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Homologous gene targeting in *Caenorhabditis elegans* by biolistic transformation

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ABSTRACT

Targeted homologous recombination is a powerful approach for genome manipulation that is widely used for gene alteration and knockouts in mouse and yeast. In *Caenorhabditis elegans*, several methods of target-selected mutagenesis have been implemented but none of them provides the opportunity of introducing exact predefined changes into the genome. Although anecdotal cases of homologous gene targeting in *C.elegans* have been reported, no practical technique of gene targeting has been developed so far. In this work we demonstrate that transformation of *C.elegans* by microparticle bombardment (biolistic transformation) can result in homologous recombination between introduced DNA and the chromosomal locus. We describe a scaled up version of biolistic transformation that can be used as a method for homologous gene targeting in the worm.

INTRODUCTION

Homologous recombination between exogenous and chromosomal DNA, mediated by the cell's repair and recombination machinery, provides a means for precise alterations of a target locus and is referred to as gene targeting. For years homologous gene targeting has been a method of choice for gene knockout and modification in such model organisms as mouse (1), yeast and *Escherichia coli*. Recently this technique was also developed for *Drosophila melanogaster* (2). Yet in another powerful model organism, the nematode *Caenorhabditis elegans*, a few cases of gene alteration via homologous recombination have been reported but no standard protocols have been established (3).

The success of *C.elegans* as a model organism in biological research is attributed to a number of biological and technical properties of the worm, such as short generation time, fixed cell lineage, transparent body, ease of maintenance and cryopreservation, sequenced genome and a wide spectrum of tools for genome manipulation. Besides the classic forward genetics approach for isolation of *C.elegans* mutants obtained by radiation, chemical or insertional mutagenesis (4), there are several reverse genetics methods of isolating gene knockouts in the worm. These target-selected mutagenesis approaches rely on screening large mutant libraries produced by either transposon insertions (5,6) or chemical mutagenesis (7,8). In addition, dsRNA-mediated RNAi can be used to generate a hypomorphic phenocopy of a mutant phenotype, thus allowing rapid examination of possible gene function (9,10). None of the above methods, however, is capable of introducing exact predefined changes into the target genome sequence, which would be a desirable approach in many cases. Therefore development of a homologous gene targeting technique for *C.elegans* would be a valuable addition to the existing genetic toolkit.

One of the limiting steps in homologous gene targeting is the introduction of donor DNA into the germ-line cells of the organism. In *C.elegans*, microinjection of DNA into the cytoplasm of the syncytial part of the gonad results in formation of extrachromosomal high-copy-number arrays of injected DNA, leading to germ-line transformation (11,12). These extrachromosomal arrays can be further integrated in random genomic loci by γ irradiation of transformed progeny (13). Co-injection of single-stranded oligonucleotides along with the transforming plasmid can stimulate non-homologous integration of the injected DNA and formation of low-copy-number integrated arrays (12). Alternatively, direct integrative transformation is possible through injection of plasmids containing the *sup-7* gene into meiotic oocyte nuclei rather than into the syncytial cytoplasm (14). *sup-7* is a suppressor tRNA gene and serves as a transformation marker and as a selection against high-copy-number arrays, since high levels of *sup-7* product are toxic.

The principal possibility of homologous recombination between introduced and chromosomal DNA in *C.elegans* was demonstrated a decade ago by Broverman *et al*. (3), who used the integrative transformation approach to study regulation of the *vit-2* gene. During the course of their work the authors produced 63 integrated *C.elegans* lines by injecting reporter constructs into nuclei of meiotic oocytes and discovered that two of these lines were homologous integrants as opposed to random integration of the transgene in the rest of the lines. The authors suggested that integrative transformation should be considered as a method of gene inactivation in *C.elegans* since

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the frequency of homologous recombination was a reasonable 3% of all integration events. The method has not gained use, however, probably because microinjection of DNA into meiotic oocyte nuclei is a laborious technique that requires substantial skills from a researcher (much more than the injection into the large syncytial gonad, commonly done for transgenesis).

