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Strongyloides stercoralis: Cell- and tissue-specific transgene expression and co-transformation with vector constructs incorporating a common multifunctional 3′ UTR ☆

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Abstract

Transgenesis is a valuable methodology for studying gene expression patterns and gene function. It has recently become available for research on some parasitic nematodes, including *Strongyloides stercoralis*. Previously, we described a vector construct, comprising the promoter and 3′ UTR of the *S. stercoralis* gene *Ss era-1* that gives expression of GFP in intestinal cells of developing F1 progeny. In the present study, we identified three new *S. stercoralis* promoters, which, in combination with the *Ss era-1* 3′ UTR, can drive expression of GFP or the red fluorescent protein, mRFPmars, in tissue-specific fashion. These include *Ss act-2*, which drives expression in body wall muscle cells, *Ss gpa-3*, which drives expression in amphidial and phasmidial neurons and *Ss rps-21*, which drives ubiquitous expression in F1 transformants and in the gonads of microinjected P0 female worms. Concomitant microinjection of vectors containing GFP and mRFPmars gave dually transformed F1 progeny, suggesting that these constructs could be used as co-injection markers for other transgenes of interest. We have developed a vector "toolkit" for *S. stercoralis* including constructs with the *Ss era-1* 3′ UTR and each of the promoters described above.
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Index Descriptors and Abbreviations: Nematode; *Strongyloides stercoralis*; Transgenesis; Vector; Expression; Neuron; Muscle; GFP, green fluorescent protein; RFP, red fluorescent protein; 3′ UTR, 3′ untranslated region

1. Introduction

Transgenesis is an essential tool in modern molecular and cellular biology. In experimental models amenable to classical genetic study, this approach can facilitate cloning and characterization of novel genes by genetic complementation and in less tractable subjects can be used to infer gene function by assessing phenotypes resulting from overexpression or from expression of dominant loss-of-function constructs. Expression of transgenes carrying specific, targeted mutations allows structural/functional study of regulatory and other functional domains in DNA, RNA and protein (Evans, 2006). Transgenesis in the model organism *Caenorhabditis elegans* was pioneered in the mid 1980s (Fire, 1986; Fire and Waterston, 1989; Fire et al., 1990a,b), and its application to the study of parasitic nematodes began approximately a decade later. As recounted in a recent comprehensive review (Kalinna and Brindley, 2007), initial attempts at transgenesis in parasitic nematodes, which resulted in transient

☆ *Note.* GenBank accession numbers for the *Ss act-2, Ss gpa-3* and *Ss rps-21* promoter sequences are EF587761, AF292562, EF589665, respectively. The GenBank accession number for the *Ss era-1* 3′ UTR is DQ333398. Plasmid distribution for all constructs described is available at http://www.addgene.org/James_Lok.

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transformation, involved biolistics as a means of gene transfer into relatively large organisms such as adult filariae (Davis et al., 1999; Jackstadt et al., 1999; Higazi et al., 2002) or stages such as the egg of *Ascaris suum*, which can be obtained in large quantities (Davis et al., 1999). This approach has been used to study mRNA processing and translation in *Ascaris suum* (Cohen et al., 2004; Lall et al., 2004; Cheng et al., 2007) and for structural and functional analysis of an HSP70 promoter and of message transplicing in *Brugia malayi* (Shu et al., 2003; Higazi and Unnasch, 2004).

The ovaries of free-living females of the nematode superfamily Strongyloidoidea (De Ley and Blaxter, 2001; Dorris et al., 2002) are very similar in structure to the gonads of *C. elegans* hermaphrodites. This has made it possible to directly apply standard methods for gonadal microinjection of *C. elegans* (Evans, 2006) to *Strongyloides* spp. and *Parastrongyloides* spp. (Lok and Massey, 2002; Grant et al., 2006; Li et al., 2006). Using this approach we have developed a reliable method for gene delivery in *Strongyloides stercoralis* (Lok and Massey, 2002; Li et al., 2006), and we have observed tissue-specific expression of a *gfp*-based reporter construct in viable F1 transformant larvae (Li et al., 2006). Such regulated transgene expression requires both 5′ and 3′ flanking regulatory sequences from *S. stercoralis*. By contrast, *gfp*-based reporter constructs incorporating regulatory elements from *C. elegans* are not expressed in the first- through third-stage larval progeny of microinjected free-living female *S. stercoralis* but only in dysregulated fashion in degenerating embryos (Li et al., 2006). We have previously detected DNA of the *C. elegans*-derived plasmid construct pTG96_2 (Gu et al., 1998) in transgenic *S. stercoralis* up to the F5 generation (Li et al., 2006), but we have not yet succeeded in deriving a stable transgene expressing line; that is, one that transmits an expressed transgene to its progeny at a constant frequency through successive generations. Grant et al. (2006) have achieved heritable transformation of *Parastrongyloides trichosuri* with β-gal-based reporter constructs, and have succeeded in maintaining transformant lines through multiple *in vitro* and *in vivo* passages, providing proof of principal for our studies in *S. stercoralis*.

