Nucleic acid transfection and transgenesis in parasitic nematodes

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SUMMARY

Transgenesis is an essential tool for assessing gene function in any organism, and it is especially crucial for parasitic nematodes given the dwindling armamentarium of effective anthelmintics and the consequent need to validate essential molecular targets for new drugs and vaccines. Two of the major routes of gene delivery evaluated to date in parasitic nematodes, bombardment with DNA-coated microparticles and intragonadal microinjection of DNA constructs, draw upon experience with the free-living nematode *Caenorhabditis elegans*. Bombardment has been used to transiently transfect *Ascaris suum*, *Brugia malayi* and *Litomosoides sigmodontis* with both RNA and DNA. Microinjection has been used to achieve heritable transgenesis in *Strongyloides stercoralis*, *S. ratti* and *Parastrongyloides trichosuri* and for additional transient expression studies in *B. malayi*. A third route of gene delivery revisits a classic method involving DNA transfer facilitated by calcium-mediated permeabilization of recipient cells in developing *B. malayi* larvae and results in transgene inheritance through host and vector passage. Assembly of microinjected transgenes into multi-copy episomal arrays likely results in their transcriptional silencing in some parasitic nematodes. Methods such as transposon-mediated transgenesis that favour low-copy number chromosomal integration may remedy this impediment to establishing stable transgenic lines. In the future, stable transgenesis in parasitic nematodes could enable loss-of-function approaches by insertional mutagenesis, *in situ* expression of inhibitory double-stranded RNA or boosting RNAi susceptibility through heterologous expression of dsRNA processing and transport proteins.

Key words: Transgenesis, transfection, parasitic nematode, biolistics, microinjection, transposon.

INTRODUCTION

Over three decades following the first reports of transgenesis in the free-living nematode *Caenorhabditis elegans* (Stinchcomb *et al*. 1985), this methodology has found only limited applications in studies of the biology of parasitic nematodes. Nevertheless, some recent successes with heritable transgenesis in the Strongyoididae (Grant *et al*. 2006*a*; Li *et al*. 2006; Junio *et al*. 2008) and filariae (Xu *et al*. 2011) and some creative applications of transient nucleic acid transfection in studies of gene regulation in ascarids (Cohen *et al*. 2004; Cheng *et al*. 2007) and filariae (Higazi *et al*. 2002, 2004, 2005; Shu *et al*. 2003; Higazi and Unnasch, 2004; Higazi *et al*. 2005; Liu *et al*. 2007; Oliveira *et al*. 2008; Liu *et al*. 2010*a*; Liu *et al*. 2010*b*; Bailey *et al*. 2011) highlight the potentialities of even the limited methods for parasitic nematodes and set the stage for more rapid progress in the near future. This review will examine the methods and routes of nucleic acid transfer explored so far in parasitic nematodes and briefly recount applications of these in studies of molecular, cellular or developmental biology of these organisms. It will also explore the alternative modes of transgene

inheritance – chromosomal or episomal – and discuss the gene transfer methods, both present and proposed, that bias transgene fate towards one of these modes or the other. It will discuss some problems and pitfalls associated with transgenesis in parasitic nematodes. Finally, the review will project general future applications of transgenesis in parasitic nematodes, including one suggested by studies in *C. elegans* (Winston *et al*. 2007) that could boost RNAi sensitivity in parasitic nematodes.

For purposes of this review, the term **transfection** will be used to denote the transient, non-heritable transfer of nucleic acids into cultured parasitic nematode cells or embryos or into localized cells or tissues of whole worms and their transient expression there. **Transgenesis** will denote genetic transformation of whole, live parasitic nematodes accompanied by promoter-regulated, tissue-appropriate transgene expression, and **heritable transgenesis** will refer to this same type of genetic transformation that is stably inherited and expressed from one parasitic nematode generation to the next. In discussions of non-heritable and heritable transgenesis in this review, the parental or P0 generation will be understood to be the generation of parasitic nematodes into which genes are initially transferred. The F1 and subsequent generations will represent the progeny of these.

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Table 1. Summary of gene delivery methods used to date in parasitic nematodes with notes on resulting transgene fates and technical advantages and disadvantages of each

Gene delivery method	Episomal arrays formed?	Relative frequency of spontaneous chromosomal integration	Heritable transgenesis achieved?	Stable transgenic lines derived?	Technical advantages	Technical disadvantages	Key references
Gonadal microinjection	Yes	Low	Yes	Yes	*C. elegans* methods easily adapted to *Strongyloides* and *Parastrongyloides*	Specialized equipment required; learning curve for microinjection technique	Li *et al.* 2006; Grant *et al.* 2006a; Junio *et al.* 2008
Microparticle bombardment	Yes	High	?	No	DNA transfer protocols are rapid and easy. Potential to transfect large numbers of parasites in a single bombardment	Specialized equipment required. Risk of physical damage to bombarded parasites. Selectable co-transformation markers not yet available	Jackstadt *et al.* 1999; Davis *et al.* 1999; Higazi *et al.* 2002; Praitis *et al.* 2001, 2006
Chemical transformation	Unkown	Unknown	Yes	No	DNA transfer is easy	Maximum gene transfer efficiency in *Brugia* requires administration during larval moulting	Xu *et al.* 2011

ROUTES AND MODES OF NUCLEIC ACID DELIVERY

Genetic modification of parasitic nematodes has been accomplished by delivering DNA or RNA constructs using biolistics (Davis *et al*. 1999; Jackstadt *et al*. 1999; Higazi *et al*. 2002), gonadal microinjection (Lok and Massey, 2002; Grant *et al*. 2006a; Li *et al*. 2006) and recently, chemical transfection (Xu *et al*. 2011) (Summarized in Table 1). Biolistics and gonadal microinjection have been used for both transient transfection with DNA or RNA and for DNA transgenesis. While most definitive for gonadal microinjection and chemical transfection, there is evidence that DNA transfer by any of these methods can result in heritable transgenesis in certain parasitic nematodes. Selection of one method over another will generally be based on the parasite species in question, the availability of stages in that species that can be cultured *in vitro* or *ex vivo* and the numbers of parental transformants that are required for establishment of stable transgenic lines. The mode of DNA delivery into nematodes also appears to affect the relative frequencies of episomal transgene incorporation or integration into the chromosome (Mello and Fire, 1995; Praitis *et al*. 2001; Praitis, 2006).

Biolistic transfer of nucleic acids

Transfection or transformation of living tissues or organisms may be achieved by bombardment with nucleic acid-coated tungsten, glass or, most often, gold microparticles driven by pressurized helium. Biolistics was first used in nematodes for DNA transformation of *C. elegans* (Jackstadt *et al*. 1999; Wilm *et al*. 1999) and for transient transfection of *Ascaris suum* embryos with both DNA and RNA constructs (Davis *et al*. 1999). The work employing biolistic transfer of DNA and RNA in *Ascaris* provides an excellent example of how well planned applications of transient transfection can yield important new insights into mechanisms of gene regulation in parasites. Specifically, these studies revealed the nature of a minimal splice leader promoter in *Ascaris* (Davis *et al*. 1999) and provided important new insights into aspects of pre-mRNA processing (trans-splicing and capping) that affect translation efficiency (Cohen *et al*. 2004).