Recently an alternative method of *C.elegans* transformation has been developed which relies on microparticle bombardment for delivery of DNA into the gonad rather than on microinjections (15,16). In this method, biolistic transformation, transgene DNA is first coated on gold microparticles and then microparticles are shot into the worms by means of helium pressure. Although the absolute efficiency of biolistic transformation (i.e. number of transformed progeny relative to the total number of bombarded worms) is low, strong selection markers allow easy identification of transformants (16). The bombardment procedure itself is a straightforward and scalable technique. The important difference between conventional microinjection and biolistic transformation is that the latter can produce both extrachromosomal and low-copy-number integrated lines. In this respect biolistic transformation as an entry route for DNA may be more similar to intranuclear injection, as performed by Broverman *et al*. (3), than to syncytial injection. Whether the transformant would be extrachromosomal or integrated is a stochastic process that probably depends, among other factors, on the penetrance of the microparticles into the germ-line nuclei. Praitis *et al*. (16) report that depending on the nature of the construct ~9–35% of transformants obtained by microparticle bombardment are integrated low-copy-number transgenic lines.

The data from Broverman *et al*. (3) and Praitis *et al*. (16) on frequencies of homologous recombination and integrative transformation events suggest that microparticle bombardment could possibly be used for homologous gene targeting in *C.elegans*, since the limiting step in gene targeting is production of a large number of integrative transformants, among which rare homologous recombination events can then be detected. In this work we demonstrate that it is possible to produce homologous integrants by microparticle bombardment with sufficient frequency, and report a protocol for homologous gene targeting in *C.elegans*.

MATERIALS AND METHODS

Strains

The *unc-119*(*ed3*) strain was received from the Caenorhabditis Genetics Center.

DNA constructs

The pDPMM016b plasmid that rescues *unc-119*(*ed3*) mutant phenotype (17) was received from J. Austin. The *unc-22* ends-in construct was made by cloning the HindIII–XhoI fragment of the *unc-22* gene (contains exons 13–19, amplified from N2 genomic DNA with primers unc-22-1 unc-22-2) into HindIII and XhoI sites of pDPMM016b. Plasmid pRP2511 for Gateway cloning of PCR products into *unc-119*(+)-containing vector was made by ligating rfB adapter (Invitrogen) into pDPMM0160b plasmid. The *unc-22* ends-out construct was made in several amplification steps. First, two PCR fragments of the *unc-22* gene were amplified with primers unc-22-7-5 and unc-22-Afl-GWR or unc-22-Afl-GWF and unc-22-7. Next, two PCR products were mixed and reamplified with unc-22-Afl-GWF and unc-22-Afl-GWR primers. The resulting 6.4 kb PCR product was cloned into the plasmid pRP2511 by Gateway technology.

The *unc-54* ends-in construct was made by Gateway cloning of PCR product amplified with unc-54-GWF and unc-54-GWR primers into pRP2511 plasmid.

Biolistic transformation

We have developed a scaled up version of the bombardment protocol described by Praitis *et al*. (16) that utilizes *unc-119*(+) gene as a selection marker. The PDS-1000/He system with the Hepta adaptor (Bio-Rad) was used for microparticle bombardment with the following parameters: 4 mm distance between exits of the Hepta adaptor gas splitter and macrocarriers, 20 mm distance between stopping screens and target shelf, 1350 p.s.i. rupture disks and 28 inches of Hg vacuum. The distance between macrocarriers and stopping screen is 14 mm and cannot be changed in the Hepta adaptor.

Gold particles (0.3–3 μm, Chempur, Germany) were prepared as described in the PDS-1000/He user's manual: 60 mg of microparticles were weighed into a 2 ml tube, soaked for 15 min in 70% ethanol, washed three times in sterile water and resuspended in 1 ml of 50% sterile glycerol.