The purpose of the present study was to determine whether the *Ss era-1* 3′ UTR can act as a multi-purpose terminator, allowing regulated expression of reporter gene constructs incorporating other tissue-specific promoters. We also evaluated an alternate *in vivo* reporter gene for *S. stercoralis*, mRFPmars, and used this to ascertain whether co-injected transgenes are incorporated together in transformant worms. We undertook *in vivo* experiments to determine whether *S. stercoralis* L3i expressing transgene constructs could infect the host, establish as parasitic adults in the gut and transmit the transgenes to their progeny. Finally, we sought to develop and make available for distribution a vector "tookit" for *S. stercoralis*, similar in concept to that developed and distributed for *C. elegans* by A. Fire and colleagues (Stanford University) (Fire et al., 1990a,b). We envisioned such a "toolkit" as comprising a series of modular vectors incorporating the Ss *era-1* 3′ UTR either alone or in combination with various reporter genes and tissue- and cell-specific promoters.

2. Materials and methods

2.1. Parasite maintenance and culture

As in previous studies on transgenesis (Lok and Massey, 2002; Li et al., 2006), all experiments were conducted with the UPD strain of *S. stercoralis*. General methods for maintenance of *S. stercoralis* in dogs, for experimental infections in gerbils and for derivation of free-living adults, L1 (both heterogonic and homogonic) and L3i from charcoal coproculture or from cultures on NGM agar plates were as described previously (Lok, 2007).

2.2. Genomic DNA preparation

Genomic DNA (gDNA) was prepared from L3i isolated by the Baermann technique from charcoal coprocultures incubated for seven days at 22 °C and purified by percoll density gradient centrifugation as described (Lok, 2007). Purified worms were washed with 10 mL TEN solution (50 mM Tris, 20 mM EDTA, 100 mM NaCl, pH 7.5), centrifuged for 3 min at 107*g* and the supernatants discarded. Worm pellets were resuspended in 2 mL TEN with 200 μg/mL Proteinase K, 1% SDS (w/v) and 1% 2-mercaptoethanol (v/v) and incubated at 65 °C for 30 min with periodic agitation until the solutions became viscous. Using standard techniques, DNA was extracted with phenol/chloroform and precipitated in sodium acetate and ethanol. DNA precipitates were wound out using sealed Pasteur pipets. DNA was dissolved in 0.4 mL TE buffer and the samples digested for 30 min at 37 °C with 20 μg/mL RNAseA. The phenol–chloroform extractions and ethanol precipitations were repeated, and resulting DNA samples were microdialysed against water for 30 min using MF-Millipore membrane filters (pore size = 0.025 μM). DNA was recovered as the filter retentate and stored at 4 °C.

2.3. Preparation of regulatory sequences from S. stercoralis

Elements referred to as "promoters" in this section and throughout the paper are, in fact putative promoters comprising at least 1000 bp of 5′ sequence flanking the coding region of the specified gene. No attempt has been made to date to define a minimal promoter for *S. stercoralis*. The *Ss act-2* promoter, comprising 1183 bp of 5′ flanking sequence was amplified by inverse PCR (iPCR) from a circularized BglII digest of *S. stercoralis* DNA as described (Lok and Massey, 2002). The *Ss gpa-3* promoter, comprising approximately 3323 bp of 5′ flanking sequence was amplified by iPCR from a circularized BglII digest of *S. stercoralis* gDNA as described (Massey et al., 2001). The promoter for *Ss rps-21*, a gene that encodes ribosomal small subunit

protein S21, comprised 3301 bp of 5′ flanking sequence amplified by iPCR from a circularized Swa1 digest of *S. stercoralis* gDNA using primers Ssrps21-hind3F TCTTCTAAGCTTAGCTCAACGTGACGGATTG and Ssrps21-age1R AGTTCAACCGGTTCTCCTTTGTCGT TTTGCATGA. These primers contained HindIII and AgeI sites (underscored), respectively, for cloning. The iPCR protocol used to amplify the *Ss rps-21* promoter was as described for *Ss gpa-3* (Massey et al., 2001) except that gDNA was microdialyzed using a MF-Millipore Membrane Filter (pore size = 0.025 μM) for 30 min against nuclease free water prior to use and that 800 U T4 DNA ligase (New England BioLabs, Ipswich, Massachusetts, USA) were used in the circularization reaction. Also, *Pfu-Turbo*® Hotstart DNA Polymerase (Stratagene, La Jolla, California, USA) was used in a slightly modified protocol for long-range PCR in which 25 μL reactions (manufacturer's protocol) contained 3 μL circularized template and were subjected to cycling regimes of 94 °C for 1.5 min for initial denaturation, followed by 35 cycles of denaturation at 94 °C for 45 s, primer annealing at 62 °C for 1 min, extension at 68 °C for not less than 3 min/1000 bp expected fragment length, followed by final extension at 68 °C for 10 min. The 593 bp *Ss era-1* 3′ UTR was prepared previously and cloned into transformation vector pPV230.13 (Li et al., 2006). The UTR was cut from pPV230.13 with restriction enzymes appropriate for cloning into new transformation vectors as described below.

