Title:

Extrachromosomal markers for bacteriophage phiC31 recombinase actions on C. elegans genome.

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ABSTRACT

We report on the experimental implementation of the transgenic phiC31 recombinase *C. elegans* intron-split system. The three-component plasmid-based phiC31 recombinase system consists of *i)* two intron-split segments of the *C. briggsae-unc-119* gene, and *ii)* the plasmid that provides phiC31 recombinase activity. Described results constitute the proof-of-concept assay for the implementation of bacteriophage phiC31 integrase in *C. elegans*.

INTRODUCTION

C. elegans genetic analysis (Brenner 1974) is conveniently facilitated by efficient microinjection of purified solutions of nucleic acids into the syncytial part of the hermaphrodite ovary (Mello et al. 1991). While the system is elegant and conceptually simple, in C.elegans injected DNA molecules spontaneously form intrinsically large heritable extrachromosomal arrays (Stinchcomb et al. 1985) rather than directly integrate into chromosomes. This rather peculiar property of heritable extrachromosomal array formation affords for the implementation of transgenic co-transformation markers that label the transformed individuals (Evans 2006). Heritable arrays of injected molecules maintained as extrachromosomal elements behave genetically as minichromosomes or free-duplications that segregate in a non-Mendelian fashion. Transgenic co-transformation markers seem incorporated into extrachromosomal arrays, as a result of the apparent (albeit poorly characterized) process of DNA concatenation.

Whereas the experimental use of heritable extrachromosomal arrays in C. elegans is widely accepted and broadly applied, the process of extrachromosomal array formation (i.e. concatenation and maturation) is understood only marginally. Injected DNA solution, once administrated into the cytoplasm of gonadal syncytia, seems eventually imported into germline nuclei while arrayed to form fairly stable extrachromosomal molecules consisting of many copies of the transformed DNA. The mechanisms of formation of heritable extrachromosomal arrays remain largely speculative; still, the repetitive extrachromosomal structures - once formed and matured - at reasonable frequencies tend to replicate along with nuclear DNA complement.

In terms of genome engineering, the extrachromosomal and repetitive nature of arrayed DNA has been regarded as procedural bias. Additional methods for extrachromosomal transgene integration have been sought. Either spontaneously sparse or also infrequent mutagen-induced

events of integration of extrachromosomal molecules have been reported and widely used (Mello & Fire 1995). Alternative methods of DNA administration, promoting low-copy transgene integration, have also been considered (Praitis et al. 2001). The advances of genome engineering resulted in improved protocols involving the transposon excision-induced DNA integrations (Frøkjaer-Jensen et al. 2008) and CRISPR-Cas9-stimulated DNA homology-directed repair mediated insertions at specific sites of loci targeted (Tzur et al. 2013).

Site-specific DNA recombinases have been proposed as available instruments for dedicated genomic surgery (Akopian &Stark 2005). Several site-specific recombinases have been derived from bacteriophages and plasmids to catalyze desired transgene integration events (Kilby et al. 1993, Turan &Bode 2011, Coates et al. 2005). Genome manipulations mediated by the transgenic phiC31 integrase perform in a number of animal systems; most notably reported in the Drosophila fly (Bateman et al. 2006, Venken et al. 2006, Bateman et al. 2013), mice (Belteki et al. 2003), and man (Groth et al. 2000), but also in zebrafish (Hu et al. 2011), chicken (Leighton et al. 2008), porcine (Bi et al. 2013) and bovine systems (Ma et al. 2006) acting as site-specific recombinase. Here, we report on the proof-of-concept assay for the bacteriophage phiC31 recombinase use in C. elegans.

