Chapter 5

Transposon-Assisted Genetic Engineering with Mos1-Mediated Single-Copy Insertion (MosSCI)

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Abstract

Transgenesis in model organisms is necessary to determine the function, expression, and subcellular localization of gene products. In *Caenorhabditis elegans*, injected DNA can be propagated as multicopy extrachromosomal arrays but transgenes in arrays are mosaic, over-expressed in some tissues and silenced in the germline. Here, a method to insert a transgene into a specific genomic location called Mos1-mediated single-copy insertion (MosSCI) is described. Single-copy insertion allows transgene expression at levels that approximate endogenous gene expression as well as expression in the germline.

Key words Mos1 transposon, Transgenesis, Mos1 single-copy insertion (MosSCI), Universal insertion sites, Germline expression, Endogenous levels of gene expression

1 Introduction

Transgenic *C. elegans* are most often generated by injection of DNA into the gonad of adult hermaphrodites [1]. Injected plasmids form repetitive extrachromosomal arrays that contain between 100 and 200 copies. In each cell division extrachromosomal arrays can be lost and the DNA is therefore transmitted at variable frequency to progeny. Arrays have the advantage that they are easy to generate and transgene expression is often high, which is beneficial for fluorescence microscopy for example. Arrays have the disadvantages that expression is variable between different strains and transgenes are frequently over-expressed relative to the endogenous expression level. Furthermore expression from extrachromosomal arrays in the germline [2] is efficiently silenced through small RNA pathways [3–6]. Several alternatives to extrachromosomal array transgenesis have been developed to overcome these limitations: biolistic transformation [7], low-copy transgene insertion by ultraviolet and trimethylpsoralen mutagenesis [8], and more recently Cas9-mediated genome engineering [9, 10].

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Here, I describe the method Mos1-mediated single-copy insertion (MosSCI) that allows insertion of single copies of transgenes into well-defined genomic locations throughout the genome. The method was developed based on the observation that the Mos1 DNA transposon from *Drosophila mauritiana* is active in the *C. elegans* germline [11] and that transgenic DNA can be inserted into the genome following Mos1 excision [12]. With MosSCI, transgene insertions are generated by transient expression of the Mos1 transposase by injection and insertions are identified based on positive and negative selection markers (Fig. 1) [13, 14]. Transgenes can be inserted into different genomic locations by cloning the transgene into distinct targeting vectors [14] or into a single, universal targeting vector [15]. Inserted transgenes can be expressed at levels that approximate endogenous expression levels and in tissues that are typically refractory to expression from arrays, such as the hermaphrodite and male germline [14].