2.4. Construction of the promoterless gfp-expression vectors

Starting materials for promoterless *gfp* expression vectors were one of the following standard *C. elegans* vectors pPD95.75, pPD95.77, pPD95.79 and pPD95.81 from the kit provided as a gift from Andrew Fire (Stanford University) and currently distributed on a nonprofit basis (Addgene, Cambridge, Massachusetts, USA, http://www.addgene.org). The GFP variant S65C encoded in these and all vectors described subsequently in this paper contains a serine-to-cysteine mutation at position 65, enhancing its fluorescence intensity (Reichel et al., 1996). Fire vectors were digested with the restriction enzymes EcoRI and EagI to remove the *Ce unc-54* 3′ UTR, and the *Ss era-1* 3′ UTR was cut from the vector pPV230.13 (Li et al., 2006) with the same restriction enzymes and ligated to the above Fire vectors to make pAJ01, pAJ02, pAJ03 and pAJ04, respectively (Table 1). Modified versions of the pAJ constructs containing a new multi-cloning site or "stuffer" were made by cutting with EcoRI and ligating with an excess of the short DNA sequence StuAvrMLuF/R containing three novel restriction sites. This sequence was made by annealing the following complementary oligonucleotides: StuAvrMLuF (5′-AATTCAGGCCT AGGACGCGT-3′) and StuAvrMLuR (5′-AATTACGCG TCCTAGGCCTG-3′). Restriction sites thus introduced were MluI, AvrII, and StuI, and the resulting constructs were designated pAJ12, pAJ13, pAJ14 and pAJ15, respectively (Table 1).

Table 1
Composition and derivation of plasmid vectors

Vector	Parent vector	Promoter	Reading frame[b]	Marker	Stuffer	3′ UTR
pAJ01	pPD95.75[a]	None	0	GFP(S65C)	—	*Ss era-1*
pAJ02	pPD95.77[a]	None	2	GFP(S65C)	—	*Ss era-1*
pAJ03	pPD95.79[a]	None	1	GFP(S65C)	—	*Ss era-1*
pAJ04	pPD95.81[a]	None	S	GFP(S65C)	—	*Ss era-1*
pAJ08	pAJ04	*Ss act-2*	S	GFP(S65C)	—	*Ss era-1*
pAJ09	pAJ01	*Ss gpa-3*	0	GFP(S65C)	—	*Ss era-1*
pAJ12	pAJ01	None	0	GFP(S65C)	+	*Ss era-1*
pAJ13	pAJ02	None	2	GFP(S65C)	+	*Ss era-1*
pAJ14	pAJ03	None	1	GFP(S65C)	+	*Ss era-1*
pAJ15	pAJ04	None	S	GFP(S65C)	+	*Ss era-1*
PAJ20	pAJ04	*Ss rps-21*	S	GFP(S65C)	+	*Ss era*-1
pAJ50	pAJ08	*Ss act-2*	S	mRFPmars	—	*Ss era-1*

[a] See 1995 Fire kit documentation http://www.addgene.org/ for plasmid details.

[b] Frames 0, 1 and 2 are the possible reading frames relative to GFP. Frame S is identical to frame 1 except for the removal of the TAG codon, restoring HindIII, SphI, PstI and SalI fusion junctions.

2.5. Synthesis of reporter DNA constructs

The *Ss act-2* promoter was transferred as a HindIII–PstI fragment from vector pPV101.1 (Lok and Massey, 2002) to pAJ04 to make pAJ08 (Table 1). The *Ss gpa-3* promoter was transferred as a HindIII–SalI fragment from the PCR-Script™ (Stratagene) clone (Massey et al., 2001) to pAJ01 to make pAJ09 (Table 1). The *Ss rps-21* promoter was transferred as a HindIII–AgeI fragment (detailed in Section 2.3.) to pAJ04 to make pAJ20 (Table 1). We constructed an expression vector containing the alternate reporter mRFPmars (Fischer et al., 2004) as follows. The mRFPmars coding sequence (AY679163) was amplified from pBsrH, kindly provided in by Dr. Jakob Franke at the Dicty Stock Center, Columbia University (http://dictybase.org/stockcenter/stockcenter.htmL), using primers mRFPmarsAgeF AGTACCGGTAAAAATGGCATC ATCAGAAGATG and mRFPmarAvEcR TTGGAATTC AGGCCTAGGAGATCCTGCACCTGTTGAATG, tagged with restriction sites AgeI and AvrII (underscored), respectively. The resulting PCR product was transferred as an AgeI–AvrII fragment to pAJ08 digested with the same enzymes to replace *gfp* and make pAJ50 (Table 1).