The filariae *Litomosoides sigmodontis* (Jackstadt *et al*. 1999) and *Brugia malayi* (Higazi *et al*. 2002) are also susceptible to transformation by biolistic DNA transfer. In the case of *L. sigmodontis*, DNA transferred by bombardment was apparently spread systemically in parental adult worms and expressed in promoter-regulated fashion in specific tissues of male and female worms. Intriguingly, small numbers of F1 microfilariae expressing the *lacZ* reporter transgene were recovered following implantation of bombarded *L. sigmodontis* adults into rodent hosts (*Mastomys coucha*) (Jackstadt *et al*. 1999). The ability

of these microfilariae to cycle through vector mites and rodent hosts was not ascertained, and further work involving transgenesis in *L. sigmodontis* has not been forthcoming to date. Biolistic transfection has been used to transfer DNA into embryos of *Brugia malayi,* and this system has yielded a wealth of information on the characteristics of minimal and optimal promoters for a number of genes in this parasite (Shu *et al.* 2003; Higazi *et al.* 2005; Bailey *et al.* 2011), the primary and secondary structural characteristics of trans-splicing elements (Higazi and Unnasch, 2004; Liu *et al.* 2010*a*) and the existence of operons in *Brugia* and their structural and functional characteristics (Liu *et al.* 2010*b*). This approach has also demonstrated the presence of an intact ecdysone signaling system in *Brugia* in which the ecdysone receptor homologue interacts *in vivo* with response elements in transfected gene constructs to upregulate reporter expression, thus underscoring the potential for ecdysteroid regulation of moulting or reproduction in this parasite (Tzertzinis *et al.* 2010). Thus, biolistic transfection, when used to induce transient expression in embryos, has proven a highly successful system for basic studies of gene regulation in *Brugia.* However, heritable transformation of parasitic nematodes by the biolistic route has not been observed. Culture conditions that allow development of transfected *Brugia* embryos to developmentally competent microfilariae have not yet been identified, and attempts to transfect later larval stages via the biolistic route have resulted in such severe damage to the cuticles of these forms as to render them non-viable (Higazi *et al.* 2002; Xu *et al.* 2011). As indicated above, transgenic microfilarial progeny of *L. sigmodontis* transfected by microparticle bombardment were not assessed for their ability to undergo further development in vectors and hosts and to transmit the transgenes to their progeny (Jackstadt *et al.* 1999).

In contrast to the results to date with parasitic nematodes, biolistic DNA transfer results in a high frequency of heritable transformation in *C. elegans,* albeit with high mortality in the P0 generation (Jackstadt *et al.* 1999; Wilm *et al.* 1999). Unlike gonadal microinjection, which favours assembly of transgenes into high-copy number episomal arrays in *C. elegans* (Mello and Fire, 1995), microparticle bombardment appears inherently to favour low-copy number chromosomal insertions (Praitis *et al.* 2001) and also may result in a significant frequency of homologous recombination (Berezikov *et al.* 2004) (Table 1). As noted by Praitis and colleagues, the ability of bombardment to integrate relatively small numbers of transgene copies into the *C. elegans* chromosome mitigates against the confounding physiological effects of transgene overexpression in experimental settings, this being particularly important with transgenes whose products are toxic when expressed at high levels. In demonstrating stable integrative transformation following biolistic DNA transfer, Praitis *et al.* (2001) also used a strategy of *unc-119* mutant rescue to select transformants from among large populations of bombarded worms. This strategy, which is now standard in methodology for *C. elegans* (Evans, 2006) involves gene transfers (bombardments) in the *unc-119* mutant background and incorporation an *unc-119* rescuing plasmid as a co-transformation marker with the transgene of interest. Rescue of the motility-deficit phenotype in *unc-119* worms, conferring normal progressive motility, greatly facilitates isolation of viable transformants from the large excess of non-transformed worms following bombardment.

In summary, the biolistic route of nucleic acid transfer has been used for transient transfections to study aspects of gene regulation and mRNA processing in parasitic nematodes. It has not, however, been used successfully to generate stable, heritable DNA transformation in these organisms. In general, this has been attributed to the inability to identify bombardment conditions that yield developmentally competent parasite stages for establishment in animal hosts. Nevertheless, modern biolistic systems allow for some flexibility in modifying conditions under which microparticles are propelled into recipient cells or animal tissues, and with sufficient study, such conditions might be identified for some parasitic nematodes. The rewards for such an achievement could be significant given the advantages of biolistic gene transfer in *C. elegans.* First, this method results in a high frequency of spontaneous, low-copy transgene insertions into the chromosomes of recipient worms, giving transgenic lines that 'breed true' and are not subject to the confounding effects of high-copy incorporation into episomal arrays that are the predominant result of gonadal microinjection. Second, the potential of biolistics to transfer DNA into small stages such as embryos or developing larvae could provide an entré for transgenesis in the majority of parasitic nematode species in which adult worms are essentially inaccessible within the host.

Gonadal microinjection

The syncytial nature of the *C. elegans* gonad and the overall transparency of this worm, allowing easy visualization of this region by differential interference contrast (DIC) microscopy, has made gonadal micro-injection the most widely used approach to transgenesis in this model organism (Mello and Fire, 1995; Evans, 2006). The presence of free-living females in the life cycles of *Strongyloides* and related genera of parasitic nematodes and the similarity of these free-living females to *C. elegans* hermaphrodites in terms of size, cuticular transparency and the syncytial character of their gonads (Schad, 1989; Viney and Ashford, 1990; Viney, 1999; Grant *et al.* 2006*b*;

Viney and Lok, 2007), has facilitated adaptation of gonadal microinjection as a method for gene transfer in these parasites (Table 1). Expression of reporter constructs in embryos of *S. stercoralis* following intragonadal microinjection was achieved almost a decade ago (Lok and Massey, 2002). However, it was not until these constructs were modified to contain an *S. stercoralis*-derived 3′ UTR that promoter-regulated, tissue-specific expression of transgene constructs was attained in F1 progeny of parental *S. stercoralis* transformed by gonadal microinjection (Li *et al.* 2006). Subsequently, an expanded number of additional fluorescent reporter constructs was developed, each incorporating a promoter with a unique tissue-specific pattern of activation, and the 3′ UTR from the *S. stercoralis* gene *Ss-era-1* as a standard, multifunctional terminator (Junio *et al.* 2008). These have been collected, along with promoterless vectors encoding either red or green fluorescent protein in all possible reading frames, into a small vector kit that is available to the research community through AddGene at http://www.addgene.org/pgvec1? identifier=Lok&f=c&cmd=searchpl&x=28&y=12. This mode of transgenesis was recently applied in a functional study that demonstrated the requirement for *Ss-daf-16* (previously denoted *fktf-1*) function in normal morphogenesis of infective third-stage larvae (L3i) in *S. stercoralis* (Castelletto *et al.* 2009). *Ss-daf-16* encodes the orthologue of a synonymous insulin-regulated transcription factor that brings about dauer larval development in *C. elegans* (Lin *et al.* 1997; Ogg *et al.* 1997; Massey *et al.* 2003). Recently, we showed that vector constructs designed for *S. stercoralis* are also expressed in F1 larvae of *Strongyloides ratti* derived from parental free-living females transformed by gonadal microinjection (Li *et al.* 2011). Anatomical expression patterns of these reporter constructs are virtually identical in the two parasites.

