carries a complex array containing the pJH4.52 plasmid [a GFP::H2B(F54E11.4) fusion that is expressed in the adult germline under control of *pie-1* promoter and 3′ untranslated region sequences; G. Seydoux, personal communication]. Strains produced in this study are listed in Table 1. The transformation plasmid pAZ110, used to create integrated lines that express a GFP transgene under control of the *sma-1* promoter, was constructed using pPD95.79 (A. Fire, S. Xu, J. Ahnn and G. Seydoux, personal communication). *unc-119(ed3)* mutants used for bombardment were grown at 20° on 100 mm Opti-gro plates [nemotode growth media (NGM plates; Lewis and Fleming 1995) supplemented with cholesterol, peptone, and Nystatin] seeded with OP50 *Escherichia coli*.

TABLE 1

Integrated transformants generated using microparticle bombardment

Strain	Transforming plasmid	Genotype[a]
AZ60	pAZ81	*ruIs1 IV*
AZ61	pAZ81	*ruIs2/+*[b]
AZ62	pAZ81	*ruIs3 IV*
AZ63	pAZ81	*ruIs4*
AZ64	pAZ81	*ruIs5/+*[b]
AZ65	pAZ81	*ruIs6/+*[b]
AZ66	pAZ81	*ruIs7/+*[b]
AZ68	pAZ81	*ruIs9*
AZ69	pAZ81	*ruIs10/+*[b] *III*
AZ71	pAZ81	*ruIs12*
AZ173	pDP#MM016b	*ruIs59*
AZ199	pDP#MM016b	*ruIs25 V*
AZ200	pDP#MM016b	*ruIs26 III*
AZ204	pDP#MM016b	*ruIs28*
AZ205	pDP#MM016b	*ruIs27 I*
AZ206	pDP#MM016b	*ruIs29*
AZ210	pAZ132	*ruIs30*
AZ211	pAZ132	*ruIs31*
AZ212	pAZ132	*ruIs32 III*
AZ213	pAZ132	*ruIs33/+*[b] *V*
AZ214	pAZ132	*ruIs34/+*[b] *I*
AZ215	pAZ132	*ruIs35*
AZ216	pAZ132	*ruIs36*
AZ217	pAZ119	*ruIs37 III*
AZ218	pAZ119	*ruIs38 III*
AZ219	pAZ110	*ruIs39*
AZ220	pAZ110	*ruIs26*

[a] All integrative transformants produced for this study are in an *unc-119(ed3)* background.

[b] These lines are propagated as obligate heterozygotes; animals homozygous for the transgene insertions in these lines are either inviable or sterile.

Bombardment methods: Microparticle bombardment of *C. elegans unc-119(ed3)* hermaphrodites was carried out using a BioRad Biolistic PDS-1000/HE with 1/4″ gap distance, 9 mm macrocarrier to screen distance, 28 inches of Hg vacuum, and 1350 p.s.i. rupture disc. These settings were based on a protocol for microparticle bombardment of Chlamydomonas (L. Mets, personal communication); alternative settings were not extensively tested.

For each bombardment, 1 μl of 1–2-μg/μl plasmid DNA was coupled to 0.6 mg of 1.0-μm microcarrier gold beads, as described in the PDS-1000/HE user's manual, and bombarded onto a monolayer of ∼10,000 *unc-119(ed3)* L4 and adult hermaphrodites (a 75-μl pellet) placed on a 20-mm diameter lawn of OP50 on 60-mm NGM plates. Worms were allowed to recover for 0.5 to 2 hr after bombardment and were then transferred onto two 100-mm seeded Opti-gro plates and grown at 24°. Because *unc-119* mutants cannot form dauers, they die in the absence of food (Maduro and Pilgrim 1995), making it easy to identify the non-Unc rescued transformants 7–14 days after bombardment. From each plate containing animals rescued for the *unc-119* mutation, individual transformed animals were cloned and their F_1 progeny scored for presence of *unc-119* mutants. Homozygous stable lines were identified by the complete absence of *unc-119* mutant progeny over several generations. Heterozygous lines were identified based on the presence of three distinct classes of progeny: heterozygous transformed animals, homozygous untransformed animals, and a third class of sterile or inviable animals. To ensure that each line was the result of an independent transformation event, we retained only one transformed line from each Opti-gro plate.

Mapping integrative transformants: Chromosomal location of integrated DNA in pDP#MM016b and pAZ81 transformed lines was determined using single worm PCR (Williams *et al.* 1992) to assay linkage between integrated Bluescript vector sequences and marker mutations on each chromosome. PCR primers 5′GGCCCCAGTGCTGCAATGATAC3′ and 5′AACACTGCGGCCAACTTACTTCTGA3′ were used to assay the presence of Bluescript sequences. For lines transformed with pAZ132 and pAZ119, chromosomal location of the integrated DNA was determined by linkage of GFP expression to marker mutations.