2.6. Gonadal microinjection of DNA constructs into S. stercoralis and C. elegans

Gonadal microinjections into *S. stercoralis* free-living females and *C. elegans* hermaphrodites were carried out as described (Mello and Fire, 1995; Evans, 2006). Injection mixes for *S. stercoralis* consisted of expression construct alone, or with pBlueScript II SK(+) (Stratagene) as carrier DNA, in distilled water with a total combined plasmid

concentration of 100 ng/μL. For promoter analysis the construct was injected into P0 worms at 100 ng/μL. For gerbil and dog infection trials 20 ng construct and 80 ng carrier DNA were injected, and for co-injection experiments 20 ng primary construct, 20 ng co-injection marker construct and 60ng carrier DNA were injected. Injection mixes for *C. elegans* consisted of 20 ng/μL of primary construct and 80 ng/μL empty vector plasmid SK+. Microinjected *C. elegans* hermaphrodites were plated singly on NGM agar plates with lawns of *Escherichia coli* OP50 (NGM/OP50) and cultured at 20 °C. Beginning 48 h after microinjection *C. elegans* broods were observed with an Olympus SZX12 stereomicroscope with coaxial epifluorescence and *gfp*-expressing transformants were picked from among the progeny of P0 hermaphrodites and replated. Microinjected *S. stercoralis* females were plated in pairs along with four or five males. At intervals of 22 and 48 h following injection, parental (P0) females and their broods were observed with the epifluorescence stereomicroscope and both total and GFP-positive or mRFPmars-positive F1 larvae were counted. P0 females and males were transferred to fresh plates following each daily count.

2.7. Co-transformation with two reporter constructs

The availability of mRFPmars as an alternative reporter gene to *gfp* allowed us to examine whether co-injected transgene constructs are inherited together or independently in F1 transformant *S. stercoralis*. Plasmids pAJ08 and pAJ50, and in another experiment plasmids pAJ09 and pAJ50, were combined at concentrations of 20 ng/μL each, together with 60 ng/μL SK+ plasmid, and injected into the gonads of *S. stercoralis* free-living females. These female worms were reared at 20 °C with three male worms each and their progeny checked for both *gfp* and mRFPmars expression over the next 72 h. Each co-injection experiment was repeated once.

2.8. Culture and passage of transgenic worms in gerbils and dogs

Host passage of transgenic worms was carried out by standard methods in Mongolian gerbils, with or without immunosuppressive prednisolone treatment (Nolan et al., 1993), or in an immunosuppressed dog (Schad et al., 1984). In gerbil infection trials 1–3, 7 and 8, transgenic L3i designated for host passage were hand selected based on *gfp* fluorescence using the epifluorescence stereomicroscope. In gerbil infection trials 4–6, an unselected population of transformed and untransformed larvae was inoculated, and the number of transgenic L3i delivered in each case was estimated by averaging counts of GFP positive individuals in 3–5 measured aliquots. In the case of a single canine infection (Trial 9, Table 4) L3i were automatically sorted from a mixed population using a COPAS BioSorter® (Union Biometrica, Holliston, Massachusetts).

2.9. Detection of transgene DNA and transgene expression in the F1 generation and beyond

Initial screening of F1 larvae for transgene expression was by epifluorescence stereomicroscopy. Detailed imaging of transgenic larvae was by Nomarski Differential Interference Contrast (DIC) microscopy or by epifluorescence using an Olympus BX60 compound microscope. Filter cubes used to detect GFP and mRFPmars, respectively, were Chroma Technology Corporation (Rockingham, VT 05101 USA) 41001 (510–560 nm emission band width) and 41002 (578–642 nm emission band width). Imaging was done with the BX60 microscope using a Spot RT Color® digital camera and the Spot Advanced® image analysis software package (Diagnostic Instruments, Inc., Sterling Heights, Michigan, USA).

Larvae from the F2 generation following transformation were isolated from the feces of infected hosts by the Baermann funnel technique (Lok, 2007) and screened for the presence of transgene DNA via PCR. All constructs used in host passage attempts included the coding region of *gfp*, a gene that does not occur in *C. elegans* nor, presumably, in *S. stercoralis*. Therefore, transgene DNA was detected in worms from the F2 generation using primers specific for the *gfp* coding region. These primers, CeGFP-476F CCCTTGTTAATAGAATCGAGTT and CeGFP-noTerR TTTGTATAGTTCATCCATGCC, yielded a 413 bp product. All PCR screening of F2 progeny from host passages included a negative control reaction conducted on pools of non-transformed *S. stercoralis* larvae, a positive control reaction conducted on purified vector plasmid and a loading control reaction using primers that amplify a 316 bp fragment of the gene *Ss rps-21,* which encodes the ribosomal small subunit protein in *S. stercoralis* (Li et al., 2006).

3. Results

3.1. 1. Cell- and tissue-specific expression of reporter transgenes in S. stercoralis

Having found that the *Ss era-1* 3′ UTR could give regulated expression of reporter transgenes under the control of its cognate promoter, we sought to determine whether this element could also function in combination with other promoters. To this end, we made a series of reporter constructs (pAJ08, pAJ09, pAJ20 and pAJ50) by cloning 5′ flanking sequence from various *S. stercoralis* genes upstream of the *gfp* coding sequence in either of the promoterless vectors pAJ01 or pAJ04 (Table 1). Both of these promoterless vectors contain the *Ss era-1* 3′ UTR fused downstream of *gfp*. After microinjection of the promoter-containing reporter constructs into free-living females, >5% of F1 progeny showed tissue-specific GFP expression patterns (Tables 2 and 3). These expression patterns remained consistent from the L1 to the L3i stage.