Plasmid DNA was linearized with appropriate restriction enzymes and restriction digests were used directly for coating the gold particles without prior purification. For one bombardment with Hepta adaptor, 50 μl of gold particles (60 mg/ml) were aliquoted into an Eppendorf tube, briefly centrifuged and supernatant removed. After that, the following components were added to the gold particles with resuspension by pipetting after each step: 50 μl of DNA (10–15 μg), 50 μl of 2.5 M $CaCl_2$ and 20 μl of 0.1 M spermidine. The mixture was incubated for 30 min on ice with periodic resuspensions, briefly centrifuged, washed in 300 μl of 70% ethanol followed by 1 ml of 100% ethanol, resuspended in 170 μl of 100% ethanol, and 20 μl of DNA-coated microparticles were spread onto each of seven macrocarriers. Bombardments were performed within 5–10 min after the macrocarriers had dried out.

We used egg plates (18) for growing large quantities of *unc-119*(*ed3*) worms. On average five egg plates are sufficient to grow worms for one bombardment. To prepare ~50 egg plates, yolks from 10 chicken eggs were separated into a sterile bottle and shaken vigorously, after which two volumes of LB were added. The mixture was incubated at 60°C for at least 1 h, cooled on ice and 40 ml of overnight OP50 culture were added. The mixture (5–8 ml) was distributed on 2% agar NGM plates (9 cm) and allowed to sediment overnight. Then the supernatant was decanted and plates were allowed to dry for another day before seeding with bleached *unc-119*(*ed3*) worms.

To prepare worms for a bombardment, egg plates with a mixed population of worms (7–10 days of growing) were washed with Egg Salts buffer (0.12 M NaCl, 0.05 M KCl) and allowed to sediment by gravity for 5–10 min in a 50 ml tube. The sedimentation procedure was repeated 3–5 times to remove most of the larvae and debris. Then most of the supernatant was removed and 2 ml of worms were spread on

the 9 cm dry NGM plate. The plate was incubated on ice for 10–30 min to allow most of the liquid to be absorbed by agar, and then subjected to bombardment.

After bombardment worms were left to recover at room temperature for 20–40 min, washed off with Egg Salts buffer and distributed onto 20–25 regular NGM plates. Inspection for survived transformants was performed 10–14 days after bombardment and worms with wild-type phenotype were singled out for further analysis. Only one worm per plate was selected to avoid redundancy and ensure independence of transformed lines.

Southern blot analysis

Genomic DNA was digested with HindIII or XbaI restriction enzymes for *unc-22* and *unc-54* homologous recombinant strains, respectively, electrophoresed and blotted onto Hybond-XL nylon membrane (Amersham Biosciences). The probes were made by PCR as shown in Figures 1A and 2A, and labeled with [^{32}P]dCTP using Megaprime DNA Labelling System (Amersham Biosciences). Hybridization and wash steps were carried out under high stringency conditions.

PCR analysis

Verification of homologous recombination events was performed by PCR with vector- and locus-specific primers. The following oligonucleotide combinations were used: unc-22-1/M13R and unc-119-seq/unc-22-2 for *unc-22* ends-in recombinants; unc-22-9/M13R for *unc-22* ends-out recombinant and unc-54-2/M13R for *unc-54* recombinant. All PCRs were performed with Expand Long Template PCR System (Roche) according to the manufacturer's protocol.

Genetic linkage analysis

Genetic analysis was performed for *unc-54* homologous recombinant strain. Hermaphrodite worms with a typical *unc-54* phenotype were crossed with N2 males, and F_2 progeny of both *unc-54* and wild-type phenotypes were analyzed by single-worm PCR (19) for the presence of the transgene using vector-specific primers amp-1 and amp-2.