To map autosomal insertions, transformed hermaphrodites were crossed with marker/+ males; F_1 progeny were allowed to self-fertilize, and individual F_2 progeny homozygous for the marker mutation were scored for presence of Bluescript sequences or GFP expression. If unlinked, 75% of the marker mutation homozygotes should be positive for Bluescript or GFP expression in homozygous transformed lines; 67% of the marker mutation homozygotes should be positive for Bluescript or GFP expression in obligate heterozygous transformed lines. If the transforming DNA is linked to a marker mutation, the frequency of Bluescript- or GFP-positive animals will vary according to the map distance between the integrated DNA and the marker mutation. In this study, we considered a map distance of <25 cM between the marker mutation and the transforming DNA as evidence of linkage.

To test for presence of transforming DNA inserted into the X chromosome, hermaphrodites from homozygous transformed lines were crossed to wild-type males and the resulting F_1 males were crossed with *dpy-6* hermaphrodites. If the integrated DNA is unlinked to the X chromosome, 50% of the F_2 hermaphrodite progeny from this cross should be Bluescript or GFP positive. If the integrated DNA is linked to the X chromosome, all of the F_2 hermaphrodite progeny should be Bluescript or GFP positive.

For two transgenic lines in which we initially mapped the integrated DNA to LGIII, we used the *unc-119(ed3)* mutation present in the background of the bombarded hermaphrodites as a second marker to confirm linkage to LGIII. We crossed transformed hermaphrodites to wild-type males, allowed the F_1 cross-progeny hermaphrodites to self, and cloned out non-Unc F_2 animals. If the integrated *unc-119* rescuing DNA is linked to LGIII, these F_2 animals will segregate 1/4 Unc progeny only when a recombination event has occurred between the *unc-119(ed3)* allele at the endogenous locus and the integration site of the transgenic *unc-119* rescuing DNA. Using this strategy, we found that for the heterozygous line AZ69, 19 out of 207 F_2 progeny were recombinant, corresponding to a distance of 10 cM between the *unc-119* locus and the integrated DNA. For the homozygous line AZ200, 20 out of 284 F_2 progeny were recombinant, corresponding to a distance of 7 cM between the *unc-119* locus and the integrated DNA. Since the original *unc-119* mutation could be re-isolated from these strains, these data also confirmed that *unc-119* rescuing activity in these transformants was not the result of gene conversion or reversion of the *unc-119(ed3)* mutation.

Southern blotting: Southern blots of *Hin*dIII or *Xba*I digested genomic DNA were carried out by standard techniques using the DIG/CPSD system (Boehringer Mannheim, Indianapolis) and hybridized to probes containing either the Bluescript vector sequence (Stratagene, La Jolla, CA) or the 5.7-kb fragment of *unc-119* genomic DNA from pDP#MM016b.

Examination of GFP expression patterns: GFP expression in transformed animals was examined using a Zeiss Axioplan microscope equipped with a 100X Plan-APOCHROMAT lens. In the case of extrachromosomal array lines, only animals positive for the cotransformation marker were scored for GFP expression. Presence or absence of variation in the level of GFP expression from animal to animal within a transformed line was determined by comparing GFP expression levels of individual animals within groups of 5–15 animals. Presence or absence of mosaicism in GFP expression patterns was determined by comparing GFP expression patterns of individual animals to the expected pattern of expression for each GFP expression construct.

RESULTS

Integrative transformation in *C. elegans* using microparticle bombardment: Microparticle bombardment, a technique in which DNA-coated beads are accelerated to high speeds, allowing them to penetrate cells of the target organism, has been used for transformation of plants, animals, and microorganisms (Klein *et al.* 1987, 1988; Christou *et al.* 1988; Kindle *et al.* 1989; Armaleo *et al.* 1990; Zelenin *et al.* 1991; Cassidy-Hanley *et al.* 1997). We reasoned that microparticle bombardment would have several advantages over germline injection for the creation and detection of chromosomal insertions in *C. elegans.* First, since each bead can deliver only a small amount of DNA to the *C. elegans* germline, the probability of creating large extrachromosomal arrays would be decreased. Second, we observed gold beads in both germline and oocyte nuclei of bombarded animals (data not shown), indicating that bombardment can introduce transgenic DNA directly into the nucleus, which may be essential for integrative transformation (Fire 1986). Finally, since a large number of animals are bombarded simultaneously ($\sim 10^4$/bombardment), even rare events such as chromosomal integration could be expected to occur at a detectable frequency.

To identify transformants, we used a selection based on rescue of the *unc-119(ed3)* mutant phenotype. *unc-119* animals are unable to form dauers (Maduro and Pilgrim 1995), an alternative larval stage that normally allows *C. elegans* to survive for months without food (Cassada and Russell 1975). As a result, *unc-119* mutants transformed with plasmids containing *unc-119* rescuing DNA (Figure 1) survive and reproduce while the untransformed animals starve and die. Although positive transformants from microparticle bombardment occur at a relatively low frequency in the total population of bombarded animals ($\sim 0.5 \times 10^{-4}$), this *unc-119*-based selection permits the surviving non-Unc transformed animals to be easily identified.