Table 2
Summary of reporter transgene expression frequencies among F1 progeny resulting from gonadal microinjection of vector constructs into free-living *S. stercoralis* females

Construct[a]	Replicate	No. P0 female worms injected	No. F1 larvae obs.	No. (%) F1 larvae GFP+
pAJ08	1	18	445	51 (11.5)
(*Ss act-2::gfp*)	2	20	349	59 (16.9)
pAJ09:SK+[b]	1	16	137	8 (5.8)
(*Ss gpa-3::gfp*)	2	15	176	10 (5.7)
pAJ50 (*Ss act-2::mRFPmars*)	1	16	560	55 (9.8)
	2	21	435	66 (15)
pAJ20	1	21	280	98 (35.0)
(*Ss rps-21::gfp*)	2	21	194	83 (42.8)

[a] All constructs injected at a concentration of 100 ng/μL.
[b] Vector pAJ09 was injected in combination with pBluescript II SK+ as carrier DNA.

Table 3
Expression patterns of GFP reporter constructs incorporating *S. stercoralis* promoters compared to those of GFP reporters incorporating orthologous promoters from *C. elegans* and to those seen following heterologous transfer of the parasite-based constructs into *C. elegans*

Orthologous promoter pair[a]	GFP reporter expression pattern	
	S. stercoralis	*C. elegans*
Ss era-1	Intestine	Not expressed
Ce cdc-48.2	ND	Intestine
Ss gpa-3	Amphidial, phasmidial neurons	Not expressed
Ce gpa-3	ND[b]	Amphidial, phasmidial neurons
Ss act-2	Body muscle	Pharynx (59)[c], pharynx + body muscle (39), body muscle alone (2) in F1 transformants; pharyngeal muscle pm6 + body muscle (90) in stable line
Ce act-1	ND	Body muscle
Ss rps-21	Ubiquitous	A few neurons in stable line
Ce rps-21	ND	Ubiquitous

[a] Designated *S. stercoralis* promoters (*Ss*) were cloned upstream of *gfp* in either pAJ01 or pAJ04 (Table 1) to derive the reporter constructs giving the expression patterns described.
[b] ND, not done.
[c] Numbers in parentheses indicate the percentages of 175 worms that exhibited the specified expression pattern.

The reporter construct designated pAJ09 (Fig. 1 and Table 1) contains 5′ flanking sequence from *Ss gpa-3*, the structural ortholog of *C. elegans gpa-3* (Massey et al., 2001), which encodes the α subunit of a G protein necessary for transduction of the pheromone signal triggering dauer development in *C. elegans* (Zwaal et al., 1997; Massey et al., 2001). Approximately 5.8% of F1 progeny of *S. stercoralis* free-living females microinjected with pAJ09 expressed GFP in a neuron-specific pattern (Tables 2 and 3). All such F1 first-stage larvae exhibited GFP expression in a cluster of amphidial neurons and in the area of the nerve ring (Fig. 1A and B). Dendritic processes of GFP expressing amphidial neurons could be traced from their origins in the lateral ganglion forward to the amphidial channnel. Some transformant larvae also showed expression in at least one and frequently two pairs of phasmidial neurons (Fig. 1A and B). High-magnification images (Fig. 1C and D) appear to show expression in eight amphidial cell bodies.

The *Ss act-2* promoter drove *gfp* expression almost exclusively in body wall muscle of F1 larvae transformed with construct pAJ08 (Table 1, Table 3 and Fig. 2A and B). This same promoter drove *mRFPmars* expression in an identical anatomical pattern in F1 larvae transformed with construct pAJ50 (Table 1 and Fig. 2C and D). The frequencies of *gfp* expression among F1 progeny of worms microinjected with the two constructs containing the *Ss act-2* promoter were similar: 13.9% on average for pAJ08 and 12.2% for pAJ50 (Table 2).

The reporter construct pAJ20 contains 5′ flanking sequence from the *Ss rps-21* gene, which encodes a ribosomal protein. F1 pAJ20 transformant *S. stercoralis* expressed *gfp* beginning early in embryogenesis (Fig. 3A and B) and continuing through larval development. Expression of pAJ20 occurred in virtually all tissues of *S. stercoralis* L1 and was particularly intense in the genital primordium (Table 3 and Fig. 3C and D). The mean frequency of *gfp* expression among F1 progeny of worms microinjected with pAJ20 was 38.2%, the highest of all constructs tested (Table 2). Unlike the other constructs examined in this study, pAJ20 was also highly expressed in the gonads and eggs of microinjected P0 free-living female worms (Fig. 3E and F).

In summary, the promoters described here function with the *Ss era-1* 3′ UTR to drive sensory neuron-specific, muscle-specific or ubiquitous expression of fluorescent markers. Given their modular structure, these constructs could easily be modified to drive expression of any gene sequence of interest.