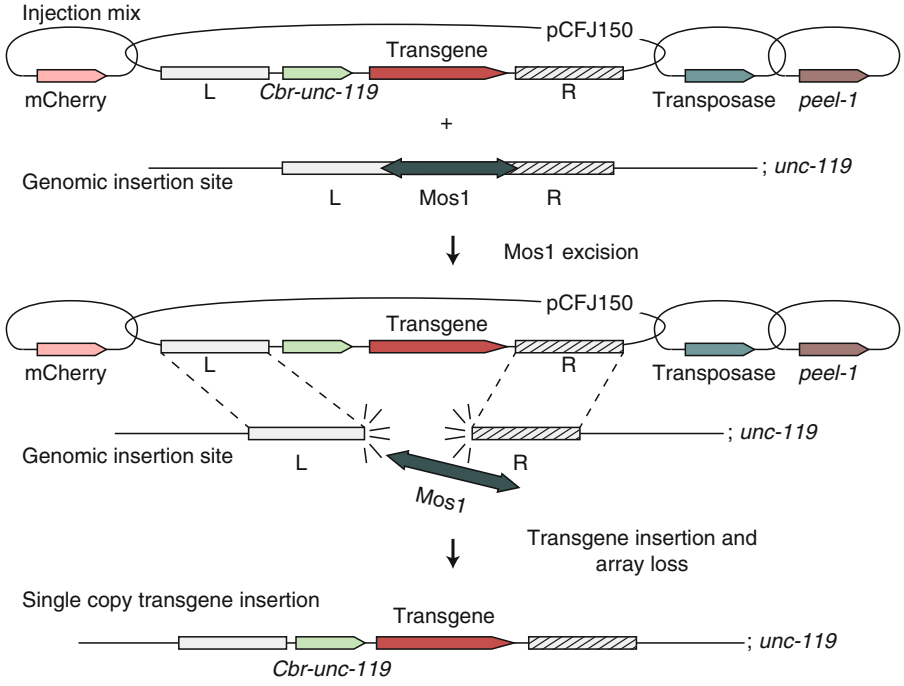

Fig. 1 Schematic of Mos1-mediated single-copy insertion (MosSCI). Targeted transgene insertion relies on generating a double-strand break (DSB) at a specific chromosomal location and a repair template that contains homology to the DSB. Mos1 mobilization by transposase injection creates the DSB. The targeting vector (here pCFJ150) contains homology to the DSB. DNA repair copies the transgene and the selection marker (*Cbr-unc-119*) into the genome. The fluorescent (mCherry) and inducible (*peel-1*) selection markers are used to select against the array

2 Materials

To generate MosSCI insertions it is necessary to have access and familiarity with standard *C. elegans* equipment and reagents, including NGM plates, a microinjection setup, a pipette puller to make injection needles, a fluorescence dissection microscope, and incubators at 20, 25, and 37 °C. All injection strains should be maintained at 20 °C on NGM plates seeded with HB101 bacteria (*see* **Note 1**). Additional information about strains, plasmids, and protocols can be found at the website www.wormbuilder.org.

2.1 MosSCI Insertion Strains

1. Injection strains: Request a strain corresponding to the desired transgene insertion site (Table 1). All strains are available from the *Caenorhabditis elegans* Genetics Center (CGC, www.cbs.umn.edu/research/resources/cgc). Choose the insertion site based on whether germline expression is required and what experiments you plan to do (*see* **Note 2**). For example, do not choose a site that is genetically linked to any mutant backgrounds you plan to cross the transgene into.

2.2 MosSCI Targeting Vectors

1. Generate a targeting vector corresponding to the chosen insertion site (Table 2). All vectors are available from Addgene (www.addgene.org/Erik_Jorgensen/). Vectors for three-fragment Gateway multisite cloning™ (Life Technologies) or for standard restriction fragment cloning are available. Use standard molecular biology protocols to insert the transgene of choice into the targeting vector.
2. Make transfection-grade DNA preparations of the targeting vector(s) (*see* **Note 3**).

Table 1
MosSCI insertion strains

Locus	Genetic position	Chromosomal position	Strain	Germline expression
unc-119 selection				
ttTi4348	I:–5.32	I: 2.85 MB	EG6701	Yes
ttTi4391	I:7.93	I: 11.27 MB	EG6702	No
ttTi5605	II:0.77	II: 8.42 MB	EG6699	Yes
cxTi10816	IV:1.41	IV: 5.01 MB	EG6703	Yes
cxTi10882	IV:–0.05	IV: 4.24 MB	EG6700	Yes (variable)
ttTi14024	X:22.84	X: 15.57 MB	EG6705	Late stages only
unc-18 selection				
ttTi4348	I:–5.32	I: 2.85 MB	EG6032	Yes

Table 2
MosSCI targeting vectors

Locus	Selection marker	Three-fragment Gateway™ vector	Multiple cloning site vector
ttTi4348	*Cbr-unc-119*	pCFJ210	pCFJ352
	unc-18	pCFJ448	pCFJ676
ttTi4391	*Cbr-unc-119*	pCFJ604	pCFJ353
ttTi5605	*Cbr-unc-119*	pCFJ150	pCFJ350
cxTi10816	*Cbr-unc-119*	pCFJ212	pCFJ356
cxTi10882	*Cbr-unc-119*	pCFJ201	pCFJ351
ttTi14024	*Cbr-unc-119*	pCFJ606	pCFJ355

Table 3
Universal insertion sites

Locus	Genetic position	Chromosomal position	Strain	Genomic environment	Germline expression
NeoR and unc-18 landing site					
oxTi185	I:1.17	I: 6.50 MB	EG8078	Intergenic	Yes
oxTi179	II:1.73	II: 9.83 MB	EG8079	In ZK938.3	Yes
oxTi444	III:–0.85	III: 7.01 MB	EG8080	In *lgc-38*	Yes
oxTI177	IV:7.43	IV: 13.05 MB	EG8081	In *scl-10*	Yes
oxTi365	V:1.52	V: 8.64 MB	EG8082	In *asp-13*	Yes
Pmyo-2:GFP:H2B and unc-18 landing site					
oxTi354	V:5.59	V: 13.78 MB	EG8083	In F53C11.3	Yes

2.3 Universal MosSCI Insertion Strains

1. Universal injection strains: A set of strains contain a universal landing site (Table 3), which are all compatible with a single targeting vector (Table 4). The strains contain a Mos1 transposon flanked by two selection markers (NeoR and *unc-18*(+) or *Pmyo-2*:GFP:H2B) (Fig. 2). The selection markers are convenient for moving a transgene insertion into other genetic backgrounds by following neomycin resistance or pharyngeal GFP fluorescence.