Oligonucleotide sequences used: unc-22-1: TGGCGCTGGTGGACTGGAAAAGTC; unc-22-2: GGTTCTCCAGCCTTTGCTTCGATT; unc-22-7-5: CATCAAGGATCTTCGTCTTAAGTGTTGTACAAGAAATGATTGC; unc-22-AflGWR: ggggaccactttgtacaagaaagctgggtAAGTAATGCCAACGCCACAGTATG; unc-22-AflGWF: ggggacaagtttgtacaaaaaagcaggctAAGTGCCGATCCCAGGTTTGA; unc-22-7: CTTAAGACGAAGATCCTTGATG; unc-22-9: ACATTCTGGGAGTGGTTCTC; unc-54-GWR: ggggaccactttgtacaagaaagctgggtTCATTCTCGAGTTCCTCTTC; unc-54-GWF: ggggacaagtttgtacaaaaaagcaggctGCCTTGTAATCCTTGTAATC; unc-119-seq: GAAGAATTTAGATATGAGGTTA; M13R: AGCGGATAACAATTTCACACAGGA; amp-1: CATCGAACTGGATCTCAACA; amp-2: GCCTCCATCCAGTCTATTAA.

RESULTS

Scaling up of biolistic transformation

Since production of a large number of integrated lines may be necessary for isolating homologous recombination events, we first took several steps to scale up published biolistic transformation protocols. Biolistic transformation of *C.elegans* was first described by Wilm *et al*. (15) and Praitis *et al*. (16). The principal difference between the two protocols is that the first one uses an in-house-made device for microparticle bombardment while the second one utilizes for this purpose the PDS-1000/He system from Bio-Rad. Although efficiency of transformation is comparable in both protocols, in our study we used the PDS-1000/He device since it is easier to standardize and scale up. Several changes to the bombardment protocol of Praitis *et al*. (16) were made: (i) we used the Hepta adapter with PDS-1000/He, which allows coverage of an area of an entire 9 cm Petri dish in a single shot, thus bombarding a large number of worms at a time; (ii) we used egg plates for growing *unc-119*(*ed3*) worms since large amounts of worms are required for bombardments; (iii) all constructs were linearized prior to coating the gold particles because free DNA ends can stimulate homologous recombination. Using these modifications to the protocol we routinely obtained 10–18 independent transformants per bombardment. The fraction of integrated lines among transformants produced by our method is comparable to published data with an average of 30%.

Ends-in gene targeting

There are two principal configurations of vectors for gene targeting, which differ in the location of double-stranded breaks (DSB) relative to the region of homology (20). In insertion, or ends-in type vectors, DSBs are located within a region of homology (Fig. 1A) and homologous recombination results in insertion of the vector and partial duplication of the target locus. In replacement, or the ends-out type of vector, DSBs are located at the ends of the homologous region and in this case homologous recombination results in replacement of the target locus with vector sequence (see Fig. 3A).

We created ends-in constructs for targeting *unc-22* (Fig. 1A) and *unc-54* (Fig. 2A) genes that contain 4.4 kb long regions homologous to coding parts of the genes. The constructs were designed to contain unique restriction enzyme sites approximately in the middle of the region of homology so that the two homologous arms of equal length were created by linearization of the vectors with these restriction enzymes. *unc-22* and *unc-54* genes were selected as models to test homologous gene targeting in *C.elegans* because these genes have clear null phenotypes that can be recognized by quick and simple inspection of the worms. We produced 400 and 274 independent transformants for *unc-22* and *unc-54* constructs, respectively. Transformed progeny were examined under the microscope and three lines with *unc-22* phenotype and one line with *unc-54* phenotype were found (Table 1). PCR and Southern blot analysis confirmed that the mutant phenotype in all four lines was due to homologous recombination in the target gene (Fig. 1B and C; Fig. 2B and C). Genetic analysis of *unc-54* homologous recombinants also showed linkage between transgene and *unc-54* phenotype: among F_2 progeny of a cross between an *unc-54* homologous recombinant and N2 strains all 21 analyzed worms with the *unc-54* phenotype contained the transgene, whereas 10 out of 21 wild-type F_2 worms did not contain the transgene. All the 11 positive wild-type worms segregated F_3 progeny with the *unc-54* phenotype.