MATERIALS and METHODS:

E. coli plasmids. Standard methods were used to construct recombinant plasmids. Two separate plasmids pS000121 and pTH627 procured entail intron-split segments of C. briggsae unc-119 gene; providing respectively, i.) unc-119 promoter and N-terminal UNC-119 CDS and ii.) C-terminal UNC-119 followed by unc-119 3′ utr. C. briggsae unc-119 gene (Maduro &Pilgrim 1996) is split at the third intron and flanked with the appropriate phiC31 recognition sites, hereafter dubbed the phiC31-recombinase-mediated linking of Att-flanked intron-split system. Plasmid pS000121 (3774bp) incorporates the I-CeuI site to facilitate the transgene engineering demanding DNA linearization prior to injection, as used in some experiments including inserted GFP reporter derivatives thereof. Plasmid pTH627 (8540bp) is a MosSCI compatible (chr. II, targeting the ttTi5605 locus) construct providing a segment of pCFJ151, and confers additional selection marker neo-R (the resistance to G418-Geneticin). The third construct used is intended to provide the phiC31 integrase activity into the C. elegans germline cells. This construct (pS00061) is essentially based on glh-2 modified vector (pJL43.1), where the Mos1 transposase coding sequence (Bessereau et al. 2001) is replaced with the codon-optimized phiC31 integrase gene (codon swaps designed according to Redemann et al. 2011; https://worm.mpi-cbg.de/codons/cgi-bin/optimize.py). Maps generated with ApE- A plasmid editor (https://jorgensen.biology.utah.edu/wayned/ape/ by Wane Davis).

C. elegans lines. Homozygous line intended as phiC31 integrase RMCE (Recombination-Mediated Cassette Exchange) docking-site was constructed with MosSCI (integrated at chromosome II). This was done by replacing ttTi5605 Mos transposon insertion upon mobilization of Mos1 insert with plasmid providing Mos1 transposase activity (Frokjaer-Jensen et al. 2012) as required for protocol established for transposon excision-triggered recombination (Frokjaer-Jensen et al. 2008). Briefly microinjection of pTH627 (entailing excision-triggered recombination cassette derived from pCFJ151) along with Mos1 transposase expressing plasmid (pCFJ601) and established selectable

markers essentially followed as described by Frokjaer-Jensen et al. 2008. DP38 unc-119 (ed3) derived hermaphrodites (Maduro &Pilgrim 1995) were injected with plasmids encoding for phiC31 integrase system components. Cohorts of 24-30 unc-119(-) animals were injected per every construct combination tested. Candidate plates prominent in F1 transgenics [cbr-unc-119('+') tagEx(?)/unc-119(ed3); where ('+') denotes intron-sealed rescue phenotype] were identified based on fluorescent markers included into injected plasmid mixtures, than starved to select based on (ed3) unc-119 (-/-) rescue. Additional neo-R selection with G418 was applied when necessary. Standard laboratory practice established for C. elegans hermaphrodite DNA microinjection methods we followed (Mello &Fire 1995).

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RESULTS:

The phiC31-recombinase intron-split system. The intron-split system consists of three plasmids. Figure 1. presents annotated maps of two separate plasmids pTH627 and pS000121 (described in method section) that constitute the intron-split constructs. The intron-split segments flanked with cohesive phiC31 integrase recombination sites attP (pTH627) and attB (pS000121) attached to segments derived from the Cbr-unc-119 gene. The labeled are phiC31 integrase recombination sites specifically concerned with the restoration of the functional ed3 complementing marker rescued from the intron-split segments of the 5' and 3' portions of the unc-119 gene provided on the separate plasmid molecules. The attached phiC31-attP and phiC31-attB recombinase sites, (see Figure 1. description for the details) define the structural component of the phiC31 integrase-based marker system.

In addition, the system utilizes the third plasmid providing the catalytic component of the intron-split marker system i.e. a bacteriophage-derived gene encoding for the site-specific phiC31 recombinase. Germinal provision of bacteriophage phiC31 recombinase activity is ensured by the use of appropriate Germ line helicase 2 (glh-2) based vector entailing glh-2 regulatory modules (glh-2 promoter and glh-2 3'utr). The glh-2 regulatory modules are arranged into a plasmid vector (Materials and Methods) to render the germline-specific expression of codon-optimized phiC31 recombinase. In the subsequent section (below) we demonstrate the intended use of the phiC31 recombinase activity providing plasmid as an essential component of the system, required to seal phiC31 recombination-sites that flank Cbr-unc-119 intron-split segments leaving the phiC31 recombination scar embedded into silently modified restored intron.