2.4 Universal MosSCI Targeting Vectors

1. Generate a universal targeting vector (Table 5) by your preferred cloning method. Transgenes in the universal vectors can be inserted into any of the six universal insertion sites and the insertion location only depends on which strain is injected.
2. Make transfection-grade DNA preparations of targeting vector(s).

Table 4
Universal targeting vector

Locus	Selection marker	Three-fragment Gateway™ vector	Multiple cloning site vector
Universal insertion sites	*Cbr-unc-119*	pCFJ150	pCFJ350

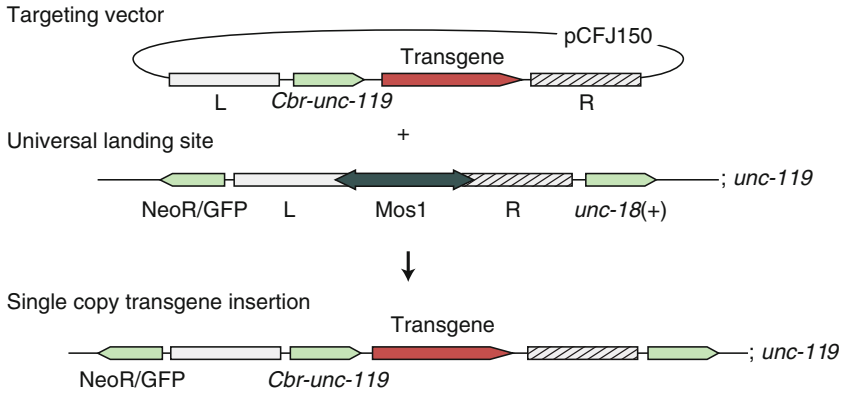

Fig. 2 Schematic of universal MosSCI sites. The universal MosSCI system relies on a set of landing sites that were introduced into locations across the genome. Because the sequence flanking the Mos1 transposon is identical for all insertion sites the same targeting vector (pCFJ150) can be used for insertion. The universal landing sites furthermore contain a selection marker adjacent to the insertion sites (NeoR or P*myo-2*:GFP) that facilitates following a MosSCI insertion in genetic crosses

Table 5
Co-injection markers

Plasmid	Description	Transgene
pCFJ601	Induces Mos1 transposition by injection	P*eft-3*:Mos1 transposase
pMA122	Negative selection, heat-shock inducible	P*hsp-16.41*:*peel-1*:*tbb-2* UTR
Red fluorescent markers		
pGH8	Visual array marker: Red, nervous system, cytosolic	P*rab-3*:mCherry:*unc-54* UTR
pCFJ90	Visual array marker: Red, pharynx, cytosolic	P*myo-2*:mCherry:*unc-54* UTR
pCFJ104	Visual array marker: Red, body wall muscle, cytosolic	P*myo-3*:mCherry:*unc-54* UTR
Green fluorescent markers		
pCFJ420	Visual array marker: Green, ubiquitous, nuclear	P*eft-3*:GFP:H2B:*tbb-2* UTR
pCFJ421	Visual array marker: Green, pharynx, nuclear	P*myo-2*:GFP:H2B:*tbb-2* UTR

2.5 Co-injection Markers

1. Make transfection-grade DNA preparations of all co-injection markers.
2. 2× red co-injection mix: 100 ng/μl pCFJ601, 20 ng/μl pMA122, 20 ng/μl pGH8, 5 ng/μl pCFJ90, 10 ng/μl pCFJ104 (*see* **Note 4**). Make 2× stock solution and store at −20 °C.
3. 2× green co-injection mix: 100 ng/μl pCFJ601, 20 ng/μl pMA122, 20 ng/μl pCFJ420, 5 ng/μl pCFJ421. Make 2× stock solution and store at −20 °C.