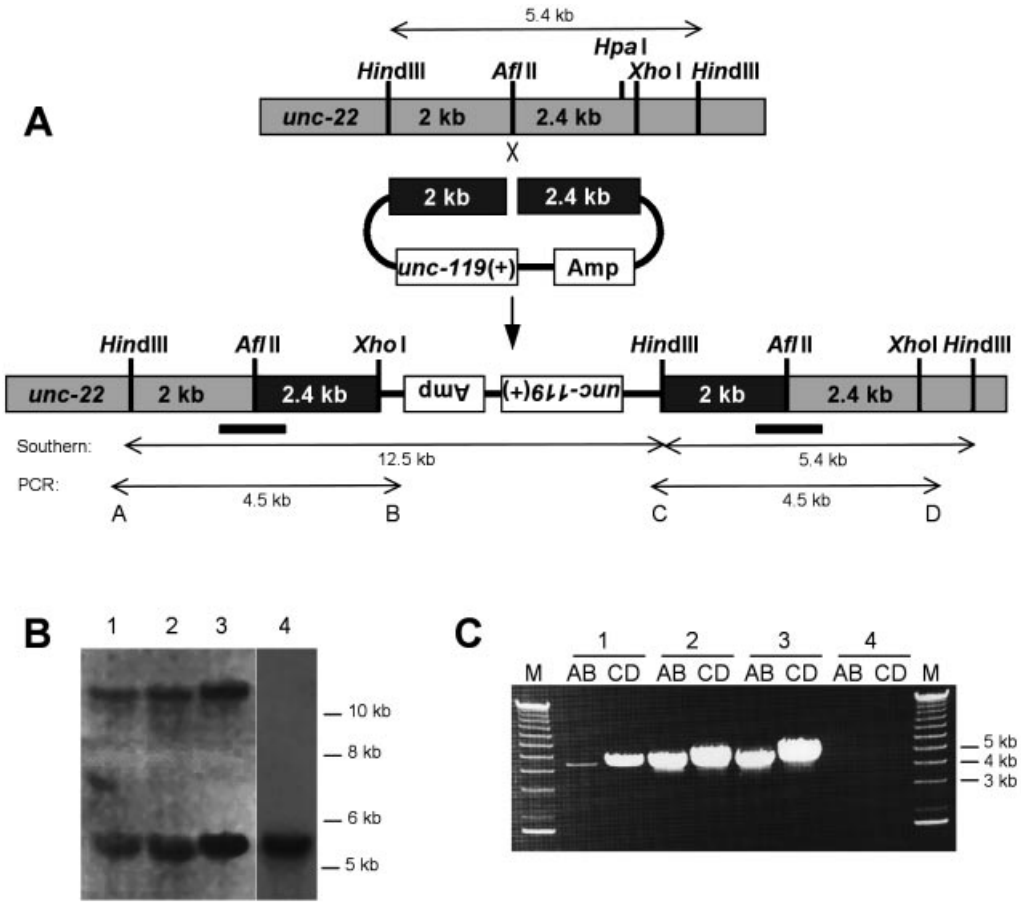

Fig. 1. Ends-in targeting of *unc-22* gene. (**A**) Vector design. Genomic sequence of *unc-22* is shown in light gray, homologous sequence included in the targeting vector is dark gray. Two-sided arrows indicate PCR fragments amplified with vector-specific primers B and C and *unc-22*-specific primers A and D located outside the regions of homology. Bold lines below AflII sites indicate positions of a probe for Southern blot analysis, and expected fragment lengths are shown below the probes. (**B**) Southern blot of *unc-22* homologous recombinant (lanes 1–3) and *unc-119*(*ed3*) (lane 4) strains. (**C**) PCR verification of homologous recombination events. Lanes 1–3, independent *unc-22* homologous recombinant strains; AB, CD, combination of primers (see A); lane 4, negative control on *unc-22* ends-in nonhomologous integrated line; M, marker line.

The observed frequency of homologous recombination was 0.75% of transformation events for the *unc-22* gene and 0.38% for the *unc-54* gene. We do not know how many of 400 and 274 transformants were integrated lines, but from separate experiments (data not shown) we know that the proportion of integrated lines produced by biolistic transformation is usually in the range of 10–50% of transformed lines. Therefore we can estimate that the frequency of homologous recombination events for *unc-22* and *unc-54* genes is ~0.7–7.5% of integration events. This estimate is in good agreement with the data of Broverman *et al*. (3), who reported a 3% frequency of homologous recombination among integrated lines created by injection of DNA into meiotic oocyte nuclei.