Previous work had shown that it was possible to generate low-copy integrated lines by injecting plasmids con-

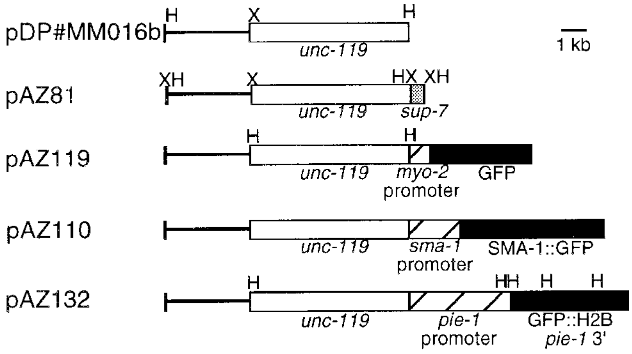

FIGURE 1.—Transforming plasmids. pDP#MM016b contains a 5.7-kb *Hin*dIII-*Xba*I fragment cloned into BS(SK−) that rescues the phenotype of *unc-119(ed3)* mutants (MADURO and PILGRIM 1995); this fragment was included in all plasmids used for transformation. pAZ81 is a derivative of pDP#MM016b that also contains the *sup-7* suppressor tRNA gene (FIRE 1986). pAZ119, derived from pOK100.03 (THATCHER *et al.* 1999), contains a multimerized *myo-2 C* subelement enhancer oligo fused to a *myo-2* minimal promoter and GFP. pAZ110 contains a SMA-1::GFP protein fusion that is expressed in the embryonic epidermis under the control of the *sma-1* promoter. pAZ132 contains a GFP::H2B protein fusion derived from pJH4.52, which is expressed in the germline under control of the *pie-1* promoter and localizes to chromosomes (G. SEYDOUX, personal communication). Solid line, plasmid vector: pAZ119 and pAZ132, BS(SK+); pDP#MM016b and pAZ81, BS(SK−); pAZ110, pCRII-TOPO. Open box, *unc-119* rescuing fragment. Box with diagonals, promoter. Solid box, GFP construct. Restriction enzyme sites that would be cut during digestion of genomic DNA for Southern blotting experiments described in this article are indicated. H, *Hin*dIII; X, *Xba*I.

taining the *sup-7* suppressor tRNA gene into oocyte nuclei (FIRE 1986; SPIETH *et al.* 1988). In these studies, the primary role of *sup-7* was as a cotransformation marker; however, it apparently also acted as a "poison sequence" to suppress formation of extrachromosomal array lines. Due to the difficulty of making transformed lines using this technique, however, this approach is rarely used at present. We reasoned that microparticle bombardment, coupled with the *unc-119* selection strategy, might provide an easier method for generating *sup-7*-containing integrated lines. We bombarded *unc-119* hermaphrodites with pAZ81, a plasmid containing both the *unc-119* rescuing fragment and the *sup-7* suppressor tRNA gene (Figure 1; MATERIALS AND METHODS).

From 36 bombardments with pAZ81, we obtained 10 independent transformed lines (Table 2). Five of these transformed lines segregated only non-Unc animals, suggesting that they were homozygous for a pAZ81 integrant. Each of the other 5 lines produced three types of progeny: transformed fertile animals, nontransformed Uncs, and a mixture of sterile animals, inviable larvae, and dead embryos. This segregation pattern suggested that these were heterozygous lines in which animals carrying one copy of a *sup-7*-containing insertion were able to reproduce, but that in animals homozygous for the insertion, the increased level of *sup-7* activity was toxic or lethal. Alternatively, these obligate heterozygous lines may be the result of an insertion into an essential gene or a DNA rearrangement associated with the insertion event (see below). These bombardments did not produce any transformed lines with the characteristic behavior of extrachromosomal arrays, indicating that presence of *sup-7* in the transforming plasmid provides a strong selection against the creation and/or maintenance of extrachromosomal arrays, similar to that observed for germline injections of *sup-7*-containing plasmids (FIRE 1986; SPIETH *et al.* 1988; MELLO *et al.* 1991).

Surprisingly, we found that inclusion of the *sup-7* suppressor tRNA was not essential for creation of stable lines using microparticle bombardment. From 17 bombardments of *unc-119* mutants with the *unc-119* rescuing plasmid pDP#MM016b, we isolated 6 independent lines that produced only non-Unc progeny (Table 2). The complete absence of untransformed animals in these transformed lines suggested that these stable lines were homozygous for a chromosomal insertion of the transforming plasmid. An additional 13 independent transformed lines isolated from this set of bombardments segregated both Unc and non-Unc progeny. Although, on the basis of their segregation patterns, these lines appeared to contain extrachromosomal arrays, it is possible that some of these lines contained chromosomal insertions of the transforming DNA, but that animals homozygous for the insertion were unable to reproduce.

TABLE 2

Frequency of integrative transformants using microparticle bombardment

Plasmid	Bombardments	Independent transformants	Integrated lines	Frequency per bombardment
pDP#MM016b	17	19	6	0.35
pAZ81	36	10	10	0.28
pAZ110	15	7	2	0.13
pAZ119	22	9	2	0.09
pAZ132	20	13	7	0.35
Total	110	58	27	0.25

Additional experiments have shown that microparticle bombardment can be used to produce stable transgenic lines in a consistent and reproducible manner. From a total of 110 bombardments using five different plasmids containing *unc-119* rescuing DNA (Figure 1), we obtained 27 stable homozygous or obligate heterozygous lines, which corresponds to a frequency of ~0.25 integrants per bombardment (Table 2). These results demonstrate that microparticle bombardment coupled with *unc-119* selection is a simple, efficient means of producing stable transformed lines.