Parastrongyloides trichosuri, a parasite of the Australian brush-tailed possum, is also capable of undertaking generations of free-living development, and its free-living females may also be transformed by intragonadal microinjection of DNA vectors (Grant *et al.* 2006a). A reporter construct encoding a fusion of GFP and *β*-galactosidase under the *Pt-hsp-1* promoter was expressed in progeny of microinjected free-living females and in parasitic females explanted from the host. Although production of transgenic F1 larvae from microinjected parasitic females was inefficient and limited by the transient viability of the parental worms in culture this latter finding is significant because it may point to a feasible protocol for DNA transformation of parasitic nematodes, such as hookworms and trichostrongyles, that do not form free-living adults.

Unlike *S. stercoralis*, and most other *Strongyloides* spp., which can undergo only one generation of free-living development before reverting to parasitism, *P. trichosuri* may undergo an apparently unlimited number of free-living generations under optimum conditions, and yet retain the capacity to form parasitic L3i and invade the host under conditions of crowding or food depletion. Broadly viewed, this property gives *P. trichosuri* the potential to fulfill the role of a genetic model for the study of parasitic nematode biology (Grant *et al.* 2006b). With specific regard to transgenesis, it has allowed stable transgenic lines to be derived by *in vitro* passage whereas this process requires alternating rounds of host and culture passage in *S. stercoralis*.

The filaria *Brugia malayi* has been transiently transformed by intragonadal microinjection of plasmid vectors (Higazi *et al.* 2002). Although it was impossible in the context of this study to identify a gonadal syncytium in *B. malayi*, adult worms microinjected in several locations along the uterus expressed both GFP- and luciferase-encoding vectors. Localization studies revealed that, whereas biolistic gene transfer resulted in expression restricted to hypodermal cells of bombarded adults, embryos proximal to points of intrauterine microinjection took up and expressed GFP-encoding vector constructs, leaving open the possibility of heritable transformation via this route. Exploring this possibility would require that explanted, microinjected adult *B. malayi* be reared either *in vitro* under conditions supporting embryonic development to the microfilarial stage or by re-implantation into susceptible hosts such as Mongolian jirds. To date, the difficult experiments required to make this assessment have not been undertaken. Recent results on successful chemically mediated transformation of *B. malayi* (Xu *et al.* 2011) may obviate this process.

Chemically mediated transformation

One of the primary barriers to uptake of exogenous DNA into cells is the hydrophobicity of cell membranes relative to this polar molecule. For almost four decades it has been standard procedure to render bacterial cell membranes permeable to exogenous naked DNA by pretreating with high concentrations of calcium (Mandel and Higa, 1970; Cohen *et al.* 1972). Similarly, eukaryotic cells may take up exogenous naked DNA directly when it is presented as a precipitate with $CaPO_4$ (Sambrook *et al.* 1989). Recently, this simple approach was shown to mediate heritable DNA transformation of *B. malayi* (Xu *et al.* 2011) (Table 1). This was accomplished by co-injecting infective *B. malayi* larvae and $DNA/CaCl_2$ precipitates into the peritoneal cavities of susceptible Mongolian jirds. A large proportion of developing parasites took up the DNA constructs as indicated by detectable transgene expression in over 60% of adults recovered from jirds treated in this way. Most significantly, these transgenic adults produced F1

transgenic microfilariae that survived cryopreservation and, upon thawing, demonstrated developmental competency by infecting mosquitoes at a frequency equal to that of non-transformed cryopreserved microfilariae. While the frequency of germline transformation among these F1 progeny remains to be determined, this finding represents a highly significant advancement towards a stable DNA transformation in *B. malayi*, which represents a complex of lymphatic-dwelling filariae that cause disease in over 200 million persons. Moreover, it demonstrates the utility of a long-standing method for cell transfection in transforming whole parasitic nematodes and may indicate a simpler method of DNA transfer into parental worms of many species than either microparticle bombardment or gonadal microinjection.

POSSIBLE FATES AND MODES OF INHERITANCE OF ADMINISTERED TRANSGENES IN PARASITIC NEMATODES

Based upon experience with *C. elegans* and other nematodes for which transgenesis has been achieved, the fate of transgene sequences administered as plasmid DNA depends to a great degree upon the route of DNA transfer. While transgenes may be stably transmitted as extrachromosomal elements, we submit that single- or low-copy chromosomal integration of transgenes is the goal of efforts towards transgenesis in parasitic nematodes.

Formation of episomal arrays

In up to 90% of *C. elegans* transformed with plasmid-encoded transgenes introduced by gonadal micro-injection, DNA is simply partitioned among somatic cells, expressed transiently and not inherited. In the remaining 10% of transformants, the great majority have transgenes assembled into tandem, multi-copy episomal arrays (Mello and Fire, 1995). These have been aptly described as behaving like mini-chromosomes that may be stably transmitted, though not in Mendelian fashion, through multiple generations in *C. elegans* (Schlager *et al*. 2009). It is assumed, but has not been rigorously proven, that microinjected transgene sequences are also assembled into repetitive episomal arrays in parasitic nematodes (Grant *et al*. 2006a; Junio *et al*. 2008). There are several disadvantages to maintenance of transgenes as episomal arrays. First, the arrays themselves may be lost after repeated generations of passage (Schlager *et al*. 2009) or transcriptionally silenced, especially in the germline (Kelly *et al*. 1997). Second, the highly repetitive nature of these arrays may result in levels of overexpression that are either toxic to transformed worms or exert non-physiological effects in them (Praitis *et al*. 2001).

Although transient transfection and transgenesis have found application in a number of studies of gene function and regulation in parasitic nematodes (Unnasch *et al*. 1999; Higazi *et al*. 2002, 2005; Higazi and Unnasch, 2004; Oliveira *et al*. 2008; Castelletto *et al*. 2009; Liu *et al*. 2010b; Tzertzinis *et al*. 2010; Bailey *et al*. 2011), transgenesis will only become a robust functional genomic tool when it becomes generally practical to derive stable transgene-expressing lines of parasitic nematodes. This goal has been technically accomplished in *P. trichosuri* which, by virtue of its ability to cycle continuously in free-living culture, has allowed stable transgene-expressing lines to be established by culture passage and transferred to the possum host for future study (Grant *et al*. 2006a). By contrast, consolidation of recent successes with production of F1 transgenic *S. stercoralis*, *S. ratti* (Li *et al*. 2006; Junio *et al*. 2008; Li *et al*. 2011) and *B. malayi* (Xu *et al*. 2011) by establishment of transgenic lines will require alternating generations of host and culture passage for *Strongyloides* spp. and serial rounds of passage in both vector and host for *B. malayi*. It is anticipated that this will also be true for other medically important parasitic nematodes for which transgenesis is achieved in the future.

In the strict sense, stable transgenesis was achieved some years ago in *S. stercoralis* when F1 transgenic larvae, derived by gonadal microinjection of circular plasmid vector encoding GFP under the *Ss-act-2* promoter, were subjected to four alternating passages in susceptible gerbils and free-living culture to an F5 generation. Transgene (*gfp*)-specific sequences were detected in each generation by PCR, indicating stable transformation, but GFP expression, as evidenced by visual detection of the fluorescent protein or by transcription of specific mRNA, could be detected only in the F1 generation (Junio *et al*. 2008). This silencing may be due to epigenetic mechanisms incited by some aspect of the configuration of transgene DNA in the parasite.