3.2. Expression of *S. stercoralis*-based constructs in *C. elegans*

Reporter constructs containing *S. stercoralis* promoters showed expression patterns similar to those of their orthologs in *C. elegans*. However, these *Strongyloides*-based constructs either exhibited markedly different patterns of expression or were not expressed at all when used to transform *C. elegans* (Table 3). The construct pAJ09, which contains the *Ss gpa-3* promoter, failed to express in *C. elegans* altogether. *C. elegans* transiently transformed with pAJ08, containing the *Ss act-2* promoter, expressed *gfp* but did so in a pattern different from that seen in *S. stercoralis*. In the majority of F1 *C. elegans* transformants, the Ss *act-2* promoter drove *gfp* expression in the pharynx, and in the pharynx and body wall (Table 3 and Fig. 4A and B). Only a small fraction (2%) of *C. elegans* F1 transformed with pAJ08 expressed *gfp* exclusively in the body wall (Table 3

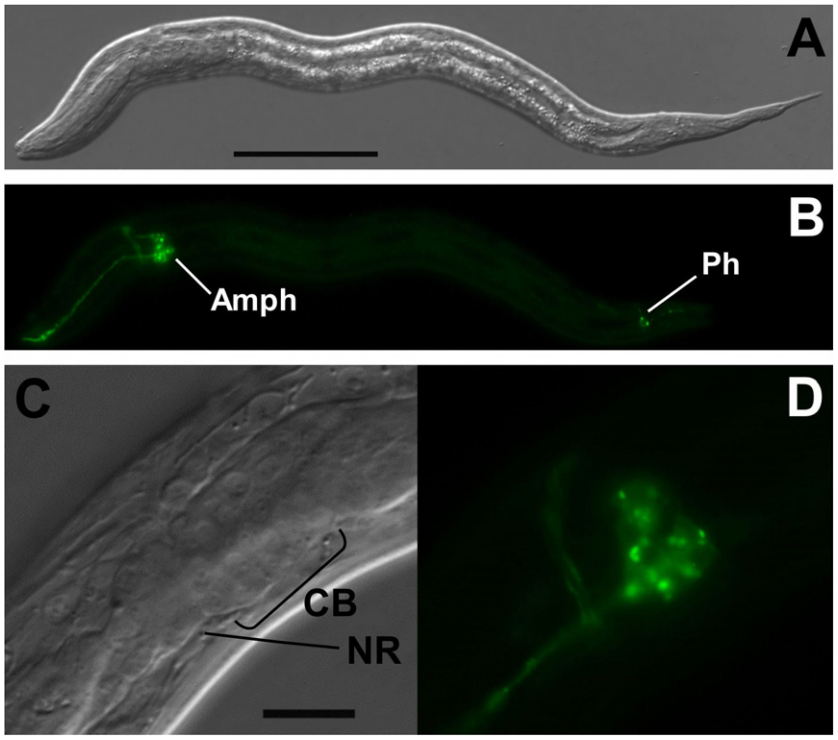

Fig. 1. Neuron-specific expression of *gfp* under the *Ss gpa-3* promoter in *S. stercoralis* transformed with construct pAJ09 (Table 1). (A and B) DIC and fluorescence images, respectively, of a transformant L1 showing GFP localization in amphidial cell bodies (Amph), in their dendritic processes leading into the amphidial channel and in phasmidial neurons (Ph), (C and D). High-magnification DIC and fluorescence images, respectively, of specimen in panels (A and B) showing GFP localization in nerve ring (NR) and eight amphidial cell bodies (CB). Scale bars: A = 50 μM; C = 20 μM.