Mapping sites of chromosomal integration: To confirm that the stable lines created by microparticle bombardment were the result of insertion of transgenic DNA into a chromosome, we mapped the location of the transforming DNA relative to known marker mutations (MATERIALS AND METHODS). For 11 stable lines, created using four different transforming plasmids, we found that each line mapped to a single linkage group (Table 3). In each case, the initial linkage group assignment was subsequently confirmed by recombination with a second marker mutation in the same linkage group. These results unambiguously demonstrate that the stability of these lines is due to integration of transgenic DNA into the *C. elegans* genome.

In the process of mapping these lines, we observed that integration can affect recombination in the region surrounding the site of integration. For two lines containing GFP-expressing DNA insertions, AZ213*(ruIs33/+)V* and AZ212*(ruIs32)III*, we found that there was an unexpectedly low frequency of recombination between the integrated transforming DNA and genetic marker mutations on the same chromosome (Table 3). In the progeny of *ruIs33/dpy-11* and *ruIs33/sma-1* hermaphrodites, 0 out of 36 animals homozygous for *dpy-11* (map position 0.0) were recombinant for *ruIs33*; 0 out of 73 animals homozygous for *sma-1* (map position +3.5) were recombinant for *ruIs33*. In the progeny of *(ruIs32)/dpy-17* and *(ruIs32)/dpy-18* hermaphrodites, only 1 out of 65 animals homozgyous for *dpy-17* (map position −2.2) and 0 out of 62 animals homozygous for *dpy-18* (map position +8.6) were recombinant for *ruIs32*. We calculated a lowest expected recombination frequency, based on the genetic map distance between each pair of genetic markers (*dpy-11* and *sma-1*, 3.5 map units; *dpy-17* and *dpy-18*, 10.8 map units), and used a chi-square test to determine if the recombination rates we observed were within normal statistical variance. For both AZ213*(ruIs33/+)V* and AZ212 *(ruIs32)III* we observed a statistically significant reduction in the recombination frequencies ($P < 0.01$).

It is likely that the decrease in recombination observed between AZ212 and AZ213 integrated DNAs and marker mutations are the result of DNA rearrangements associated with integration of the transforming DNA. Regions of decreased recombination have been observed for a variety of DNA rearrangements including

TABLE 3

Mapping stable transformants to single linkage groups

		pAZ81			pDP#MM016b			pAZ132			pAZ119	
Linkage group	Marker mutation	AZ60 cM (*n*)	AZ62 cM (*n*)	AZ69 cM (*n*)	AZ199 cM (*n*)	AZ200 cM (*n*)	AZ205 cM (*n*)	AZ212 cM (*n*)	AZ213 cM (*n*)	AZ214 cM (*n*)	AZ217 cM (*n*)	AZ218 cM (*n*)
I	*dpy-5*	U	U	U	U	U	6 (18)		U	5 (27)		
	dpy-14						9 (18)			8 (33)		
II	*dpy-10*	U	U		U	U	U	U	U	U	U	U
III	*dpy-18*	U	U	12 (18)	U	6 (18)	U	0 (62)			U	11 (70)
	unc-119			10 (207)		7 (284)						
	dpy-17							1 (65)	U	U	21 (43)	4 (28)
	dpy-1							19 (47)			5 (51)	24 (42)
IV	*dpy-13*	5 (10)	5 (10)		U	U	U					
	dpy-4	15 (21)	0 (10)									
V	*dpy-11*	U	U		6 (18)	U	U	U	0 (36)	U	U	U
	sma-1				16 (17)				0 (73)			
X	*dpy-6*	U	U		U	U						
Linkage group		*IV*	*IV*	*III*	*V*	*III*	*I*	*III*	*V*	*I*	*III*	*III*

Recombination frequency in centimorgans. U (unlinked) indicates a recombination frequency of >25 cM. See MATERIALS AND METHODS for description of crosses.

translocations, inversions, and deficiencies (Rosenbluth and Baillie 1981; McKim *et al.* 1988; Rosenbluth *et al.* 1990; Zetka and Rose 1992). Of the 27 stable lines obtained in this study, 7 are obligate heterozygous lines. These heterozygous lines may have resulted from direct insertion of DNA into essential genes or, in the case of lines transformed with pAZ81, from the presence of too many copies of the *sup-7* suppressor tRNA gene (Fire 1986). Alternatively, these obligate heterozygous lines may contain DNA rearrangements associated with the integrated DNA that result in homozygous transformed animals that are sterile or inviable.

Integrative transformants typically contain a small number of copies of the transforming DNA: We examined copy number of the transforming DNA in 22 integrated lines produced using microparticle bombardment. Genomic DNA from each line was digested with *Hin*dIII and hybridized to a Bluescript-specific probe on Southern blots (materials and methods; Figure 2). Digests of either pDP#MM016b or pAZ81 with *Hin*dIII produce a single 8.7-kb band containing Bluescript sequence, while *Hin*dIII digests of pAZ119 and pAZ132 produce 5.3-kb and 4.6-kb Bluescript-positive bands, respectively (Figure 1). Digestion of transforming DNA can also produce novel-sized bands as a result of plasmid concatemerization and rearrangement or plasmid breakpoints created during insertion into a chromosome.

