**piggyBac**

A vehicle for integrative DNA transformation of parasitic nematodes

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In addition to their natural role in eukaryotic genome evolution, transposons can be powerful tools for functional genomics in diverse taxa. The piggyBac transposon has been applied as such in eukaryotic parasites, both protozoa and helminths, and in several important vector mosquitoes. piggyBac is advantageous for functional genomics because of its ability to transduce a wide range of taxa, its capacity to integrate large DNA 'cargoes' relative to other mobile genetic elements, its propensity to target transcriptional units and its ability to re-mobilize without leaving a pattern of non-excised sequences or 'footprint' in the genome. We recently demonstrated that piggyBac can integrate transgenes into the genome of the parasitic nematode Strongyloides ratti, an important model for parasitic nematode biology and a close relative of the significant human pathogen *S. stercoralis*. Unlike transgenes encoded in conventional plasmid vectors, which we assume are assembled into multi-copy episomal arrays as they are in Caenorhabditis elegans, transgenes integrated via piggyBac are not only stably inherited in *S. ratti*, they are also continuously expressed. This has allowed derivation of the first stable transgene expressing lines in any parasitic nematode, a significant advance in the development of functional genomic tools for these important pathogens.

**Introduction**

Parasitism by nematodes is often regarded as peripheral in human medicine, but, in fact, these infections account for an enormous burden of disease and disability when viewed globally. In the aggregate, parasitic nematodes infect roughly a quarter of the world’s population and cause acute illness, chronic debilitation, disfigurement, blindness and failure to thrive in children. The burden of disease due to nematode parasitism rests disproportionately on developing nations, but some of these agents are endemic in economically stressed communities in the USA. *S. stercoralis* is a significant human pathogen, infecting approximately 200 million people. It is unique among nematodes parasitizing humans in its ability to autoinfect and thus self-replicate in its host. This can result in potentially fatal disseminated hyperinfection in patients immunocompromised by HTLV-1 infection or undergoing immunosuppressive drug treatment. There are no practical vaccines against parasitic nematode infection, and the armamentarium of drugs effective against these parasites is small and threatened by resistance. Modern approaches to discovery of new drug or vaccine targets in parasitic nematodes are hampered by the lack of functional genomic tools for these organisms. Furthermore, there are many interesting basic biological questions, among them ones pertaining to the genetic bases for the evolution of parasitism among nematodes and to the extreme longevity of parasitic nematodes compared with their free-living counterparts, which might be addressed at the functional level if more robust molecular genetic tools were in hand. *Strongyloides*
and related genera would be particularly well suited to basic studies of the kind just described because their life cycles include alternative pathways of development leading to relatively short-lived free-living adults or relatively long-lived parasitic ones (Fig. 1).5 Methods for transgenesis and for disruption or silencing of specific genes in parasitic nematodes, which could facilitate both basic studies and the discovery of novel drug and vaccine targets, have been very slow to develop owing to the very complex and protracted life histories of these parasites and to the fact that none of them can be propagated in vitro.

To address this methodological gap, our laboratory has pursued techniques for transgenesis in Strongyloides, which, because of some unique aspects of its life cycle, to be discussed below, is a more tractable model for parasitic nematode biology. Methods for gene transfer into S. stercoralis, involving only slight modifications of standard techniques for gonadal microinjection in C. elegans,9,10 were developed a decade ago11 and promoter-regulated, tissue-specific transgene expression was achieved in 2006.12 Despite these early successes, lines of Strongyloides that stably inherit and express transgene sequences were not achieved until 2012, when Shao et al. reported integrative transformation and establishment of stable transgenic lines in S. ratti.13 This significant result hinged upon chromosomal integration of transgene sequences, and this was achieved using elements of the piggyBac transposon system. This paper is the subject of this Commentary.

Mobile Genetic Elements (MGEs) Occur Naturally in the Genomes of Parasitic Nematodes and Some Likely Originated via Horizontal Transfer from the Host