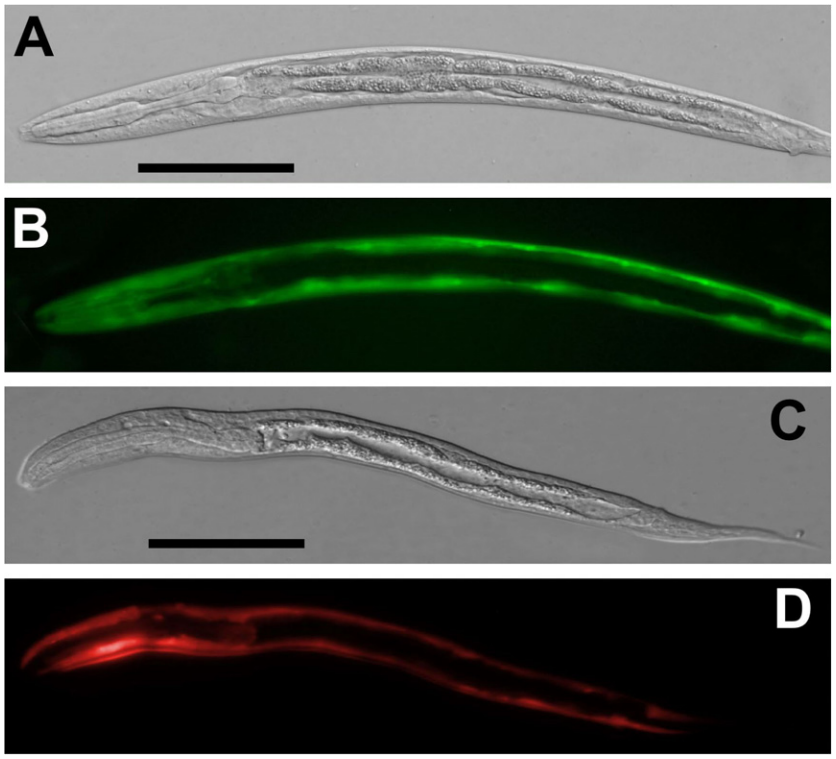

Fig. 2. Body wall-specific expression of *gfp* and *mRFPmars* under the *Ss act-2* promoter in *S. stercoralis* transformed with construct pAJ08 and pAJ50, respectively (Table 1). (A and B) DIC and fluorescence images, respectively, showing GFP localization in F1 transformant first-stage larvae (L1) transformed with the reporter construct pAJ08. (C and D) DIC and fluorescence images, respectively, showing mRFPmars localization in F1 transgenic L1 transformed with the reporter construct pAJ50. All scale bars = 50 μM.

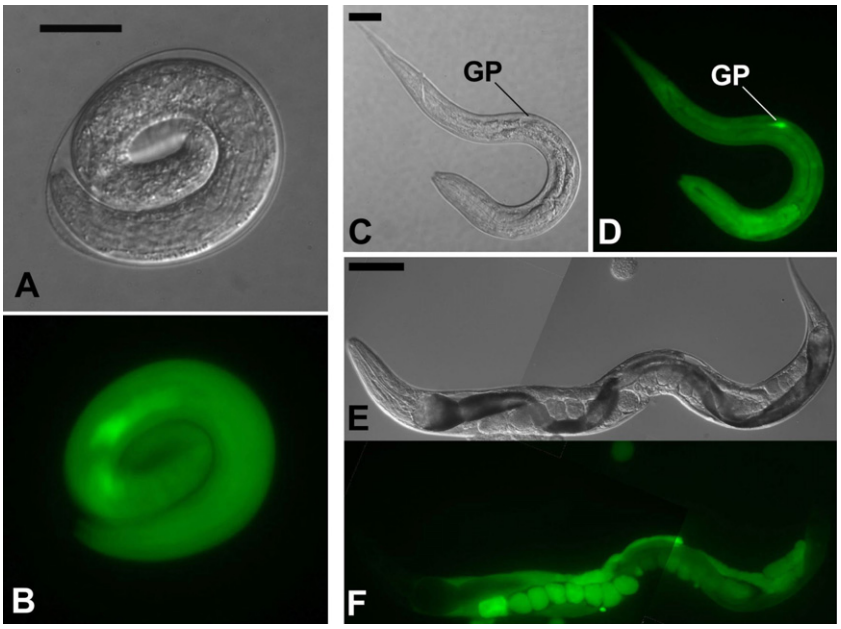

Fig. 3. Ubiquitous expression of *gfp* under the *Ss rps-21* promoter in *S. stercoralis* transformed with construct pAJ20 (Table 1). (A and B) DIC and fluorescence images, respectively showing ubiquitous distribution of GFP in a vermiform embryo transformed with pAJ20. (C and D) A pAJ20 transformant first-stage larva showing *gfp* expression in virtually all body tissues with the highest level of expression occurring in the genital primordium (GP). (E and F) Composite DIC and fluorescence images, respectively, of a P0 free-living female microinjected with pAJ20. Note the high level of *gfp*-expression in the gonad and eggs. Scale bars in A and C = 20 μM; E = 100 μM.