Southern blots of genomic DNA from lines created by microparticle bombardment using the transforming plasmids pDP#MM016b, pAZ81, pAZ119, or pAZ132 showed that these integrated lines typically contain only a small number of copies of the transforming DNA. The pDP#MM016b transformed lines AZ200 and AZ205 contained two and three low-intensity DNA bands, respectively, indicating the presence of only a few copies of the plasmid (Figure 2A, lanes 1 and 3); similar patterns containing two or three DNA bands were also found in AZ173, AZ204, and AZ206 (data not shown). Analysis of the 10 lines created by bombardment with the pAZ81 *sup-7*-containing plasmid indicated that they also contain only a small number of copies of the transforming DNA (Figure 2B, lanes 1–3 and data not shown). Similar results were observed for lines created by transformation with pAZ119 or pAZ132 (Figure 2C, lanes 1 and 2; Figure 2D, lanes 1–4). These data clearly demonstrate that these stable lines do not carry extrachromosomal arrays, which would contain one to two orders of magnitude more copies of the transforming DNA (Stinchcomb *et al.* 1985; Fire and Waterston 1989; Mello *et al.* 1991; MacMorris *et al.* 1994). Out of 22 stable lines examined, we observed a high number of copies of the transforming plasmid in only 1 line, AZ199 (Figure 2A, lane 2). Since the transforming DNA in this line mapped to LGV (Table 3), we know that it is integrated into the chromosome. It is possible that the unusually high plasmid copy number in AZ199 is the result of a two-step process in which an extrachromosomal array was formed and then integrated into the chromosome.

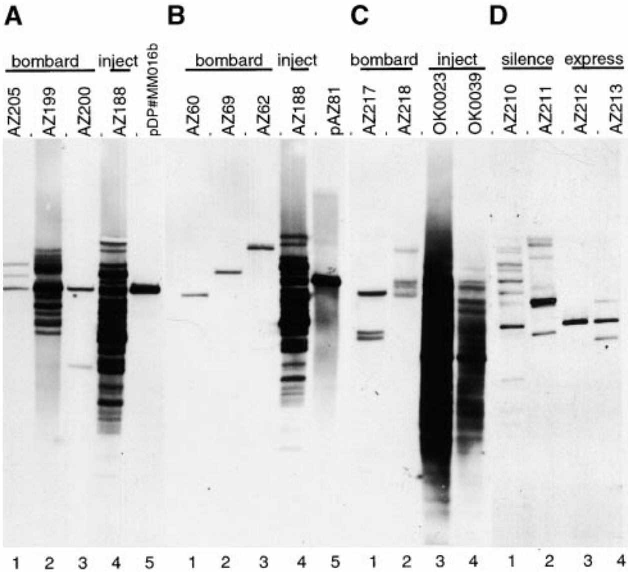

Figure 2.—Plasmid copy number in transformed lines. Genomic and plasmid DNAs were digested with *Hin*dIII and hybridized to a probe containing the full sequence of Bluescript (materials and methods). (A) Homozygous stable lines created by microparticle bombardment with pDP#MM016b (lanes 1–3); AZ188, a transformed line carrying an extrachromosomal array created by germline coinjection of pDP#MM016b and pAZ75 (lane 4); pDP#MM016b (lane 5). (B) Homozygous lines AZ60 and AZ62 (lanes 1 and 3) and a heterozygous line AZ69 (lane 2) created by microparticle bombardment with pAZ81; AZ188 (lane 4); pAZ81 (lane 5). (C) AZ217 and AZ218 (lanes 1 and 2), created by microparticle bombardment, contain only a few copies of the pAZ119 *myo-2* promoter::GFP expression construct. The extrachromosomal array line OK0023 (lane 3) and the integrated array line OK0039 (lane 4), which contain the same *myo-2* promoter::GFP construct, have a much higher number of copies of the transforming DNA. (D) Lines created by microparticle bombardment of the *pie-1* GFP::H2B fusion plasmid pAZ132. Lines AZ210 and AZ211 (lanes 1 and 2), which are silenced in the germline, have a more complex digest pattern and contain more copies of the transforming plasmid pAZ132 than lines in which germline expression is maintained (AZ212 and AZ213, lanes 3 and 4).

In at least two cases, we have obtained transformed lines that contained only a single copy of the transformation plasmid. Southern blots of *Hin*dIII-digested DNA from AZ60 and AZ69, which were transformed with the *sup-7*-containing plasmid pAZ81, each contained a single band distinct in size from the 8.7-kb band observed when pAZ81 is digested with *Hin*dIII (Figure 1; Figure 2B, lanes 1 and 2). These data are consistent with the insertion of a single copy of pAZ81: if multiple copies of pAZ81 were present in this line, there would be multiple *Hin*dIII sites in the transgenic DNA (Figure 1), which would create a more complex digest pattern. We confirmed this result using Southern blots of *Xba* I-digested AZ60 and AZ69 DNA. Hybridizing these blots with either

Bluescript- or *unc-119*-specific probes also produced only a single band for each line (data not shown).