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The phiC31-recombinase actions on MosSCI modified site. Having secured the above plasmid-based reagents as a minimal three-component intron-split system, established at our disposal, we attempted to challenge the above assumption with a two-step phiC31-RMCE protocol. In a first step, we transformed a MosSCI compatible plasmid pTH627 (perceivably integrated at chromosome II by replacing ttTi5605 Mos transposon insertion) to generate a selected (neo-R) homozygous phiC31-RMCE receptive C. elegans line (see Materials and Methods). That line harbors the 3' portion of the split Cbr-unc-119 gene (itself non-functional) flanked with phiC31-attB recombination site. In a second step the above (neo-R) homozygous phiC31-RMCE receptive C. elegans line, we transformed with several plasmids derived from entry vector pS000121 providing 5' portion of the split unc-119 gene (itself non-functional) flanked with appropriate phiC31-attP recombination site (as described in method section), co-injected into hermaphrodite gonads with the third plasmid providing the catalytic component of the phiC31 recombinase. Plasmids derived from pS000121 used in that step were recognized by the distinct pattern of GFP reporter fluorescence

(Sarov et al.); were modified to facilitate the insertion of the fluorescent reporters into pTH627 based phiC31 RMCE landing pad pre-integrated into the aforementioned site on Chr. II. This was done because RMCE could be a preferred phiC31 variant since it tends to precisely exchange the target sequences inserting the reporter cassette along with the 5' portion of the intron-split unc-119 gene (see Fig.1.). An initial detour exercised the phiC31-mediated insertion via cassette exchange of reporter plasmids derived from pS000121 (modified with several distinct fluorescent reporters entailed into the above phiC31-RMCE compatible entry vector; not shown) co-injected as closed circular supercoiled molecules, along with phiC31 recombinase encoding plasmid. No movement competent unc-119('+') consistent with RMCE-compatible phiC31-mediated integrants were found. Provided the initial concerns regarding the overall transformation efficiency observed in phiC31 RMCE-compatible line we repeated the above experiments including an extrachromosomal marker (myo-2p:RFP) into injected plasmid mixtures. This iteration demonstrated that extrachromosomal arrays formed as judged by observed marker phenotype in phiC31-RMCE-compatible C. elegans line at an adequate frequency (estimated at 8-12% of heritable array formation; not shown). However, no movement competent unc-119('+') consistent with RMCE-compatible phiC31-mediated integrants were found. Next, to exclude that the entry vector plasmid linearization could have affected the overall efficiency of the phiC31-RMCE process we addressed the requirement for I-CeuI treatment of reporter plasmids derived from pS000121 prior to injection. Since again no movement competent unc-119('+') transformants consistent with RMCE-compatible phiC31-mediated integration were found, after screening of the progeny of >800 P0's (P0= quality injected parental hermaphrodite) we concluded both injected plasmid-based forms (i.e. closed circular vs. I-CeuI linear) of reporter modified entry vector were equally inefficient rendering the desired RMCE integrants.

The phiC31-recombinase actions on extrachromosomal arrays. Since no conclusive evidence for phiC31 recombinase actions on C. elegans genome could be drawn from the above efforts focused on phiC31-RMCE, we repurposed our intron-split system to develop an extrachromosomal assay, likely useful as a genetic marker for phiC31 activity.

For this purpose, we implemented the classic assay for unc-119(-/-) mutant rescue (Maduro& Pilgrim 1995; 1996). In that assay injection of homozygous uncoordinated (ed3) hermaphrodites with DNA preparation supplements the wild type unc-119 gene (derived of either C.elegans or C. briggsae). That assay provides a robust and convienient phenotypic readout for the restored unc-119 function. In a given example of the unc-119 intron-split split system a single phiC31 integrase-mediated recombination event could be detected as inferred based on the phenotypic readout. Expectedly the unc-119(-/-) background based assay should be sensitive enough to detect even an isolated recombination event between a single pair of phiC31-attB and phiC31-attP recognition sites, hence a preferred assay for phiC31 recombinase activity.