MGEs are important drivers of genome evolution in eukaryotic organisms, and genomic study of nematodes (including parasitic species) reveals that this is no less true for these worms.14 Both long-terminal repeat retrotransposons (LTRR) and Non-LTRR have been have been discovered in Ascaris lumbricoides,15,16 and a family of non-LTRR, dubbed the Dingo retrotransposons, was recently reported from the genome of the hookworm Ancylostoma caninum.17 In addition to retrotransposons, Class II MGEs, which are DNA transposons, have been discovered in C. elegans (active transposons Tc1-Tc5)18 and a number of parasitic nematodes, particularly within phylogenetic Clade V.19 These parasites include the trichostrongyles, which are important parasites of ruminant livestock, and hookworms, which are important pathogens of both humans and domestic animals. The Class II MGEs discovered in Clade V parasites include Tc1-like transposons such as hctcl from the trichostrongyle Hemonchus contortus and Mariner-like elements, including mle-1 from the trichostrongyle Trichosymylylus colubriformis and Bandit from the hookworm A. caninum.20 We have also discovered SMART from the threadworm Strongyloides stercoralis, a Clade IV parasite, only distantly related to the hookworms and trichostrongyles (Lok et al. unpublished). A defining characteristic of DNA transposons is their propensity for horizontal transfer between species. Phylogenetic analysis revealed that, at the time of its discovery, the closest known relative of the Bandit transposon from A. caninum was the Mariner-like element.
Hsmar-1 from humans, strongly suggesting that Bandit originated from horizontal transfer of a Mariner-like element to hookworms from their human hosts. Our own phylogenetic study of SMART in S. stercoralis (unpublished) reveals, similarly, that its closest relatives are Bandit from A. caninum and Hsmar-1 suggesting that this transposon was also acquired by S. stercoralis via horizontal transfer from its human host. It is interesting to note that retrotransposons have evidently been involved in horizontal transfer of other sequences to nematodes from other species. One prominent example of this is the apparent transfer of genes of insect origin, such as those encoding Diapausins, to the nematode Pristionchus pacificus, which lives in necromenic association with beetles.

**DNA Transposons have Practical Applications in Functional Genomic Study of Parasitic Nematodes and Other Parasitic Helminths**

The ability of MGEs to integrate into eukaryotic genomes makes them potential vehicles for experimental transformation of parasitic nematodes and other modifications of their genomes. Recent papers from Brindley and colleagues demonstrate the capabilities of pseudotyped retroviruses to integrate transgenes into the genomes of parasitic flatworms, the blood flukes Schistosoma mansoni and S. japonicum. The Brindley lab has also achieved integrative transformation of S. mansoni using a modified DNA transposon, piggyBac, by transducing larval stages (schistosomules) with plasmid vectors encoding a reporter transgene flanked by truncated piggyBac inverted terminal repeats (ITRs) and capped mRNA encoding the piggyBac transposase. Demonstrating the feasibility of using MGEs as vectors for integrative transgenesis in schistosomes proved to be crucial in the development of methods for stable transgenesis in parasitic nematodes.

As previously indicated, our overall approach to transgenesis in parasitic nematodes was to select a subject species, preferably one of medical significance, that was most amenable to adaptation of standard methods for gene transfer in *C. elegans*, which involve infusing solutions of vector DNA into the gonadal syncytium by microinjection. The unique life cycles of Strongyloides spp, comprising both free-living and parasitic generations, provide access to free-living female worms of a similar size and body plan to *C. elegans* hermaphrodites, including a conspicuous gonadal syncytium that can be easily microinjected with nucleic acid solutions. Once it was determined that endogenous 3' and 5' regulatory sequences were necessary for promoter-regulated expression of transgenes in *S. stercoralis*, it became feasible to isolate transformed progeny of microinjected free-living females, rear them to infective stage larvae in vitro and to propagate these F1 transgenics through repeated rounds of alternating host and culture passage. As indicated, host passage is required for establishment of transgenic lines of any obligately parasitic helminth, as none can be cultured continuously in vitro. *Strongyloides* offers the advantage of one or more generations of free-living development, enhancing its utility as a subject for initial attempts at transgenesis. However, even in these most tractable of parasitic nematodes, free-living cycles must alternate with parasitic ones in deriving and maintaining transgenic lines. This notwithstanding, our first attempt at host passage of F1 transgenic *S. stercoralis* revealed that a significant proportion of the progeny of parasitic females transformed with plasmid vectors by gonadal microinjection retained transgene sequences through at least three generations of host and culture passage, indicating that germline transformation had occurred. F1 transgenic *S. stercoralis* larvae generated by this technique express transgenes in anatomical patterns that are consistent with their *C. elegans* orthologs. However, parasites transformed in this manner show no evidence of transgene expression in the F2 generation of passage and beyond. We hypothesize that this represents active silencing of transgenes in the F2 and subsequent generations of transgenic *S. stercoralis* (Fig. 2).

In assessing possible explanations for transgene silencing in *S. stercoralis*, we assumed that like *C. elegans*, these parasites assemble the preponderance of microinjected plasmid-based vectors into tandem, multi-copy arrays located in episomes (Fig. 2). Based upon this assumption, we hypothesized that these sequences are silenced by *S. stercoralis* (and, as subsequent unpublished studies showed, by *S. ratti* as well) because of their highly repetitive nature and/or their episomal location. We resolved to test this hypothesis by integrating a reporter transgene of proven functionality into the genome of *S. stercoralis* or *S. ratti* and searching for evidence of expression in F2 transgenic worms. Because of its demonstrated capability to integrate transgenes into the chromosomes of *S. mansoni*, we selected the piggyBac transposon as a system for transgene integration. As an additional precaution against epigenetic silencing, we also tested insulator sequences from another MGE, the gypsy retrovirus, that are known to resist positional silencing of transgenes in *Drosophila*.

