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Transgenesis in parasitic nematodes: building a better array

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Abstract

In spite of recent progress in the development of transgenesis in parasitic nematodes, several impediments remain before this methodology can become a practical and widely employed tool in parasitology. Recently published studies on transgenesis in the necromenic nematode *Pristionchus pacificus* from the laboratory of Ralf Sommer highlight several leads that might be valuable as efforts to refine current systems in obligate parasites go forward.

Lessons learned from free-living nematodes

One of the great ironies of modern parasitology is that although a wealth of functional genomic methods exists for *Caenorhabditis elegans*, making it a prime model for genetic, molecular and cellular biological study, such techniques have been comparatively slow to develop for the very organisms to which *C. elegans* science would seem most readily applicable: parasitic nematodes. Transgenesis had been well established in *C. elegans* for more than two decades [1–4] before the first reports of gene delivery, heritable transgenesis and regulated, tissue-specific transgene expression in *Stongyloides stercoralis* [5,6] and *Parastrongyloides trichosuri* [7]. Because their life cycles feature generations of both free-living and parasitic individuals, these members of the superfamily Strongyloidea were likely candidates for the first forays into transgenesis for parasitic nematodes. These initial successes notwithstanding, both systems continue to have shortcomings that limit their applicability to basic studies of gene function. A recent paper [8], perhaps unnoticed by many parasitologists, reports a robust system for creating stable, transgene-expressing lines of the nematode *Pristionchus pacificus* (Figure 1a). In addition, this paper describes the preparation of a co-transformation marker for *P. pacificus* based on a dominant ‘roller’ mutation similar to the *rol-6* cuticular collagen in *C. elegans*. Together, these achievements represent a major advance towards a practical genetic system for *P. pacificus*, which, in its necromenic[†] association with scarab beetles (Figure 1b,c), undertakes many of the same crucial functions and life-cycle transitions that medically important parasitic nematodes execute in their vertebrate hosts [9,10]. Of more immediate relevance to transgenesis in medically important parasitic nematodes, it recounts the empirical optimization of conditions for founding lines of worms that stably inherit and express transgenes, including both behavioral and fluorescent reporters. The following discussion seeks to identify leads from this work that might help overcome remaining technical hurdles in the development of similar systems for parasitic nematodes.

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[†]Necromeny among soil-dwelling nematodes is an association in which dauer larvae transfer from the environment to an individual of another invertebrate species (a beetle, in the case of *P. pacificus*) and await the death of this carrier animal. When the carrier dies, the dauer larvae recommence development, feeding and propagating on the decomposing carcass. Available online 18 July 2009.

Transgenesis in *Strongyloides* and its relatives

As stated, there has been some notable, but qualified, success recently in work towards practical systems for transgenesis in *S. stercoralis* and *Ps. trichosuri*. In the case of *S. stercoralis*, F1 progeny of transformed free-living females express a variety of reporter constructs incorporating both red fluorescent protein and green fluorescent protein (GFP) that are easily detected in living worms in both the free-living and parasitic phases of development. However, whereas transgene DNA sequences have been detected through serial host and culture passage of *S. stercoralis*, expression of these sequences seems to be silenced transcriptionally in the F2 generation and beyond [6] and, to date, no stably expressing transgenic line of *S. stercoralis* has been established. Conversely, because *Ps. trichosuri* can execute repeated generations of free-living development, it has been possible to generate stable, transgene-expressing lines by serial culture passage and to maintain these with continuous transgene expression through passage in the marsupial host [7]. These experiments were performed with a plasmid-based transgene construct encoding both β -galactosidase (β -GAL) and GFP. Although β -GAL expression occurs through multiple generations of transgenic *Ps. trichosuri*, GFP expression has not been observed in these parasites and, to date, the system is without a practical *in vivo* marker for transgenesis [7].

Building complex episomal arrays in *Pristionchus*

Schlager *et al.* [8] provide an outstanding example of how careful analysis of previous experiences in the development of modern functional genomic methods for *C. elegans* can accelerate progress along the same lines in other nematode species. They set the stage by relating a simple and straightforward model of how transgene sequences delivered by microinjection into the syncytial gonad of *C. elegans* are concatenated into more or less repetitive extrachromosomal arrays, which they aptly describe as ‘...small, highly repetitive and artificial nematode chromosomes’. They note that in *C. elegans*, such multiple tandem repeats are subject to silencing, especially in the germline, and that in the case of transgenes, such silencing can be reversed by introducing complexity into these highly repetitive arrays [11]. Approaches to the formation of more complex arrays in *C. elegans* involve linearizing transgene constructs and introducing them with an excess of fragmented genomic ‘carrier’ DNA [11,12]. Schlager *et al.* [8] took a systematic approach to optimizing the configurations of transgene constructs and carrier DNA for heritable transformation of *P. pacificus*. In brief, they determined that the highest frequency of heritable transgenesis in this worm occurred when transgene DNA linearized by restriction digestion of plasmid constructs was co-injected into the gonad with an excess of *P. pacificus* genomic DNA (gDNA) having complementary restriction ends (Figure 1d,e). Stable transgenic lines were formed at much lower frequencies or not at all when circular plasmid constructs were administered, with or without excess gDNA digest. In addition, worms administered mixtures of linearized plasmid and digests of conspecific gDNA having non-complementary restriction ends were far less likely to found stable transgenic lines. Finally, it seems that conspecific gDNA is crucial to the process of forming transmissible episomal arrays because stable lines were generated in only one (0.2%) of 356 transformation attempts in which linearized plasmid constructs were co-injected with gDNA from a wide range of other animal species, including *C. elegans*, *Drosophila* sp., salmon and zebrafish (Figure 1e). This low frequency of heritable array formation with heterologous gDNA took place in spite of the presence of complementary restriction ends (Figure 1d,e).