It is assumed that, like *C. elegans*, parasitic nematodes assemble gonadally microinjected transgene sequences, especially those delivered in circular plasmid vectors, into multi-copy episomal arrays. Although this assumption has not been rigorously proven in *Strongyloides spp*. or *Parastrongyloides trichosuri*, there is indirect evidence in support of it. First, transgenic parasitic female *S. stercoralis* that are derived from free-living parents transformed by gonadal microinjection transmit transgene sequences to less than 100% of their progeny (Junio *et al*. 2008). This pattern of inheritance is also seen in serial culture passage of transgenic *P. trichosuri* (Grant *et al*. 2006a) and is typical of *C. elegans* carrying transgenes in episomal arrays (Mello and Fire, 1995). Second, inverse PCR analyses conducted to date on transgenic *S. stercoralis* derived from parents microinjected with circular plasmids have revealed only stretches of vector sequence flanking reporter transgenes and

never genomic sequence from the parasite (Lok *et al*. unpublished). Similarly, Southern blots of total DNA from transgenic *S. stercoralis* only show transgene sequences that co-migrate with the vector.

Apparent transcriptional silencing of transgenes in *S. stercoralis* and *P. trichosuri* may also be related to their assembly into repetitive episomal arrays. The induction of epigenetic silencing by tandem repeat sequences in mammalian genes (Soragni *et al*. 2008), leaves open the possibility that parasitic nematodes have a high propensity, relative to *C. elegans*, to silence coding sequences in tandem repetitive arrays by epigenetic mechanisms. The problem of transcriptional silencing and loss of episomal transgene arrays has been discussed in the context of efforts to establish stable transgenesis in the necromenic nematode *Pristionchus pacificus* (Schlager *et al*. 2009). In this case, a retrospective analysis of various protocols for transforming *P. pacificus* by microinjection of DNA into the gonadal syncytium revealed that coinjection of linearized plasmid vectors with digests of genomic DNA from the worm with complementary restriction ends resulted in the highest frequency of stable transgene-expressing lines of several protocols tested, including syncytial injection of circular plasmid. The logical interpretation of this result was that the highlighted protocol resulted in interspersing of transgene sequences with significant stretches of gDNA from *Pr. pacificus*, thus reducing the repetitive character of the episomal arrays and rendering them less susceptible to epigenetic silencing or elimination (Schlager *et al*. 2009). This finding has been reviewed and the potential of such heterogenous arrays to inhibit transgene silencing in medically important parasitic nematodes discussed (Lok, 2009).

Chromosomal integration

Beyond their likely repetitive character, the episomal location of microinjected transgene sequences *per se* may also be a deciding factor in their silencing by parasitic nematodes. For this reason, methods of gene transfer that favour low-copy chromosomal integration may be the keys to establishment of parasite lines that inherit and express transgenes stably. The standard route of microinjecting plasmid-based constructs into the gonadal syncytium of *C. elegans* strongly favours formation of episomal transgene arrays, and spontaneous chromosomal integrations are exceedingly rare (Mello and Fire, 1995). By contrast, injection of such constructs into individual oocyte nuclei results in 0·1–0·5% of *C. elegans* with transgene integrations (Fire, 1986; Mello and Fire, 1995). Similarly, microinjecting linear transgene constructs into the gonadal syncytium in the presence of an excess of random 80-base oligonucleotides appears to promote recombination and results in approximately 1% of worms with chromosomal

integrations (Mello *et al*. 1991). Other approaches to integration of transgenes in *C. elegans* involve exploiting DNA repair mechanisms to drive transgene incorporation into chromosomes at breaks induced by gamma (Mello and Fire, 1995; Evans, 2006) or UV irradiation (Evans, 2006). Although they possess the advantage of having been standardized into reliable, published 'bench protocols' for *C. elegans* (Evans, 2006), these variations on microinjection technology all have disadvantages as practical alternatives for parasitic nematodes. For example, starting material for the methods involving integration of transgenes at induced chromosomal breaks is always a stable episomal line. With the exception of *Parastrongyloides trichosuri* (Grant *et al*. 2006a), no such episomal line has been derived in any parasitic nematode. Also, the frequency of integrations, even in the facilitated system outlined by Mello (Mello *et al*. 1991), is less than 1%, suggesting that the chances of encountering an integrant among the F1 progeny of a microinjected parasitic nematode are remote. As has been discussed above, microparticle bombardment inherently favours integrative transformation of *C. elegans* relative to microinjection of transgene sequences, and the proportion of bombardments that results in an integrated transgenic line may be as high as 35% (Praitis *et al*. 2001). However, even in this case, the practicality of the system for parasitic nematodes must be assessed in light of the fact that in *C. elegans*, transformants constitute only about 0·05% of the bombarded population. To the extent that these efficiencies may be extrapolated to parasitic nematodes, they combine to predict that production of a single chromosomal integrant individual would require bombardment of about 6,000 parasites. The fact that using such integrants to establish integrated transgenic lines would require passage in a susceptible host further diminishes the likelihood of success. Clearly, the 'arithmetic' dictates that alternative means of integrating transgene sequences into the chromosomes of parasitic nematodes be explored. In other systems, vectors encoding elements such as transposons or retroviruses with intrinsic capability to integrate into chromosomal DNA have provided a means of directly and efficiently incorporating transgenes into the genomes of recipient cells, tissues or whole organisms. Both of these approaches have been employed with a high degree of success in schistosomes (Mann *et al*. 2007) (see also Beckmann and Grevelding, in this special issue of *Parasitology*) and should be examined for their feasibility in parasitic nematodes.

Transposable elements and retroviruses as vectors for integrative transformation

The development of transposon-mediated transgenesis in schistosomes provides an appropriate model

for development of this approach in parasitic nematodes. A valuable background for this work is the increasingly substantial literature on mobile genetic elements that occur naturally in the genome of *Schistosoma mansoni* (Brindley *et al*. 2003; Mann *et al*. 2007). These endogenous elements are generally thought to have been rendered inactive by accumulating mutations, but it has been pointed out (Brindley *et al*. 2003; Mann *et al*. 2007) that such degenerate sequences can, like the *Sleeping Beauty* Tc1/*Mariner*-class transposon of salmon (Plasterk *et al*. 1999), be repaired by deliberate 'back mutation' and recovered as active elements for functional genomic study. While this avenue has not yet been explored in schistosomes, a transposon of insect origin, *piggyBac*, has been used to achieve integrative transgenesis in *S. mansoni* (Morales *et al*. 2007). This successful effort involved transfection of schistosomules with a linearized donor plasmid encoding a luciferase reporter gene flanked by the *piggyBac*-specific terminal inverted repeats and with *in vitro*-transcribed mRNA encoding the *piggyBac* transposase. Schistosomules transduced with these elements expressed active luciferase, and Southern hybridization and retrotransposon-anchored PCR (RAP) confirmed chromosomal integration of the reporter transgene. Sequencing of RAP products indicated that integrations were specific for TTAA insertion sites and occurred in at least 19 locations in the *S. mansoni* genome. It is also noteworthy that the *piggyBac* transposon system has formed the basis of a robust method of integrative transgenesis and a powerful functional genomic tool in *Plasmodium* spp. (Balu *et al*. 2005, 2009; Balu and Adams, 2006; Fonager *et al*. 2011). In addition to its applicability to highly diverse taxa, the *piggyBac* transposon has the advantage of its ability to transpose large inserts or 'cargoes' relative to other transposons (Nakazawa *et al*. 2009).