The piggyBac transposon, originally discovered in the cabbage looper moth *Trichoplusia ni*, has a number of advantages that enhance its utility as a tool for transgenesis or other genome modifications in a broad range of eukaryotic taxa. Its high frequency of transposition and its capacity for transposing DNA inserts or ’cargoes’ that are large (up to 18 kb) relative to other MGEs make it an efficient vector system for transgenesis. Moreover, piggyBac has a propensity to integrate into coding sequences and this makes it an attractive agent for forward genetics using insertional mutagenesis and for ’trapping’ of genes and regulatory elements such as promoters and enhancers. Finally, piggyBac may be remobilized from the genomes of transposed organisms without leaving a ’footprint’ or characteristic pattern of unexcised nucleotides as seen with other transposons. These advantages, coupled with its ability to transpose in a wide variety taxa, have stimulated interest in piggyBac as a vector system for functional genomic study of numerous divergent parasites and their arthropod vectors. In addition to schistosomes and *Strongyloides*, piggyBac has become the vector of choice for transgenesis and mutagenesis in the malaria parasites *Plasmodium falciparum* and *P. berghei* and in *Anopheles* and *Aedes* mosquitoes. Its tendency to
**Plasmid Vector**

- **P0**: Transform with plasmid-encoded transgene.
- **F1**: Derive F1 in culture; select for transgene expression.
- **F2**: Derive F2 by host passage of selected transgene expressing larvae.

Episomal transgene array retained but silenced

Episomal inheritance of silenced array-encoded transgene; lost without selection

Array-encoded transgenes silenced in F2 generation of passage and beyond.

**piggyBac Transposon**

- **P0**: Co-transform with plasmid-encoded transposase and piggyBac-encoded transgene.
- **F1**: Derive F1 in culture; select for transgene expression.
- **F2**: Derive F2 by host passage of selected transgene expressing larvae.

Transposon vector (Transgene)

Episomal transgene array (transposase) Expressed

Mendelian inheritance of expressed integrated transgene; selection of stable lines feasible.

Integrated transgenes expressed in F2 generation of passage and beyond.
transpose into transcriptional units has also enabled its use in enhancer trapping studies in *Anopheles gambiae*.35

Whereas efforts to achieve stable, heritable expression of transgenes encoded in conventional plasmid vectors were uniformly unsuccessful, attempts to accomplish this using integrative transformation by piggyBac were immediately successful in *S. stercoralis* and *S. ratti*. Parental free-living female *S. stercoralis* or *S. ratti* were co-transformed by microinjection with a donor plasmid encoding a GFP reporter transgene flanked by the piggyBac inverted terminal repeats and the gypsy insulator sequences and a helper plasmid encoding the piggyBac transposase. Host passage of F1 co-transformed parasites resulted in the first F2 individuals observed to express the reporter transgene (Fig. 2).35,36 Subsequent experiments in *S. ratti*, which has the advantage over *S. stercoralis* of a well-adapted rodent host in which to undertake serial passage, demonstrated that these transgene-expressing F2 worms could be used to establish stable lines that uniformly transmit and express the reporter.13 These experiments also demonstrated that the gypsy insulators were not necessary to sustain transgene expression per se and that integration into the chromosome is the essential key to avoiding the active silencing of transgenes. It is noteworthy that silencing of arrays encoding the transposase enzyme. Note that silencing of arrays encoding the transposase enzyme in worms transformed with piggyBac vectors will prevent inadvertent re-mobilization of the integrated transgene copies in the F2 generation and beyond. Green and gray worm cartoons indicate parasites that express or do not express the transgene, respectively. Green and orange inserts in plasmid and transposon cartoons denote transgene and transposase coding sequences, respectively. Black arrowheads flanking the transgene insert in the transposon vector cartoon denote the piggyBac ITRs. Cartoon design is based on the concept of Mello and Fire26 in their depiction of hypothetical processing and inheritance of plasmid-encoded transgenes delivered by microinjection into oocyte nuclei of *C. elegans*.

The piggyBac system has the potential to be a robust method not only for transgenesis in *S. ratti*, but also for identification of regulatory elements and for unbiased forward genetics.