For the *unc-54* homologous recombinant line we observed spontaneous revertants after massive cultivation of the worms (estimated frequency of reversions is 10^{-5}–10^{-6}). Genetic analysis confirmed the homologous recombination nature of the reversions. The weak wild-type band in lane 1 of Fig. 2B can be explained by somatic reversions in the *unc-54* locus.

A targeting vector can be designed to contain homologous arms of unequal length with most of the homologous sequence concentrated in one arm and keeping the other arm short. This design simplifies screening for homologous recombination events by PCR. To test this approach in *C.elegans*, the ends-in *unc-22* construct was linearized by HpaI restriction enzyme instead of AflII, resulting in 4 and 0.4 kb arms (Fig. 1A). Among 275 transformants obtained with this vector we did not find a single line with the *unc-22* phenotype (Table 1). However, no definitive conclusion about targeting efficiency of the construct can be drawn from these numbers. Further experiments are required to establish the minimal length of the short arm in targeting constructs that can be reliably used for gene targeting in *C.elegans*.

Ends-out gene targeting

In insertional gene targeting, homologous recombination results in the duplication of the region of homology (Fig. 1A), which can be a disadvantage for production of gene knockouts in *C.elegans*. Since efficiency of gene targeting depends on the length of the homologous region in the targeting construct, at least a several kb long region should be included in the construct. But many worm genes are