The repurposed extrachromosomal intron-split system for phiC31 activity, therefore, becomes a streamlined one-step procedure with an advantage of simplicity.

In that system, both pS000121 and pTH627 plasmids (providing attB-P recombinations sites) are directly injected (supercoiled preparations) into the homozygous unc-119 deficient line along with plasmid providing phiC31 recombinase activity. The unc-119 deficient line (ed3) is used instead of the phiC31 RMCE-compatible line. We assume here the abnormal phenotype of unc-119, which is a consequence of the homozygous absence of functional unc-119 allele, should be corrected by the experimental introduction of the intron-sealed pS000121 and pTH627 plasmids

into mutant worms, depending on the germ-line provision of the phiC31-recombinase.

To test for the above assumption we injected, two of the above plasmids (supercoiled pS000121 and pTH627) containing intron-split segments (attB-P flanked) of the unc-119 gene into gonads of the DP38 (ed3) hermaphrodites. In the absence of the phiC31 recombinase encoding plasmid we observed at least four 4 independent array forming events in 30 injected hermaphrodites (~13%); where 4 out of 4 transgenic extrachromosomal array transmitting lines conferred non-rescued uncoordinated phenotype. Based on the segregation of the fluorescent transformation markers in the selfed progeny of transgenic hermaphrodites in this experiment, we concluded that while the injected split unc-119 segments do efficiently form the heritable transgenic arrays, however, collectively fail to correct the unc(-) phenotype.

This experiment ensures that non-targeted (phiC31 recombinase independent) linking of injected plasmids i.e. perceivably fairly stochastic co- or post- transformational recombination events involved in heritable extrachromosomal array formation (i.e. DNA concatenation, array maturation, and replicative maintenance) does not readily lead to correction of unc-119(-) phenotype. This is what would be expected for the phiC31 recombinase independent control experiment in a given experimental setting and that is what we have observed.

We contrast the above observation, with the results of the phiC31-recombinase guided experiment. In that experiment two att-flanked intron-split segments of unc-119 (plasmids pS000121 and pTH627 as above) were co-injected along with the phiC31-recombinase providing plasmid into gonads of unc-119(-/-) hermaphrodites. We observed at least 4 independent events of the formation of the heritable arrays in 28 injected hermaphrodites (~14%). In that case, 4 out of 4 transgenic array transmitting lines conferred the rescue phenotype resulting in non-uncoordinated movement observed in otherwise unc-119(-/-) animals. Those results are consistent with phiC31 mediated linking of the intron-split unc-119 segments observable at the appreciable frequency, hence constitute the proof-of-concept for the use of the phiC31 recombinase in C. elegans.

DISCUSSION

Our study demonstrates that the phiC31 integrase, site-specific recombinase derived from a Streptomyces phage, can function efficiently in transgenic C. elegans.

Here we have demonstrated that i.) intron-split constructs with cohesive phiC31-sites could be productively rearranged into a functional gene, and ii.) that this transaction depends on the transgenic provision of site-specific phiC31 recombinase. We have shown that the phiC31 recombinase expressed from codon-optimized plasmid act efficiently to join the two segments of the intron-split Cbr-unc-119 rescuing cassette provided in the context of heritable transgenic extrachromosomal arrays.

Our demonstration seems directly relevant because the intron-split system (consisting of three plasmids) could be used as a selectable marker for the transgenic phiC31 integrase activity. Our marker system for phiC31 integrase activity is based on repurposed unc-119 (-) background; a selection scheme compatible with widely established transgenic C. elegans assays (Maduro 2015, Priatis et al. 2001), including large scale transformation efforts (Dupuy et al, Sarov et al).