In contrast to the small number of copies of transforming DNA in integrated lines produced by microparticle bombardment, we found a much higher level of the transforming DNA in extrachromosomal and integrated arrays produced using germline injection. Southern blot analysis of AZ188, which contains an extrachromosomal array containing the *unc-119* gene, and of DP132, which contains an integrated array of an UNC-119::GFP protein fusion (MATERIALS AND METHODS), show that these lines both contain many copies of the transforming DNA (Figure 2A, lane 4 and data not shown). Similarly, we found that OK0023, which carries an extrachromosomal array containing the pOK100.03 *myo-2* promoter::GFP expression construct, and OK0039, which contains an integrated array derived from OK0023, both contain a high number of copies of the transforming DNA (Figure 2C, lanes 3 and 4).

Stable transgene expression using low-copy integrated lines: In extrachromosomal array lines created by germline injection, the expression pattern of GFP and LacZ reporter constructs can vary from animal to animal due to mosaic loss of the extrachromosomal array and silencing of transgene expression (STINCHCOMB *et al.* 1985; OKKEMA *et al.* 1993; KRAUSE *et al.* 1994; KELLY *et al.* 1997; HSIEH *et al.* 1999). To determine whether stable lines created using microparticle bombardment would have a more consistent pattern of transgene expression, we bombarded *unc-119* animals with pDP#MM016b plasmid derivatives containing either *sma-1* or *myo-2* promoter constructs fused to GFP (Figure 1; MATERIALS AND METHODS). In both cases, we were able to isolate stable homozygous lines (Table 2) and found that animals from these lines consistently expressed GFP in the expected patterns (Figure 3A and data not shown).

We compared the GFP expression of these stable integrated lines to transformed lines produced by germline injection of the same GFP expression constructs. We found that the level of GFP expression in transformed animals carrying an extrachromosomal array containing the *myo-2* promoter::GFP expression construct varied from animal to animal and that 42% of the transformed animals had mosaic patterns of GFP expression. In contrast, the integrated array OK0039, and the low-copy integrated lines AZ217 and AZ218, which contain the same *myo-2* promoter::GFP construct, did not exhibit variation in the level or pattern of GFP expression. Similar results were also obtained in a comparison of extrachromosomal arrays and stable integrated lines containing the *sma-1* promoter::GFP fusion (data not shown).

Although the integrated array lines we have examined exhibit a consistent GFP expression pattern, the level of GFP expression does not appear to accurately reflect the number of transgene copies present in the array. Southern blots of the integrated array line OK0039 showed that this line contained a >10-fold increase in the copies of the transforming DNA relative to the integrated bombardment lines AZ217 and AZ218 (Figure 2C, compare lanes 1 and 2 with lane 4). In contrast, the level of GFP expression in OK0039 animals was only ~2-fold higher than that of AZ218 and 4-fold higher than that of AZ217. Some of the decreased level of GFP expression per transgene copy may be due to partial or rearranged transgenes unable to express GFP. In addition, however, it is likely that the reduced GFP expression per transgene copy in integrated arrays is the result of gene silencing and/or limits on protein synthesis in the cells where it is expressed (MACMORRIS *et al.* 1994).

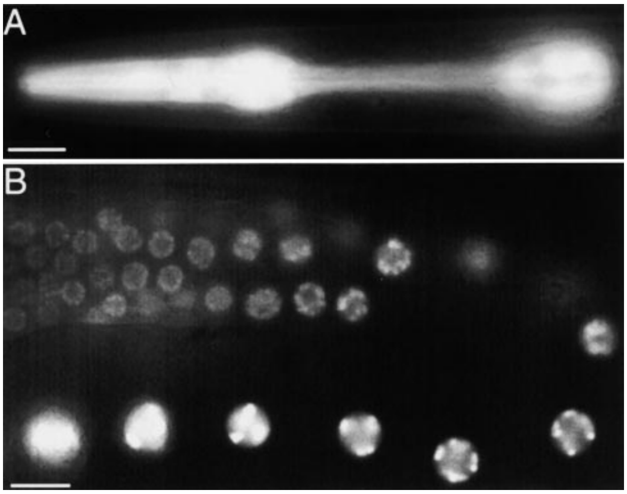

FIGURE 3.—Expression of GFP reporter fusions in stable lines created by bombardment. (A) GFP expression in pharynx of an AZ218 L1 larva. AZ218 is a stable line created by bombardment with the *myo-2* promoter::GFP plasmid pAZ119 (Figure 1; MATERIALS AND METHODS); uniform expression of GFP throughout the pharynx was observed for all animals examined for >20 generations. (B) Germline expression of GFP::H2B fusion protein in an AZ212 adult hermaphrodite. AZ212 is a stable line created by bombardment with pAZ132 (Figure 1; MATERIALS AND METHODS); consistent germline expression of GFP was observed in all animals examined for >20 generations. Bar, 10 μm.