The success of transposon-mediated transgenesis in *Schistosoma* sp. and *Plasmodium* sp. argues strongly for efforts along similar lines in parasitic nematodes. Endogenous transposons have been discovered in a number of parasitic nematode species (Brindley *et al*. 2003; Laha *et al*. 2006, 2007). The *bandit* transposon of *Ancylostoma caninum* is particularly fascinating in that its close sequence homology to human Mariner-like elements suggests the likelihood of lateral gene transfer between host and parasite (Laha *et al*. 2007). Successful systems for transfection or transgenesis are prerequisites for testing a transposon-mediated system and their existence in *Strongyloides stercoralis*, *S. ratti*, *Parastrongyloides trichosuri*, *Brugia malayi*, *Litomosoides sigmodontis* and *Ascaris suum* argues strongly for a concerted effort to achieve transposon-mediated integrative transgenesis in these parasitic nematodes. Its successful application in parasite taxa as diverse as *Schistosoma* and *Plasmodium* make a strong case for

the *piggyBac* system as an opportune subject for this effort. To this end our laboratory has recently deployed the *piggyBac* transposon system as a possible means of integrative transgenesis in *S. stercoralis* and *S. ratti* (Shao *et al*., unpublished). A donor vector encoding GFP expressed under the promoter for *Ss-act-2*, which encodes a cellular actin principally localized in the body wall, and flanked by the *piggyBac*-specific inverted terminal repeats (Fig. 1A) was injected into the gonads of free-living *S. stercoralis* or *S. ratti* females along with capped mRNA transcribed *in vitro* from a plasmid encoding the *piggyBac* transposase under the T7 promoter (Fig. 1B). It should also be noted that in the donor construct (Fig. 1A), the *Ss-act-2::gfp* sequence was flanked by the gypsy retroviral insulator sequences from *Drosophila* as a precaution against transcriptional silencing of the transgene (Markstein *et al*. 2008). GFP-positive F1 larvae derived in culture from the microinjected female worms were reared to infective third-stage larvae (L3i) and were inoculated into susceptible gerbils (*S. stercoralis*) or rats (*S. ratti*) and F2 transgenic parasites were collected from the faeces of the animals at patency. Experiments to ascertain chromosomal integration of the transgenes in this study are still underway but, for both parasites tested, this combination of constructs yielded the first GFP-expressing F2 transgenics observed by us to date (Fig. 1C; Table 2). It is significant that in *C. elegans* such F2 transgenics serve as the founders of stable transgenic lines (Mello and Fire, 1995; Evans, 2006). The yield of transgene-expressing F2 individuals as a proportion of inoculated F1 transgenic L3i was almost eight-fold higher in *S. ratti* than in *S. stercoralis* (Table 2), probably owing to the higher infection efficiency of *S. ratti* in the rat. This fact underscores the advantage of *S. ratti* as a model with which to move forward with integrative transgenesis and establishment of stable transgenic lines.

The potential of retroviral vectors for transgenesis in parasitic nematodes is indicated by their successful application to transgenesis in schistosomes (Kines *et al*. 2006; Mann *et al*. 2007; Yang *et al*. 2010) (see also Beckmann and Grevelding, in this special issue of *Parasitology*). A vesicular stomatitis glycoprotein pseudotyped Maloney murine leukemia virus was used to transform schistosomules of *S. mansoni* (Kines *et al*. 2006) and *S. japonicum* (Yang *et al*. 2010). Schistosomules transformed in this manner express active luciferase (*S. mansoni*) or human telomerase reverse transcriptase (*S. japonicum*) from reporter transgenes. Moreover, the VSVG pseudotyped leukemia virus vector serves to integrate transgenes into the chromosomes of *S. mansoni* (Kines *et al*. 2008). As with the *piggyBac* transposon system, transgene integration was confirmed by Southern hybridization and integration site boundaries were detected and sequenced by RAP. These analyses
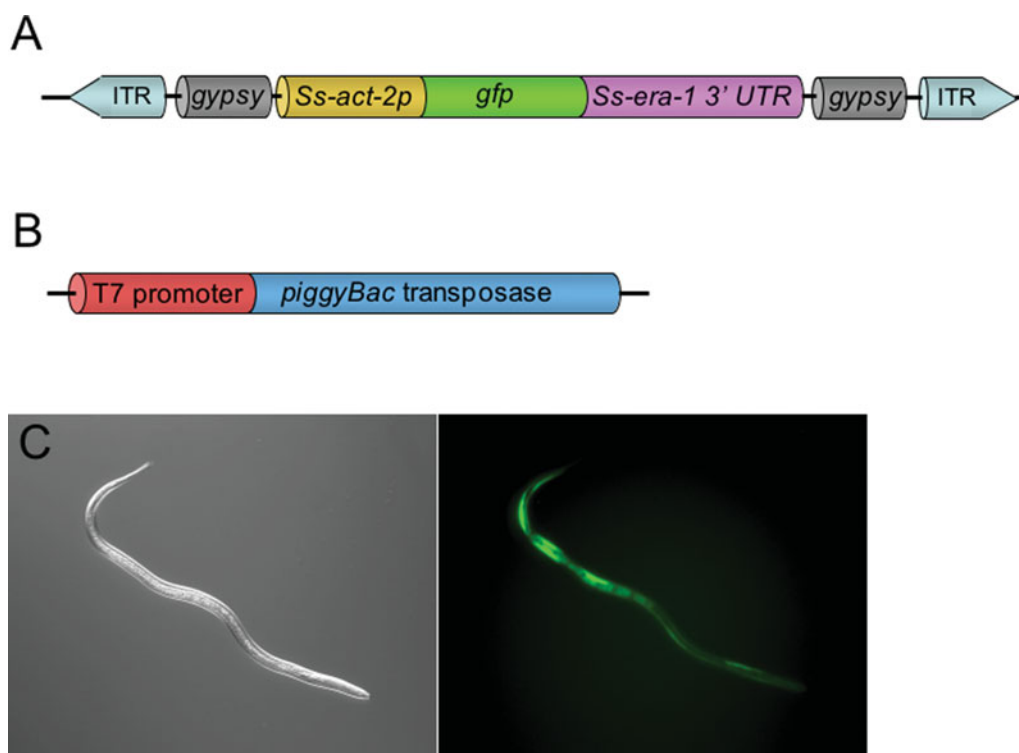
Fig. 1. Transgenesis in *Strongyloides stercoralis* and *S. ratti* mediated by the *piggyBac* transposon. **A**. Donor plasmid containing the coding sequence of *gfp* linked to the *Ss-era-1* 3′ UTR and expressed under the promoter for the *S. stercoralis* cellular actin *Ss-act-2*. Coding sequences are flanked by the gypsy retroviral insulator sequences from *Drosophila* and by the *piggyBac* inverted tandem repeats (ITR). **B**. Construct for *in vitro* transcription of mRNA encoding the *piggyBac* transposase. **C**. DIC (left) and fluorescence images (right) of an F2 transgenic *S. stercoralis* L3i expressing GFP. These and similar *S. ratti* larvae represent the first transgene-expressing individuals of the F2 generation observed to date for these parasites.

identified 16 integration sites of the retrovirus-encoded luciferase reporter in the *S. mansoni* genome with a common gGATcc motif at each. These results also call for evaluation of pseudotyped retroviruses as vectors for integrative transgenesis in parasitic nematodes.