Possible applications of the phiC31-recombinase extend beyond the extrachromosomal intron-split marker system. The ability to discern transformed progeny that engages phiC31 recombinase activity (based on the corrected unc-119(-) phenotype) provides the selectable advantage, likely useful when selecting for genetic transactions involving chromosomal DNA. This could be particularly useful as a two-step strategy used in combination with available genome editing tools. Either CRISPR-Cas9 mediated edits (Zhao et al. 2014) inserting oligonucleotide encoded phiC31 compatible docking sites into targetted chromosomal locations, or alternatively miniMos- mediated insertions (Frøkjær-Jensen et al. 2014) could be used to pre-integrate the desired phiC31 compatible recombination sites. An att-site pre-edited background (unc-119 deficient in a given example) would be used in a second (phiC31 recombinase dependent) step, warranted the background integration events could be identified and/or selected against.

Background integration events could be suspected in genome-editing experiments. For example, we reported on unexpected background array integration events we observed in the case of CRISPR-Cas9 experiments (Kapulkin et al. 2016), albeit in genetic background different from unc-119(-). Transformed DNA may integrate into chromosomes of the unc-119 background. This may happen in recombinase independent manner or alternatively may be dependent on germline phiC31-integrase provision. In the latter case, so-called phiC31 recombinase att-pseudo sites have been reported in other systems (Thyagarajan et al. 2001, Chalberg et al. 2006, Yu et al. 2014). However phiC31 recombinase pseudo-att sites have not been characterized in C.elegans. It is also possible that phiC31-integrase mediated array rearrangement promoted some secondary background integration events independent of att-pseudo sites. Together the major limitation of our study is a lack of dedicated classical genetic and sequence-level follow-up analysis.

Regardless of the above limitations, it is interesting to surmise on additional applications of phiC31-integrase mediated recombinations as instruments for the dedicated genomic surgery. Those applications will expectedly extend on the existing C. elegans genome engineering toolbox.

First, in the context of extrachromosomal arrays demonstrated targetted linking of segments flanked by the minimal phiC31 recognition sites, could be of more general use. While most of the worm genes are of size within the resolution of physical genome map (Coulson et al. 1986) i.e. fit into the size of a single cosmid or fosmid, some of the genes exceed that limitation (Spieth et al.) Moreover, certain functional modules found existing in the nematode genomes are encoded by ordered multi-cistronic transcriptional units organized within operons (Blumenthal et al. 2004; Allen et al. 2011). Certain nematode operons may again exceed the size entailed by bacterial replicons commonly used to transform C.elegans. With an aid of in vivo recombination technology, the required cohesive phiC31-sites could be pre-inserted to facilitate the downstream engineering steps, such as phiC31-recombinase-mediated assembly of the larger DNA segments.

Second, the C. elegans lines pre-edited with dedicated phiC31-recombinase compatible att-sites may lead to improved protocols facilitating the insertion of larger DNA segments (e.g. DNA segments maintained as artificial chromosomes) into designated genomic locations. Further, the strategies relying upon the C. elegans lines pre-edited with mutually compatible (or incompatible) phiC31-recombinase att-sites could also extend to expedite targetted chromosome-scale rearrangements. Together, all the above manipulations may benefit from the available phiC31-recombinase selectable co-transformation marker system - providing the particular experimental advantage in selecting the desired segregants - i.e. identifying the fraction of transgenic progeny where the activity resulting from bacteriophage phiC31 derived recombinase actions could be confidently ascertained.