3 Methods

3.1 MosSCI Injections

1. Centrifuge the 2× co-injection mix and the targeting vector at highest speed on a tabletop microcentrifuge for 2 min.
2. Make 20 μl injection solution by mixing 10 μl of the 2× co-injection mix (*see* **Note 5**) with the targeting vector to a final concentration of 10–50 ng/μl. Bring the final concentration of DNA up to 100 ng/μl with a DNA ladder without dye or another inert stuffer DNA. Add molecular grade purified water to a final volume of 20 μl.
3. Immediately before injection, centrifuge the injection mix at highest speed on a tabletop microcentrifuge for 2 min.
4. Pick relatively healthy and unstarved young adult hermaphrodites for injection from strains maintained at 20 °C (*see* **Note 6**). Pick animals from the strain corresponding to the targeting vector (Tables 2 and 4).
5. Generate transgenic animals by following standard protocols for injection ([1] and Chapter 3) (*see* **Note 7**).
6. Place one to three injected animals on individual NGM plates seeded with OP50. Place NGM plates with injected animals in 25 °C incubator (*see* **Note 8**).

3.2 Isolation of Transgenic Animal with Single-Copy Insertion

1. After approximately 1 week and the injected animals have starved out the plate, heat-shock plates with injected animals for 1 h at 37 °C. Place animals at room temperature after the heat-shock. Animals with extrachromosomal arrays die from the heat-shock whereas animals with a transgene insertion survive (*see* **Note 9**).
2. The day after heat-shock, screen plates for transgene insertions with a fluorescence dissection microscope. Animals with transgene insertions are rescued for the mutant phenotype (*unc-119* or *unc-18*) but do not contain any of the fluorescent co-injection markers (*see* **Note 10**).
3. Pick three to four animals from each plate with a transgene insertion to a single NGM plate seeded with OP50 (*see* **Note 11**).

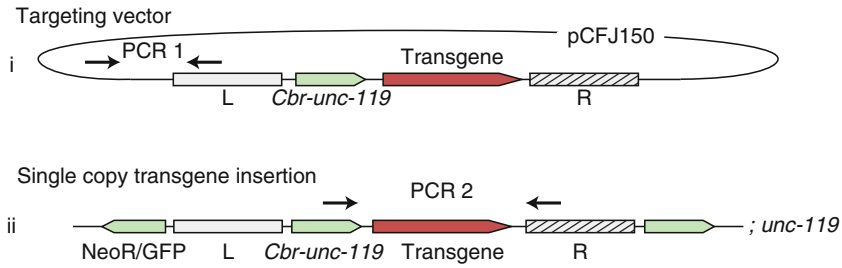

Fig. 3 Validation of MosSCI insertions. Approximately 15 % of MosSCI insertions contain one of the two errors, either dual insertion of the transgene or indels within the transgene. To validate full-length, single-copy insertions two strategies are used: (*i*) a PCR designed to detect the junction between the bacterial backbone of the targeting vector and the homology region. This DNA should not be present in the final strain and a positive PCR band therefore indicates a dual insertion. (*ii*) PCR amplification of the inserted transgene: Indels are typically large enough (approx. 1 kb [14]) to be detected by gel electrophoresis. For large transgenes, splitting the PCR into several smaller amplifications will facilitate the detection of aberrant insertions

4. 2–3 days later, verify that the selected animals do not express fluorescent co-injection markers and pick a single, clonal animal *with eggs* to a new plate from each plate. Only pick one insertion from each plate to ensure that all insertions are independent and treat each insertion as an independent allele (*see* **Note 12**).
5. In the following generation, pick four to eight clonal animals to individual plates to establish a strain that is homozygous for the insertion (*see* **Note 13**).

3.3 Validation of Single-Copy Transgene Insertion

1. If the inserted transgene contains a fluorescent marker (GFP for example), check each independent strain for fluorescence (*see* **Note 14**).
2. If insertion of only a single copy of the transgene is important then validate the strain by PCR amplification on genomic DNA derived from the strain with oligos designed to amplify the junction between the plasmid backbone and the homology regions that flank the transgene in the targeting vector (Fig. 3). Use the targeting vector as a positive control and N2 genomic DNA as a negative control for the PCR reaction. Single-copy insertions do not contain the plasmid backbone and should therefore not result in a visible amplification band.
3. Validate intact, full-length transgene insertions by PCR amplification across the entire transgene, either as a single amplicon or as separate amplicons using oligos that do not anneal in non-transgenic animals (*see* **Note 15**). Use N2 genomic DNA as the negative control for PCR reactions.
4. Give each validated MosSCI strain an allele name using the "Si" nomenclature corresponding to the lab allele designation (*see* **Note 16**).