FUTURE DIRECTIONS IN DEVELOPMENT AND APPLICATION OF TRANSGENESIS IN PARASITIC NEMATODES

The application of transient transfection in parasitic nematodes to studies of transcriptional gene regulation has already been actively pursued and the many substantial findings resulting from these studies have been discussed earlier in this review (Cohen *et al.* 1972; Davis *et al.* 1999; Higazi *et al.* 2002, 2005; Cohen *et al.* 2004; Higazi and Unnasch, 2004; Liu *et al.* 2007, 2009, 2010*a*,*b*; Tzertzinis *et al.* 2010; Bailey *et al.* 2011). Transient transfection serves satisfactorily for the experiments outlined in these studies, and it is likely and highly appropriate that these approaches continue to be used where possible for further studies of gene regulation in parasitic nematodes, especially in species that do not lend themselves to the manipulations necessary for achieving stable transgenesis. The following are applications for transient transfection or transgenesis that can presently be applied in *Brugia* spp. and in *Strongyloides* and related parasites and that will be extended to other medically important species as technical advances allow.

Analyzing spatial patterns of gene expression and trafficking of gene products in living parasitic nematodes

Constructs linking gene promoters to fluorescent, antigenic or chromogenic reporters have provided information about the patterns of gene expression in cells, tissues and organs of many organisms, including *C. elegans*, and these techniques will certainly translate to studies of the anatomical pattens of gene expression in parasitic nematodes as transgenesis is attained in these important pathogens. Reporter constructs incorporating promoters with high levels of site specificity have been reported in *P. trichosuri* (Grant *et al.* 2006*a*), *S. stercoralis* (Li *et al.* 2006; Junio *et al.* 2008; Castelletto *et al.* 2009) and *S. ratti* (Li *et al.* 2011), and consistent anatomical patterns of reporter gene expression have been seen in transiently, as well as stably, transformed individuals. A caveat in interpreting such results is the putative nature of the promoters involved. In fact, the characteristics of minimal and optimal promoters

Table 2. *Preliminary findings on heritable transposon-mediated transformation and the first sustained transgene expression in the F2 generations of* Strongyloides stercoralis *and* S. ratti

Parasite	Construct	No. passage attempts	Total no. GFP+ F1 L3i inoculated	Host	Total no. (%) parasitic females recovered	No. (%) GFP+ parasitic females recovered	No. (%) GFP+ F2 larvae recovered
S. stercoralis	* *piggyBac/ gypsy Ss-act-2p::gfp*	4	448	Gerbil	12 (2·67)	4 (0·89)	2 (0·44)
S. ratti	* *piggyBac/ gypsy Ss-act-2p::gfp*	3	88	Rat	8 (9·09)	1 (1·13)	3 (3·39)

* This construct is diagrammed in Fig. 1A. Parental free living females were microinjected intragonadally with the plasmid in circular form and capped mRNA encoding the piggyBac transposase transcribed *in vitro* from the construct diagrammed in Fig. 1B.

have been rigorously defined in only one parasitic nematode to our knowledge, *Brugia malayi* (Shu *et al*. 2003; Higazi *et al*. 2005; Bailey *et al*. 2011), and the majority of other studies employing reporter transgenes in parasitic nematodes have assumed that one to three kilobases of 5′ flanking sequence from the coding region of a gene contains an authentic promoter. The caveat notwithstanding, the overall similarities of gene expression patterns deduced by this approach in parasitic nematodes to patterns reported for orthologous genes in *C. elegans* (compare, for example, findings by Castelletto *et al*. (2009) and Lee *et al*. (2001) on *daf-16* orthologues in *S. stercoralis* and *C. elegans*) bolster confidence that this assumption is valid.

Elegant and comprehensive studies of organellar dynamics (Dzierszinski *et al*. 2004; Nishi *et al*. 2008) and secretory trafficking (Joiner and Roos, 2002) in the protozoan *Toxoplasma gondii* illustrate the potentialities of a robust system for transgenesis in the basic cell biology of a medically important parasite. Avenues such as these, which draw heavily upon the ability to express organelle-specific fluorescent reporters, will be opened for parasitic nematodes when stable transgenesis is achieved. Indeed, a transient transformation system in which a GFP-tagged Ss-DAF-16 was expressed in *Strongyloides stercoralis*, has already been used to find that nuclear versus cytoplasmic localization of this FOXO-class transcription factor is determined by phosphorylation of consensus AKT sites on the molecule (Castelletto *et al*. 2009). Stable transgenesis will enable a wide range of similar experimentation on parasitic nematodes in the future.

Facilitating RNAi as a functional genomic tool in parasitic nematodes

Given that the use of RNAi as a tool for functional genomic study was pioneered in *C. elegans* and that, with some reservations, the technique has been applied effectively in schistosomes, it is disappointing that RNAi has failed thus far to be widely applicable to studies in parasitic nematodes of animals (Knox *et al*. 2007). Very promising results have been obtained with filariae (Aboobaker and Blaxter, 2003; Lustigman *et al*. 2004; Ford *et al*. 2005, 2009; Pfarr *et al*. 2006; Tachu *et al*. 2008) and there have been some positive technical advances recently (Song *et al*. 2010; Samarasinghe *et al*. 2011). However, overall, a relatively small number of RNAi- sensitive targets have been interrogated successfully, and a relatively low efficiency in target knockdown and the consequent infrequency of informative phenotypes have hampered application of RNAi in many species of animal parasitic nematodes.

A system for stable transgenesis might go far towards surmounting the obstacles that hamper application of RNAi methodology in some parasitic nematodes. To the extent that anatomical barriers to uptake or cell-to-cell spread of exogenously applied dsRNA are limiting, vector-based RNAi, whereby dsRNA, short interfering RNA (siRNA) or short hairpin RNA (shRNA) is expressed from a transgene construct, could provide an answer for parasitic nematodes. Robust vector-based RNAi has been achieved in *C. elegans* (Tavernarakis *et al*. 2000) and adapted for large-scale screening approaches (Johnson *et al*. 2005), providing a model for the way forward in parasitic nematodes. As discussed by Beckmann and Grevelding (in this special issue of *Parasitology*), vector-based RNAi has been achieved in schistosomes (Zhao *et al*. 2008; Tchoubrieva *et al*. 2010; Ayuk *et al*. 2011), and this fact should make *in vivo* expression of interfering RNAs a top priority once stable transgenesis in parasitic nematodes is achieved.