Assuming, logically, that microinjected transgene sequences are predominantly incorporated into episomal arrays in *P. pacificus* (as they are in *C. elegans*), these findings indicate that factors favoring incorporation of transgenes into complex arrays, as opposed to homogenous

and highly repetitive ones, will mitigate against loss of transgene sequences and enhance the likelihood of establishing stable transgenic lines.

Relevance to obligate parasites

The optimization of transformation protocols in *P. pacificus* could provide an important lead in overcoming at least some of the current difficulties with establishing practical systems for transgenesis in *Strongyloides* and *Parastrongyloides*. Although the emphasis of Schlager *et al.* [8] was on the failure of highly repetitive transgene sequences to be inherited as episomes, recent findings on heterochromatic silencing of tandem repeat sequences in mammalian systems [13] also leave open the possibility that highly repetitive nematode sequences are more susceptible than others to epigenetic transcriptional silencing. It is possible, therefore, that formation of more complex arrays would prevent the transcriptional silencing of inherited transgenes that apparently occurs in *S. stercoralis* and *Ps. trichosuri*. Some tentative experiments along these lines have been attempted with *S. stercoralis* [7], but no systematic approach has been taken to optimize assembly of complex episomal arrays, and the conditions identified for *P. pacificus* – in particular, the generation of complementary restriction ends on both target transgene sequences and carrier DNA – provide an excellent starting point for such an undertaking. Finally, preparation of fluorescent reporters for *P. pacificus* stressed the importance of parasite specificity in both adenylation and splicing sequences [8]. Care has been taken to include parasite-specific 3' UTRs in transformation constructs for both *S. stercoralis* and *Ps. trichosuri* [5,7], and similar attention to upstream splice acceptors might be beneficial in deriving a fluorescent *in vivo* reporter gene for *Ps. trichosuri*.

It is logical for transgenesis in parasitic nematodes to build upon highly successful methods that favor formation of extrachromosomal elements in *C. elegans*, but the potential for methods favoring spontaneous chromosomal integration of transgenes should not be overlooked. Incorporating transgenes into chromosomes at low copy number might decrease their susceptibility to destabilization and epigenetic silencing. Furthermore, chromosomal elements would have the decided advantage of Mendelian inheritance, thus obviating the reselection necessary to maintain episomal transgenes through serial passage. In contrast to nematode systems, integrative methods are the norm in model systems such as tissue-specific human cell lines [14], mice [15], *Xenopus laevis* [16], *Drosophila melanogaster* [17] and medically important organisms such as mosquitoes [18], *Plasmodium* spp. [19] and *Leishmania* spp. [20]. Retroviral or transposon-based vectors are used frequently to integrate transgenes in these systems. In the present context, it is noteworthy that integrative transformation has recently been achieved using both types of vector in another medically important parasitic helminth, the trematode *Schistosoma mansoni* [21,22].

Concluding remarks

In presenting details of their transgenesis system for *Pristionchus pacificus*, Schlager *et al.* [8] have served the parasitology community in two ways. In a specific sense, they have underscored that conserved mechanisms for eliminating or silencing foreign DNA sequences probably operate in parasitic, as well as free-living, nematodes and have demonstrated that these mechanisms must, and can, be addressed in the development of transgenesis as a practical methodology. In a more general sense, they remind us of the wealth of practical leads that can be mined from *C. elegans* science as the quest for functional genomic methods for parasitic nematodes goes on.

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Figure 1.

Pristionchus pacificus: a model for assembly of heritable, stably expressed transgene arrays in parasitic nematodes. **(a)** Scanning electron micrograph of an adult *P. pacificus*. **(b)** *Exomala orientalis*, a scarab beetle host of *P. pacificus*. **(c)** *P. pacificus* on cuticle of an *E. orientalis* carcass. **(d)** Configurations of transgene and carrier DNA tested in *P. pacificus* [8], with hypothetical resulting episomal arrays. **(e)** Relative heritability of transgenes delivered in configurations illustrated in **(d)**. Panel (a) courtesy of Ralf Sommer, panel (b) courtesy of Matthias Herrmann and panel (c) courtesy of Dan Bumbarger. All images used with permission.