4 Notes

1. *unc-119* and *unc-18* animals propagated on HB101 bacteria are healthier than mutant animals propagated on standard OP50 bacteria. HB101 bacteria is available from the *Caenorhabditis elegans* Genetics Center (CGC). Injections into relatively healthy animals are much more efficient than into starved animals. I therefore take great care to maintain relatively healthy animals by picking three individual adult animals to three separate spots on a recently seeded HB101 plate on a daily basis. The progeny from these picked animals are ready for injection after approx. 3–5 days.
2. Expression in the germline was tested with the ubiquitous *dpy-30* promoter driving GFP:histone expression. P*dpy-30* is highly sensitive to genomic position effects (CFJ, unpublished observations) and other germline-specific promoters may behave differently.
3. Preparation of injection plasmids (co-injection plasmids as well as targeting vectors) with a plasmid purification method that yields transfection-grade plasmids significantly improves the number of F1 progeny generated from each injection [15]. I routinely use the kit PureLink HQ miniPlasmid DNA Purification kit (Life Technologies).
4. In my experience, using all three fluorescent co-injection markers significantly reduces the number of false positive insertions.
5. I recommend using co-injection markers that are not homologous to the transgene; for example, for a GFP-tagged transgene I recommend using the mCherry markers. The repair process which copies the transgene into the genomic location is likely to be inhibited by the presence of several different homologous templates in the injection mix.
6. To facilitate freezing and handling, the strains contain an extrachromosomal array with *unc-119* rescue. Before MosSCI injections this array needs to be lost and injections should be performed into *unc-119* mutant animals.
7. The number of injected animals necessary to generate a single MosSCI insertion will depend on the transgene (for example, larger transgenes >10 kb are more difficult to insert) and on how experienced the researcher is at injecting. In my experience, insertions are generated in 10–50 % of injected animals but for researchers with less experience the insertion frequency is often closer to 1–5 %. Adjust the number of injections according to the size of the transgene and your injection experience.
8. The MosSCI insertion frequency appears to be independent of temperature [15] but there are two advantages to placing

animals at 25 °C. First, the animals develop faster and can be screened for insertions faster. Second, a higher percentage of MosSCI insertions are expressed in the germline when the injected animals are placed at 25 °C (Frøkjær-Jensen, unpublished observations). Even genotypically identical insertions do not necessarily express the transgene in the same way in the germline because of stochastic silencing by small RNAs [16].

9. Most animals with extrachromosomal arrays typically die within approx. 4 h of the heat-shock and plates can be scored the same day if necessary. Generally it is easier to score the animals the following day when even highly mosaic animals have died from the heat-shock.
10. Mostly, the negative selection with *peel-1* is very efficient with only a low frequency of false positives (approx. 10 %). However some laboratories report a much higher frequency of false positives. The plates can be screened for MosSCI insertions even in the absence of the negative selection marker but I suggest trying a higher concentration of the pMA122 plasmid if false positives occur at >10 % of rescued animals after the heat-shock.
11. Following injection and heat-shock the animals show a high frequency of sterility. Picking a single L1 animal with a transgene insertion is therefore often not enough to establish a line. Instead, I pick several L1 animals from the same plate and only select a clonal animal when they have become adults and the absence or presence of eggs is obvious.
12. When the Mos1 element is excised it sometimes inserts into another genomic location. So even if each strain contains the same insertion it is possible that the genetic background is different.
13. There is strong selection towards homozygosity of the transgene insertion, so the longer a mixed population is propagated the higher the proportion of homozygous animals will be.
14. Single-copy insertions are often dimmer than extrachromosomal arrays and the strains should therefore be checked for fluorescence on a microscope with high magnification.
15. Approximately 15 % of MosSCI insertions are either dual insertions or imperfect insertions that contain indels. In the absence of phenotypic rescue or consistent GFP fluorescence between several independent alleles, it is therefore important to validate each MosSCI insertion.
16. Alleles are designated by the lab-specific designation followed by “Si.” For example, the allele designation for Erik Jorgensen’s lab is “ox” and all MosSCI alleles are therefore named oxSi together with an allele number.

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