Deficiencies in key protein components of the processing 'machinery' for exogenously applied dsRNA have been proposed as a cause of low or highly variable RNAi sensitivity in parasitic nematodes (Knox *et al*. 2007). However, a recent transcriptomic survey (Dalzell *et al*. 2011) reveals

that although parasitic nematodes generally have a less diverse repertoire of RNAi processing or transport proteins than *C. elegans*, they have conserved the major classes of them such that all are qualitatively represented. This study concludes that the low RNAi sensitivity that characterizes parasitic nematodes overall is not associated with any specific deficiency in RNAi processing or transport proteins and is more likely due to morphological barriers to dsRNA uptake or dissemination or to allelic differences in parasite populations studied to date. Nevertheless, while major classes of RNAi effectors such as the proteins affecting uptake and spreading of dsRNA or RISC silencing complex components such as the Argonaut proteins are conserved, individual proteins in these classes are conspicuous by their absence in certain parasitic nematode species (Dalzell *et al.* 2011). Therefore, the overall conclusions of Dalzell *et al.* (2011) notwithstanding, it may be rational to attempt heterologous complementation of some of these specific deficiencies by expressing such RNAi effectors from *C. elegans* in parasitic nematodes on a case-by-case basis as stable transgenesis is achieved. It is noteworthy in this regard that such heterologous expression of *C. elegans sid-2* in *Caenorhabditis briggsae* rendered that nematode susceptible to environmental RNAi (i.e. soaking) (Winston *et al.* 2007).

Studies of gene function using transgene constructs with dominant mutations

Absent a robust system for functional genomic study based on RNAi, transgenesis provides an alternative approach based on expression of transgene constructs encoding dominant loss-of-function mutations. In general, such 'dominant-negative' constructs would be designed to compete with endogenously expressed targets for binding sites or response elements but lack or actively suppress the function of the endogenous target. Such an approach has been used in transgenic parasites to demonstrate a role for the Ss-DAF-16 transcription factor (then designated FKTF-1) in morphogenesis of infective third-stage larvae of *Strongyloides stercoralis* (Castelletto *et al.* 2009). Similarly, overexpression of transgene constructs with activating mutations could, by virtue of their overexpression relative to the endogenous gene, achieve a dominant gain of function whose phenotypic effects could also shed light on gene function.

Forward genetics

In addition to their utility as vectors for integration of transgenes into parasite genomes, transposons hold great promise as agents of gene disruption through insertional mutagenesis in functional genomic studies. Transposon-based insertional mutagenesis approaches to ascertaining gene function are now

standard in *Drosophila*, mouse and human genetic systems (Thibault *et al.* 2004), and have recently been explored as a forward genetic tool to great effect in *Plasmodium* (Balu *et al.* 2005, 2009; Balu and Adams, 2006; Fonager *et al.* 2011). Fully acknowledging the challenges involved with mutagenesis as a functional genomic tool in parasitic nematodes (lack of *in vitro* systems for parasite propagation most notably) it is worth considering that a significant phenotype, ivermectin resistance, was selected in a chemically mutagenized population of *Strongyloides ratti* (Viney *et al.* 2002), supporting the feasibility of such forward genetic approaches in parasitic nematodes. As it does in the area of transgenesis, the *piggyBac* transposon has significant advantages as an agent for insertional mutagenesis. Unlike the P elements commonly used in *Drosophila* that tend to insert into 5′ regulatory sequences, *piggyBac* favours insertion into the 5′ ends of coding sequences (Thibault *et al.* 2004; Balu and Adams, 2006), thus increasing the likelihood of functional disruption of genes. Combined with this factor, *piggyBac* inserts randomly into recipient genomes at multiple sites, enhancing its efficiency as a mutagen. Documentation of at least 19 *piggyBac* insertion sites in *S. mansoni* (Morales *et al.* 2007) underscores the potential of this transposon system as a forward genetic tool in parasitic helminths and argues for its exploration in this context in parasitic nematodes.

Genetically targeted cell ablation

In aspects of cell biology, and particularly in neurobiology, the functions of individual cells have been deduced by ablating them and observing resulting effects on the organism in question. This approach, in which neuronal cell bodies are killed by microlaser surgery, has been widely adopted in *C. elegans* biology to deduce the neuron function (Bargmann and Avery, 1995; Gray *et al.* 2005; Bargmann, 2006) and was translated to the study of sensory neurobiology in parasitic nematodes by Schad and colleagues (Ashton *et al.* 1999, 2007; Lopez *et al.* 2000; Bhopale *et al.* 2001; Forbes *et al.* 2004; Ketschek *et al.* 2004). Although powerful, the technique of microlaser surgery requires a high degree of technical skill and is limited in the number of successfully operated nematodes that can be produced. Genetically targeted cell ablation, involving expression of lytic or other cytotoxic factors under the control of promoters with specificity for single or small subsets of cells, has been proposed as an alternative approach in *C. elegans* (Harbinder *et al.* 1997), with the most contemporary approach being the use of caspases reconstituted from subunits expressed under the control of promoters with specific patterns of expression in overlapping sets of cells (Fig. 2A–C) (Chelur and Chalfie, 2007). This system obviates the use of promoters with single-cell
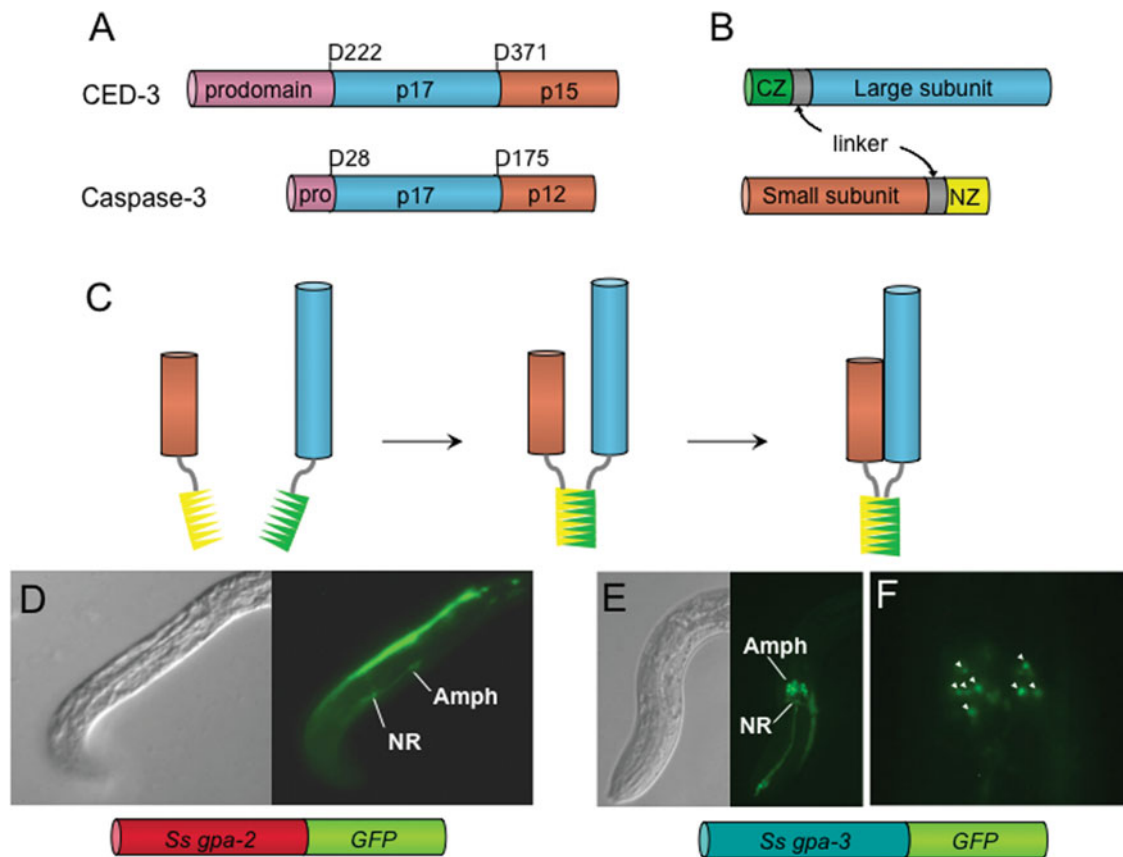
Fig. 2. Cell ablation by expression of reconstituted caspases: a potential application of transgenesis in *Strongyloides* sp. **A.** Schematics of the *C. elegans* CED-3 and caspase-3 procaspases showing prodomains (pink), large subunits (blue) and small subunits (orange). **B.** Expression constructs encoding the large and small subunits of a caspase fused to one of two antiparallel leucine zipper domains (CZ, green; NZ, yellow) and an intervening linker sequence (gray). These would be expressed under separate promoters with multi-cell specificities that overlapped in a single cell or cell type. **C.** In the single cells where the subunit constructs were co-expressed, the complementary leucine-zippers would bind, bringing the large and small subunits into proximity, reconstituting the active caspase and destroying the cell. **D – F** depict expression of neuron-specific reporter constructs in apparently overlapping subsets of amphidial neurons in transgenic *S. stercoralis*. **D.** DIC (left) and fluorescence images (right) of an F1 transgenic first-stage larva expressing GFP under the promoter of the Gα protein-encoding gene *Ss-gpa-2*. Note that a single amphidial neuron, which we hypothesize is ASK, expresses the reporter. The cell body of this neuron, labeled 'Amph', and its dendritic process track along the lower margin of the worm, posterior to the nerve ring (NR) in this image. **E.** DIC (left) and fluorescence images (right) of an F1 transgenic first-stage larva expressing GFP under the promoter of the Gα protein-encoding gene *Ss-gpa-3* (Amph – amphidial cell bodies; NR – nerve ring). **F.** High-magnification fluorescence image of amphidial cell bodies (arrow heads) expressing GFP under the *Ss-gpa-3* promoter. Note that up to eight amphidial neurons express the construct and that based on comparison to *C. elegans* we hypothesize that these include ASK. Promoters with these cell specificities would theoretically allow ablation of ASK-class neurons by transgene-based expression of reconstituted caspases. Panels A–C are redrawn from Chelur, D. S. and Chalfie, M. (2007). Targeted cell killing by reconstituted caspases. *Proceedings of the National Academy of Sciences of the United States of America*, **104**, 2283–2288, Copyright (2007) National Academy of Sciences, U.S.A., with permission.