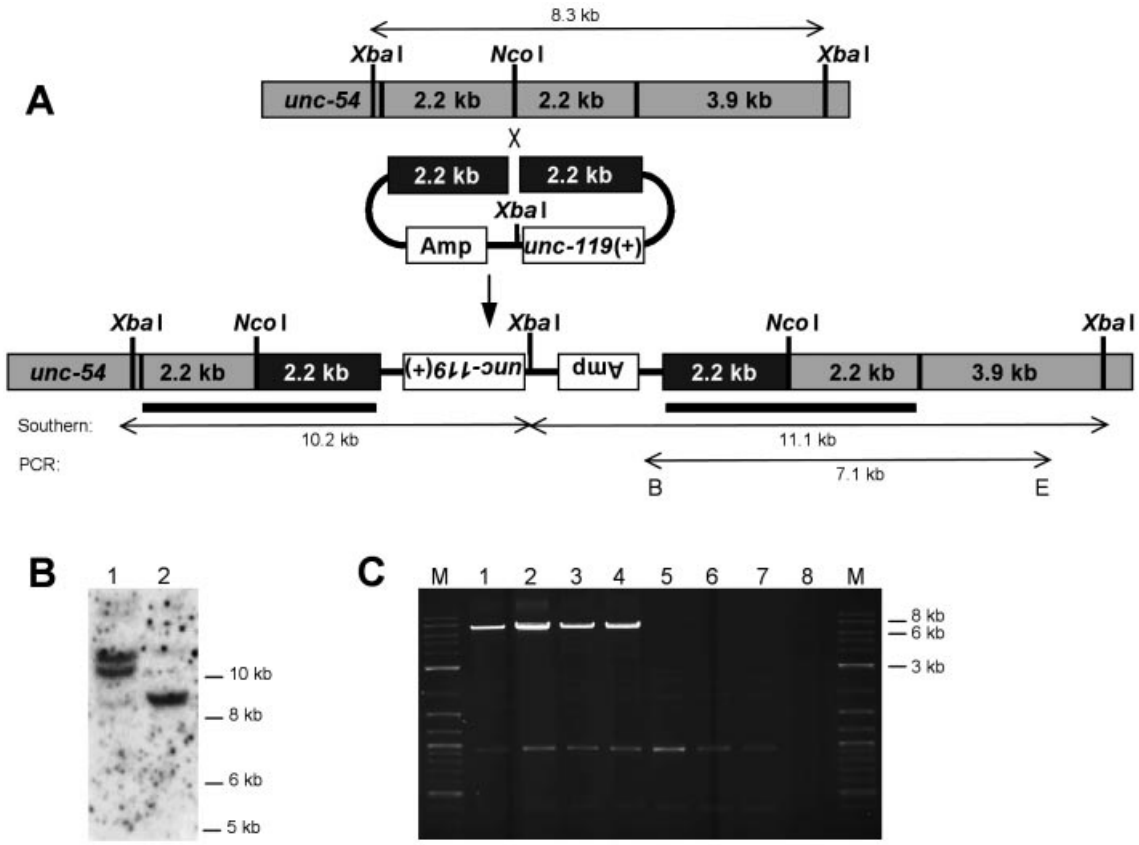

Fig. 2. Ends-in targeting of *unc-54* gene. (**A**) Vector design. Genomic sequence of *unc-54* is shown in light gray, homologous sequence included in the targeting vector is dark gray. Two-sided arrows indicate the PCR fragment amplified with vector-specific primer B and *unc-54*-specific primer E located outside the region of homology. Bold lines below NcoI sites indicate positions of a probe for Southern blot analysis, and expected fragment lengths are shown below the probes. (**B**) Southern blot of *unc-54* homologous recombinant (lane 1) and *unc-119*(*ed3*) (lane 2) strains. (**C**) PCR verification of homologous recombination events using primers B and E (see panel A). Lanes 1 and 2, homozygous *unc-54* recombinant worms (unc-54 phenotype); lanes 3 and 4, heterozygous *unc-54* recombinant worms (wild-type phenotype but segregate unc-54 progeny); lanes 5–7, independent non-homologous unc-54 transformants; lane 8, no DNA control; M, marker line.

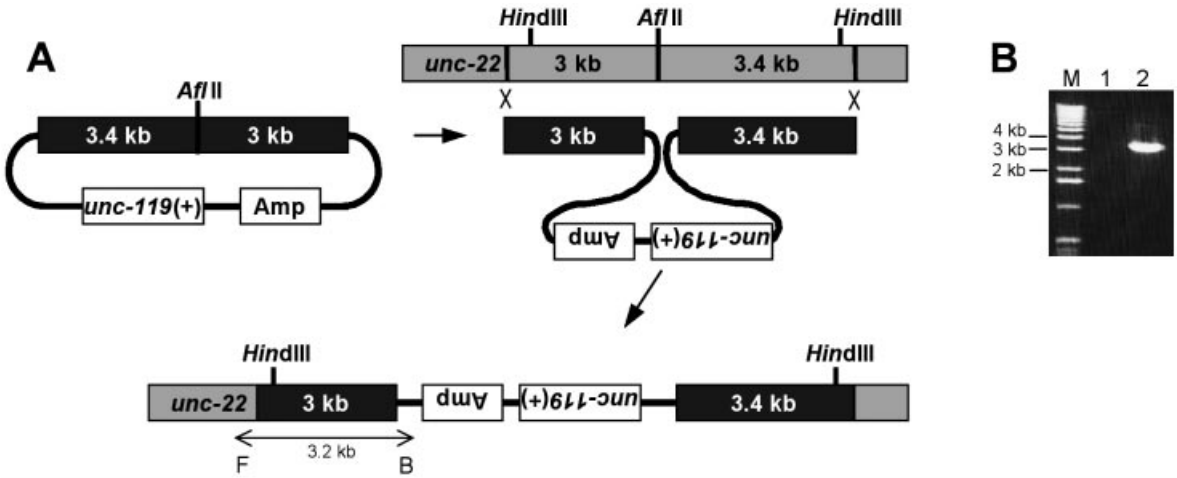

Fig 3. Ends-out targeting of *unc-22* gene. (**A**) Vector design. Genomic sequence of *unc-22* is shown in light gray, homologous sequence included in the targeting vector is dark gray. Two-sided arrows indicate PCR fragments amplified with vector-specific primer B and *unc-22*-specific primer F located outside the region of homology. (**B**) PCR verification of homologous recombination event using primers B and F (see A). Lane 1, negative control on *unc-22* ends-out non-homologous integrated line; lane 2, homologous recombinant line; M, marker line.