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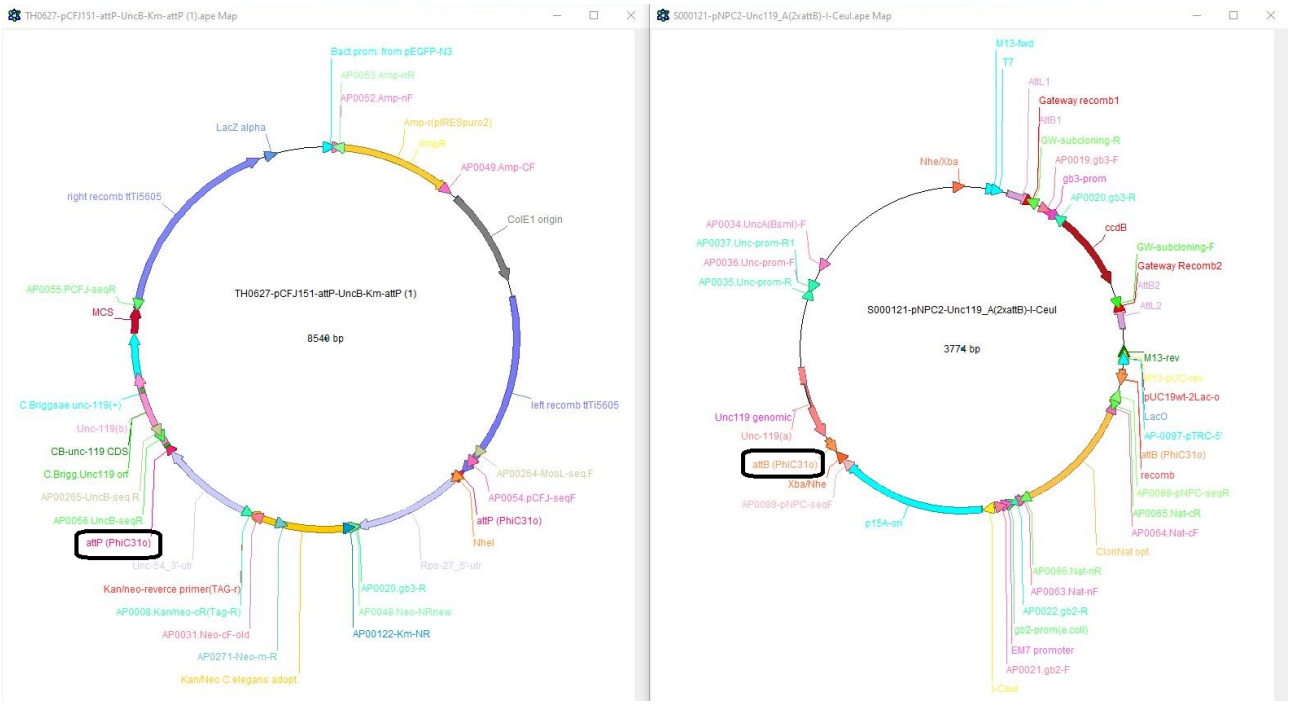
Figure 1. Annotated maps of two separate plasmids pTH627 and pS000121 (described in method section) that constitute the intron-split constructs. Black rounded rectangles indicate the phiC31 integrase recombination sites attB and attP (respectively donor and acceptor) concerned with the functional restoration of ed3 complementing marker rescued from the split segments of Cbr-unc-119 gene. The pTH627 entails a 3' portion of the unc-119 gene (C-terminal exons and 3' untranslated region) while pS000121 entails a 5' split segment (unc-119 promoter and N-terminal exons). MosSCI compatible ttTi5605 recombination sites derived from pCFJ151 are annotated with the pTH627 map. Note the presence of the second pair of the phiC31 integrase recombination sites attB and attP at the other end of the cassette segment intended as RMCE.



Additional Information:

1.) pS000121 attB sites (3774bp)

attB(PhiC31o) 1027-1077
CCGCGGTGCGGGTGCCAGGGCGTGCCCTTGGGCTCCCCGGGCGCGTACTCC

attB(PhiC31o) 2419-2469

                      ##
ggagtacgcgcccggggagcccaagggcacgccctggcacccgcaccgcgg

-->unc-119

[I-CeuI linearizes backbone between attB sites]

I-CeuI 1775-1803

CGTAACTATAACGGTCCTAAGGTAGCGAA

2.) pTH0627-CFJ151 attP sites (8540bp)

[integrated with ttTi5605 (left recombination site 1853-3188 and right 6631-8058)]

attP(PhiC31o) 3189-3238

                         ##
ctacgcccccaactgagagaactcaaaggttaccccagttggggcactac

[rps-27 driven Kan/NeoR]

attP(PhiC31o) 5547-5596

gtagtgccccaactggggtaacctttgagttctctcagttgggggcgtag