specificity to target lytic factors to individual cells. We are now attempting to use nascent systems for transgenesis to apply this system to cell ablation in *Strongyloides* spp. To this end, we have identified the promoter of *Ss-gpa-3*, a gene encoding a Gα protein orthologous to one essential for sensing the dauer pheromone in *C. elegans*, as one that can drive reporter expression in 8 amphidial neurons (including ASK) and some phasmidial neurons in transgenic *S. stercoralis* (Fig. 2E, F) (Junio *et al.* 2008). This pattern is virtually identical to the expression of *Ce-gpa-3* in *C. elegans* (Zwaal *et al.* 1997). Like its *C. elegans* orthologue, the promoter for another Gα protein-encoding gene, *Ss-gpa-2*, drives reporter expression in a single amphidial neuron (Fig. 2D) (Ranjit and Lok, unpublished). We have not conclusively identified the specific neuron in which this reporter is expressed, but based on reported expression patterns of *Ce-gpa-2*, we hypothesize that it is ASK. The neuronal patterns of expression of *C. elegans* Gα protein genes are such that the subsets of amphidial neurons in which *Ce-gpa-2* and

Table 3. Specificity of *Ce-gpa-2* and *Ce-gpa-3* promoter activation in ampidial neurons. Note that expression overlaps only in ASK class neurons (gray shading), providing a potential unicellular target of genetic cell ablation by expression of reconstituted caspases

Amphidial neurons

Promoter	ADE	ADF	ADL	ASE	ASG	ASH	ASI	ASJ	ASK	AFD	AIA	AIZ	ALA	ALN	AQR	AVM	AWA	AWB	AWC
Ce-gpa-2	X	X	X	X	X	X	X	X	X		X								
Ce-gpa-3									X			X							X

Ce-gpa-3 are expressed overlap in a single neuron ASK (Table 3). Based on the strong similarities between these patterns and those observed for *Ss-gpa-2* and *Ss-gpa-3* (Fig. 2D–F), we hypothesize that the reconstituted caspase system (Chelur and Chalfie, 2007) could be used to kill ASK and, by extrapolation, other sensory neurons in transgenic *S. stercoralis*. As proof of principle, we are currently engineering vectors to express individual CED-3 caspase subunits under control of the *Ss-gpa-2* and *-3* promoters and intend to ascertain their ability to bring about killing of ASK-class neurons when co-expressed in first stage-larval *S. stercoralis*.

Studies of the host immune response to infection with parasitic nematodes

Stable transgenesis in parasitic nematodes would greatly facilitate studies on the host immune response to these potential pathogens. Among the many possible applications of transgenic nematodes in such studies is their use in elucidating the manner in which polarized T helper cell responses conferring either immunity or chronic infection in nematode parasitism are incited by the parasite and then further differentiate. Antigens of microbial pathogens are sufficiently well characterized to have allowed dominant epitopes to be associated with receptors on specific T cell populations, but due to their greater complexity, such studies in more complex eukaryotic pathogens such as the parasitic protozoa or, to a greater degree, parasitic helminths have been slower to emerge (Lok and Artis, 2008). For example, the manner and anatomical locations in which parasite antigens stimulate populations of CD4+ T cells elaborating type 2 (Th2) cytokines to differentiate during a protective response to intestinal nematode infection are poorly understood (Lok and Artis, 2008; Maizels *et al*. 2009; Saenz *et al*. 2010). Transgenic parasites expressing standard model antigens such as avian ovalbumin (OVA) coupled with transgenic OVA-specific T cells have allowed the mechanisms of antigen presentation and CD8+ T cell differentiation and trafficking to be elucidated in *Toxoplasma gondii* infection (Pepper *et al*. 2004; Dzierszinski *et al*. 2007; Jordan *et al*. 2009, 2010; Tait *et al*. 2010). This work provides a model for how transgenic parasites expressing a model antigen like OVA could be employed in similar studies of how the Th2-polarized CD4+ T cell response develops in parasitic nematode infection.

CONCLUSION

Although transgenesis in parasitic nematodes has been slow to develop, recent advances in these worms and in parallel studies with schistosomes suggest that the long-term goal of stable transgenesis may be within reach in the near future. Among these

advances are vector modifications for transformation of *Strongyloides* spp. that employ a transposon-mediatiated system for chromosomal integration and incorporation of retroviral sequences to resist epigenetic transgene silencing, allowing heritable transgenesis with stable expression and a highly creative approach involving revival of classic chemically mediated system for gene transfer into the filaria *B. malayi* and subsequent heritable transgenesis in that important parasite and laboratory model. Establishment of these methods for species of gastrointestinal and tissue-dwelling parasitic nematodes would be a major advancement allowing myriad studies in modern biology, not least of which would be the validation of new drug and vaccine targets and the elucidation of protective and pathological immune responses to host infection.

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