Germline expression of transgenes using low-copy integrated lines: The most dramatic example of gene silencing in *C. elegans* is seen in the germline, where transgenes in conventional extrachromosomal arrays are not expressed (KELLY *et al.* 1997; KELLY and FIRE 1998; SEYDOUX and STROME 1999). Germline silencing may be the result of heterochromatic packaging of the repetitive sequences in extrachromosomal arrays. This hypothesis is supported by the requirement for functional *mes-2* and *mes-6* genes, which encode proteins related to the polycomb group of transcriptional repressors, to maintain germline silencing of extrachromosomal array transgene expression (KELLY and FIRE 1998; SEYDOUX and STROME 1999).

If germline silencing results from the presence of tandemly repeated copies of transgenes in extrachromosomal arrays, we hypothesized that low-copy integrated lines would not be silenced. To test this hypothesis, we bombarded *unc-119* animals with pAZ132, a construct containing the *unc-119* gene and a GFP::H2B fusion driven by the *pie-1* promoter, which directs expression in the adult germline (Figure 1). From 20 bombardments, we obtained five lines that expressed GFP in the germline (Tables 1 and 2). In one line, AZ212, all of the animals expressed GFP (Figure 3B). Two other lines, AZ213 and AZ214, segregated Uncs, GFP-expressing rescued animals, and dead embyos/inviable larvae in a ratio of 1:2:1; these appear to be obligate heterozygous lines. In all three of these lines, we have observed robust GFP expression for >20 generations. In contrast, animals from the other two homozygous lines, AZ210 and AZ211, were unhealthy and lost both GFP expression and *unc-119* rescuing activity over several generations. Two additional pAZ132 lines were rescued for *unc-119*, but failed to express the transgene, possibly due either to germline silencing or disruption of the *pie-1*::GFP::H2B transgene.

Using Southern blots, we found that the silenced lines AZ210 and AZ211 contained more copies of the PAZ132 transforming plasmid, as determined by complexity of the digest pattern, than AZ212 and AZ213, which continued to express GFP over many generations (Figure 2D, compare lanes 1 and 2 with lanes 3 and 4). This result indicates that the decrease in GFP expression observed for AZ210 and AZ211 is unlikely to be due to a loss of copies of the integrated transgene. We propose instead that in AZ210 and AZ211 the presence of a higher number of copies of the transgene results in silencing of the inserted transgenic DNA, while the lower number of transgene copies in AZ212 and AZ213 does not activate germline silencing.

Germline silencing can be alleviated by creating complex arrays that intersperse genomic and transgene DNA (Kelly *et al.* 1997). Although both complex arrays and low-copy integrated lines can be used for germline expression, a comparison of the two types of transformed lines suggests that low-copy integrated lines created by microparticle bombardment have several advantages for germline expression of transgenes. In the case of the plasmid pJH4.52, which contains a GFP::H2B gene fusion expressed under control of the *pie-1* promoter (materials and methods), it has been difficult to obtain complex array lines that express the GFP transgene in the germline; lines that do express in the germline often lose expression in <5 generations(G. Seydoux, personal communication). In contrast, three out of the five GFP::H2B-expressing lines that we obtained using microparticle bombardment have consistently expressed the GFP::H2B transgene in the germline for >20 generations.

When we compared the complex array line SS599, which has expressed the *pie-1* GFP::H2B gene fusion over many generations (S. Strome, personal communication), with the low-copy integrated line AZ212, which expresses the same *pie-1* GFP::H2B gene fusion, we observed several striking differences. Maintenance of the SS599 complex array line required growth at 25° and selection of GFP-positive animals in each generation. In this line, 26% of the animals expressed the Rol phenotype associated with the *rol-6(su1006)* cotransformation marker present in this array. A majority of the animals with a strong roller phenotype expressed the GFP::H2B transgene, although the expression pattern varied in some animals, suggesting that silencing and/or mosaic loss of the transgene was taking place. In contrast, AZ212 could be maintained at all temperatures between 15° and 25°, and 100% of the animals expressed GFP in a consistent pattern. The ease with which integrated lines can be obtained using microparticle bombardment and the stability of germline expression in these lines indicates that this technique is an improvement over currently available methods for germline expression of transgenes in *C. elegans.*

DISCUSSION

This study has demonstrated that microparticle bombardment is a simple and efficient technique for generating stable transgenic lines in *C. elegans.* We have found that a substantial proportion of the transgenic lines generated by microparticle bombardment contain a low number of copies of the transforming DNA integrated into a chromosome, resulting in stable transmission of the transgenic DNA over many generations. A critical factor in the success of this microparticle bombardment transformation strategy is the use of a selectable cotransformation marker to identify rare transformed animals within the population of bombarded animals and their descendants. For the experiments described in this article, we bombarded *unc-119* mutants with plasmids containing an *unc-119* rescuing fragment and were able to identify transformed animals based on their ability to survive starvation and on their non-Unc phenotype.

In some cases, the *unc-119* gene may be an unsuitable cotransformation marker due to interactions between the *unc-119* mutant phenotype and the transgenic DNA. In these instances it should be possible to use other selectable markers, such as temperature-sensitive mutants that are sterile or dead at the restrictive temperature. In preliminary experiments, we have found that the *dpy-20* gene can also be used to identify stable transformed lines created by microparticle bombardment (S. Kniss and J. Austin, unpublished results), confirming that it is not essential to use *unc-119* as the cotransformation marker.