Stable transgenesis in *S. ratti* mediated by piggyBac could provide an entrée into other functional genomic methods for parasitic nematodes. For example it could facilitate development of gene silencing by RNAi as a functional genomic method in this parasite. Like other animal parasitic nematodes, *Strongyloides* has proven largely refractory to post-transcriptional silencing of genes by administration of sequence-specific double-stranded (ds) RNA. While most components of the RNAi pathway are conserved in animal parasitic nematodes, deficiencies in dsRNA transporters homologous to *C. elegans* sid-1 and sid-2 have been noted in some,44 and this has been confirmed by genomic and transcriptomic study in *Strongyloides* (Lok et al. unpublished). It is possible that stable heterologous expression of one or both *C. elegans* dsRNA transporters could result in stable lines of transgenic parasite with enhanced sensitivity to RNAi and therefore greater utility as subjects for functional genomics.

Optimization of integrative transgenesis in *Strongyloides* will also require solution of some problems highlighted in our study. One in particular is the large number of transgene copies integrated by the piggyBac system as presently configured. This could result in nonspecific phenotypes, including loss of fitness in transgenic lines due to overexpression of transgenes or insertional mutagenesis. A clever technique, termed MosSCI (Mos induced single copy insertion), exists for single-copy transgene integration in *C. elegans*, which involves introducing a unique Mos1 transposon site into the genome and then creating episomal arrays containing a transgene encoding both the functional sequence of interest and a positive selection marker, another transgene encoding the Mos1 transposase under an inducible promoter and negative selection markers to subsequently eliminate the extra-chromosomal array.35,46 The challenge in *C. elegans* is to distinguish between worms expressing the transgene of interest from the episomal array and worms with a transgene integration. This is accomplished through the visual and negative selection markers encoded in the array. The technical challenges of creating strains of *Strongyloides* with unique transposon sites as integration targets are daunting but not insurmountable. Moreover, the selectable markers that facilitate single-copy integration in *C. elegans* are not currently available in *Strongyloides*, but alternatives exist in the form of fluorescent markers that are expressed only in integrants of the F2 generation and beyond and can be selected manually or via fluorescence activated sorting.47 More positively, the fact that *Strongyloides* appears to silence episomal transgene arrays, should eliminate the confounding factor of transgene expression from arrays that pertains in *C. elegans* and obviate the measures necessary to eliminate array-carrying worms. It is also noteworthy that the Mos1 transposon has been used in methods for gene deletion (MosDEL)48 and gene conversion (MosTIC)49 in *C. elegans*, and these systems might also be used to advantage in *Strongyloides*.

Ultimately, it will be highly desirable to apply the piggyBac transposon system to transgenesis in a wider range of animal parasitic nematodes. The chief hurdle in these studies will not be the mechanism of transgene integration but rather the physical mode of delivering DNA into the parasite. Among parasitic nematodes, availability of free-living females with
similar gonadal morphology to *C. elegans* is limited to *Strongyloides* and its relatives. It is unlikely, therefore, that gonadal microinjection will be broadly applicable for gene transfer into other medically important species of parasitic nematode. More likely is a system in which piggyBac or other integrating MGEs is introduced via chemically mediated gene transfer, as was recently accomplished for the filaria *Brugia malayi*. This system does not employ microinjection for gene delivery, but rather involves culturing worms through one or more molt cycles in the presence of DNA-calcium co-precipitates. Consequently it could be applicable to the wide range of parasitic nematodes that can be induced to develop for short intervals either in vitro or in vivo in the presence of these co-precipitates. Coupled with an efficient system for chromosomal integration of transgenes such as the piggyBac transposon, this method could make the powerful technique of transgenesis available, and thus greatly facilitate functional genomic study for a wide variety of parasitic nematodes.

**Conclusion**

MGEs, including DNA transposons and pseudotyped retroviruses have proven to be efficient vehicles for integration of transgenes into the chromosomes of parasitic helminths. The piggyBac transposon has numerous advantages as a tool for transgenics including the capacity for integrating large DNA inserts and a high frequency of transposition in a wide range of taxa. Among eukaryotic pathogens these include helminths such as *Schistosoma mansoni*, parasitic protozoa such as *Plasmodium falciparum* and several species of important vector mosquitoes. Recently, we have used the piggyBac transposon to integrate transgenes into the genome of the parasitic nematode *Strongyloides ratti*. This finding is highly significant because chromosomal integration sufficed to overcome active silencing of transgenes observed in previous attempts at stable transgenesis using conventional plasmid vectors that are presumably assembled by the parasite into repetitive episomal arrays. This has allowed derivation of the first lines of a parasitic nematode that stably transmit and express transgenes. This system will facilitate functional genomic study in parasitic nematodes and could hasten the identification of new drug and vaccine targets that are crucial to the control and elimination of these important pathogens.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**Acknowledgments**

I am grateful for advice and support from Paul Brindley on use of the piggyBac transposon system. I also thank Adrian Streit, Hongguang Shao and Jonathan Stoltzfus for critical comments on the manuscript. This work received support from US National Institutes of Health grants AI2548 to James Lok and Edward Pearce, AI50668 and AI22662 to James Lok and RR02512 to Mark Haskins.

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