Table 1. Statistics of gene targeting in *C.elegans*

Vector	Bombardments	Transformants	Homologous recombinants
unc-22 ends-in equal (2 + 2.4 kb)	28	400	3
unc-22 ends-in unequal (4 + 0.4 kb)	34	275	0
unc-22 ends-out (3.4 + 3 kb)	38	259	1
unc-54 ends-in (2.2 + 2.2 kb)	59	274	1

relatively short, with an average density of one gene per 5 kb (21) and therefore a duplication produced by insertional gene targeting may preserve functionality of one copy of the gene. In contrast, ends-out gene targeting results in replacement of chromosomal sequence with the targeting construct (Fig. 3A) thus making knockout of even very short genes possible.

To test whether ends-out gene targeting is feasible in *C.elegans*, we created the replacement vector for the same region of the *unc-22* gene as was used for ends-in targeting, but the length of homology was extended from 4.4 to 6.4 kb (Fig. 3A). From 259 transformants obtained with the *unc-22* ends-out construct one line showed *unc-22* phenotype (Table 1). PCR analysis confirmed a homologous recombination event in this line consistent with the rearrangement expected for replacement gene targeting (Fig. 3B).

The efficiency of replacement versus insertional gene targeting in other organisms is not completely clear, since some studies report that the ends-out type is less efficient than ends-in targeting (22,23) while other work shows that both types of targeting have similar efficiency (24–26). Our results show that ends-out gene targeting in *C.elegans* is possible, but a larger dataset will be required to draw a statistically supported conclusion about its efficiency.

DISCUSSION

We have demonstrated that biolistic transformation is an efficient means of gene targeting in *C.elegans*. Since homologous recombination events constitute a small fraction of all integration events in *C.elegans*, the bottleneck for successful gene targeting in the worm was in production of large numbers of integrated lines. The PDS-1000/He system with Hepta adapter for microparticle bombardment and *unc-119* gene as a transformation selection marker appears to be a robust combination for large-scale production of integrated *C.elegans* lines. We have shown for two independent loci that ~300 transformants are required to isolate a homologous recombination event. With an average efficiency of 10–15 transformants per bombardment, no more than 30 bombardments per construct are required, which can be easily accomplished in 1–2 days' work after populations of animals are cultivated. Screening for homologous recombinants can be performed by PCR or Southern blot analysis. In cases where the expected recombinant phenotype is known, simple phenotypic selection of recombination events is also possible.

Our data on targeting of *unc-22* gene suggest that both insertional and replacement gene targeting can be observed in the worm. Efficient gene targeting in murine embryonic stem cells depends exponentially on the length of homology between targeting vector and targeted locus with a plateau at ~14 kb of homology (25). The frequency of gene targeting in *C.elegans* reported in this work is based on constructs with 4.4–6.5 kb long homologous regions, and it is possible that efficiency of gene targeting can be further improved by increasing the length of homology in the targeting construct.

Besides the length of homology, frequency of gene targeting in other organisms is shown to vary several-fold between different chromosomal loci (27,28) probably as a result of chromatin structure and position effects. It is possible that gene targeting efficiency in *C.elegans* would be less variable for different loci since the worm chromosomes are holocentric, unlike their mammalian counterpart. The data on homologous gene targeting in *C.elegans* available to date (*vit-2*, *unc-22* and *unc-54* genes) do not show a dramatic difference in frequency of gene targeting between the loci.

Finally, it is worth mentioning that V. Jantsch, P. Pasierbek, M.M. Mueller, D. Schweizer, M.F. Jantsch and J. Loidl (manuscript submitted) have successfully used our protocol for homologous gene targeting of the *zip-3* gene and observed similar transformation and recombination efficiency as reported in this work. This provides independent verification of reproducibility and feasibility of our approach for homologous gene targeting in *C.elegans*.

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