The process by which chromosomal integration occurs in *C. elegans* has yet to be elucidated. Previous work had shown that creation of integrated lines containing the *sup-7* suppressor tRNA occurred only when the transforming DNA was injected directly into oocyte nuclei

(Fire 1986). Similarly, the ability to introduce transforming DNA directly into oocyte and germline nuclei by microparticle bombardment may be critical for successful integration of transgenic DNA. We originally predicted that inclusion of *sup-7* in the transformation plasmid would be essential for selection of low-copy integrants. We found, however, that we were able to obtain integrants using transforming DNA that did not contain *sup-7*, indicating this selection was not necessary. It is also clear that using *unc-119* as a cotransformation marker does not create a selection for low-copy transformation, because we have also been able to use it as a cotransformation marker in extrachromosomal arrays created both by germline injection and by microparticle bombardment. These results suggest that introduction of DNA into *C. elegans* by microparticle bombardment inherently favors the creation of low-copy chromosomal insertions.

The presence of nonrecombining DNA in some of our lines, likely due to rearrangements associated with the site of integration (Rosenbluth and Baillie 1981; McKim *et al.* 1988; Rosenbluth *et al.* 1990; Zetka and Rose 1992), suggests that integration occurs during the process of repairing double strand breaks. It is not clear whether or not microparticle bombardment plays a direct role in this process, creating double strand breaks or producing cell damage that induces DNA repair activity. In our experiments, the location of integration sites for each of our transforming plasmids appears to occur at random; we identified integration events on four different chromosomes (I, III, IV, and V) and in some cases have identified integration sites at different locations on the same chromosome. It should be noted, however, that 5 of the 11 identified integration sites map to LGIII, which is also the location of the cotransformation marker gene *unc-119*. Additional experiments will be required to determine if there are favored sites for chromosomal integration and, if so, whether sequences in the transforming plasmid play a role in determining the site of integration for the transforming DNA.

Using microparticle bombardment, we generated lines that express GFP transgenes in reproducibly consistent patterns in somatic tissues. In extrachromosomal array lines, expression patterns can vary from animal to animal due to mosaic loss of the array (Stinchcomb *et al.* 1985). In addition, it has been observed that lines containing extrachromosomal arrays with the same transgenic DNA can vary widely in their level of gene expression relative to gene copy number (MacMorris et al. 1994). These variations in the level of gene expression are likely the result of context-dependent gene silencing, in which expression of tandemly repeated sequences is repressed (Kelly *et al.* 1997). Mutations in the *tam-1* gene result in hyper-silencing of both extrachromosomal and integrated arrays, while endogenous loci and complex arrays, which intersperse genomic and transgene DNA, are able to express (Hsieh *et al.* 1999). This correlation between transgene copy number and silencing indicates that gene silencing is regulated in a copy-number-dependent manner. We have found that in our low-copy integrated lines that express GFP transgenes, the level and pattern of GFP expression does not vary from animal to animal. We propose that in these lines, the number of transgene copies is insufficient to activate context-dependent gene silencing in the soma. As a consequence, the level of transgene expression in these lines should more accurately reflect gene copy number. Use of low-copy integrated lines should permit expression of transgenes that are toxic when overexpressed as well as a more accurate analysis of protein function in cases where overexpression may alter the localization or regulation of the protein gene product.

We have found that low-copy integrated lines can be used to express transgenes in the *C. elegans* germline as well as in somatic tissues. Of five lines created using microparticle bombardment that initially expressed a *pie-1*-GFP expression construct in the adult germline, three lines have continued to express the GFP transgene for >20 generations. In the other two lines, we have observed silencing of both *unc-119* rescuing activity and transgene expression. Interestingly, the two silenced lines have only a slightly higher number of copies of the transgene than the lines that have continued to express the *pie-1*-GFP expression construct. This correlation between transgene copy number and germline expression suggests not only that the germline has a very low threshold for multicopy sequences but also that it is able to discriminate relatively small differences in transgene copy number. This sensitivity of the germline to copy number, due to germline-specific gene silencing mechanisms (Seydoux and Strome 1999), helps to explain why it has been difficult to generate lines that can consistently express transgenes in the germline. The ability to generate stable lines with consistent germline expression by microparticle bombardment represents an improvement over current methods and should increase the ability of researchers to investigate the regulation of germline development and function.

The ability to create low-copy integrated lines will dramatically increase the approaches available for analysis of gene expression and function in *C. elegans.* Low-copy integrated lines should express at levels close to that of the corresponding endogenous genes, allowing expression patterns and protein function to be determined more precisely. Previous work has shown that integration of transforming DNA via homologous recombination can occur in *C. elegans* (Broverman *et al.* 1993). Although integration by homologous recombination has been only rarely observed in *C. elegans,* a reliable method for chromosomal integration of DNA may be an important first step toward making homologous recombination a usable tool in this organism. Similarly, the ability to create integrated lines should make it possible to use enhancer traps to identify gene expression patterns, a powerful approach for gene analysis that has previously been hampered by the inability to integrate reporter constructs into the *C. elegans* genome.

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