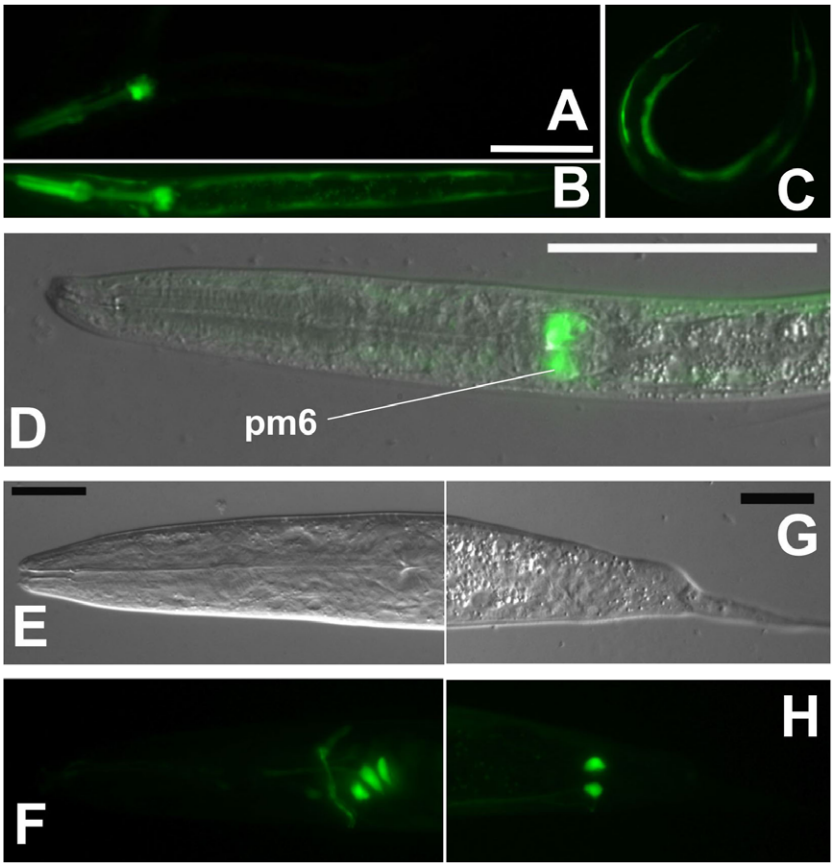

Fig. 4. Expression of *S. stercoralis*-based reporter constructs in *C. elegans*. (A–D) Variable expression patterns of *gfp* expression under the *Ss act-2* promoter in *C. elegans* transiently transformed with construct pAJ08 (Table 1). (D) DIC and fluorescence overlay image showing GFP localization in the sixth pharyngeal muscle ring (pm6). Scale bar for A–D = 50 μM. (E–H) Images of *gfp* expression under the *Ss rps-21* promoter in *C. elegans* transformed with construct pAJ20 (Table 1). Images show GFP localization in glial cells in the amphidial (E and F) and phasmidial (G and H) complexes. Scale bars in E and G = 20 μM.

and Fig. 4C) while all such *S. stercoralis* transformants observed to date have exhibited this expression pattern. *C. elegans* offers the advantage of easy establishment of stable transgenic lines, that is lines of germline transformants that transmit the transgene through many generations with a consistent frequency. In a stable line of *C. elegans* transformed with pAJ08, expression predominated in the sixth pharyngeal muscle and body wall (Table 3 and Fig. 4D). *S. stercoralis* transformed with pAJ20, which contains the *Ss rps-21* promoter, show ubiquitous expression of *gfp* (Table 3 and Fig. 3). In stark contrast, *C. elegans* transformed with this construct expressed *gfp* in a grouping of three neuronal cell bodies near the pharyngeal bulb (Table 3 and Fig. 4E and F) and in a pair of phasmidial cells (Table 3 and Fig. 4G and H). This pattern was consistent among F1 transformants and in a stable line derived from them. We conclude that a transgene's expression pattern in *C. elegans* is not a reliable indicator of its likely expression pattern in the *S. stercoralis*.

3.3. Co-transformation with two reporter constructs

Dually transformed F1 progeny were produced in both the replicates of the each plasmid combination (Table 4 and Fig. 5). In these dual transformations, the relative expression frequencies of the vector constructs, as approximated by fluorescence of the reporter gene products GFP or mRFPmars, were consistent with those seen in singly transformed worms (Tables 2 and 4). The frequencies of expression of the constructs used in the co-transformation experiments were such that pAJ08 > pAJ50 > pAJ09 ($\chi^2 = 12.53$; $P < 0.005$). In each pairing of vector constructs, the construct with the lowest frequency of expression was always seen in combination with the construct having the highest frequency and never by itself. For example, in both replicates of the pairing of pAJ09 with pAJ50, pAJ09 (giving GFP expression) was always seen in combination with pAJ50 (giving mRFPmars expression) and never alone. A small proportion of transgenic worms in this experiment (2.8%), equal to the difference in the expression frequencies of the two constructs, expressed pAJ08 alone (Table 4). These data are consistent with the hypothesis that transgene constructs co-injected into *S. stercoralis* are incorporated together in F1 transgenic progeny. From a practical standpoint, the data suggest that the fluorescent reporters described here could be used as co-injection markers for other non-fluorescent transgenes.

3.4. Host passage of transgenic S. stercoralis

We attempted to establish a stable transgene-expressing line of the parasite by passage through susceptible mammalian hosts. Because of the intensity of its expression in L3i we chose pAJ08 (Table 1) for these experiments. An esti-

Table 4
Frequencies of reporter transgene expression and co-expression in F1 progeny of *S. stercoralis* free-living females co-injected with vector constructs

Constructs co-injected[a]	Replicate	No. P0 female worms injected	No. F1 larvae obs.	No. (%) of all F1 larvae expressing GFP	No. (%) of all F1 larvae expressing RFP	No. (%) F1 larvae expressing GFP only	No. (%) F1 larvae expressing RFP only	No. (%) F1 larvae expressing GFP and RFP[b]
Ss act-2::gfp + Ss act-2::mRFPmars (pAJ08:pAJ50:SK+)	1	20	480	0	38 (7.9)	13 (2.7)	0 (0)	38 (7.9)
	2	16	162	36 (22.2)	31 (19.1)	5 (3)	0 (0)	31 (19.1)
Ss gpa-3::gfp + Ss act-2::mRFPmars (pAJ09:pAJ50:SK+)	1	21	397	12 (3.0)	19 (4.8)	0 (0)	7 (1.7)	12 (3.0)
	2	18	231	12 (5.2)	17 (7.4)	0 (0)	5 (2.2)	12 (5.2)

[a] Respective concentrations in ng/μL of construct components were 20:20:80 in all cases. SK+ denotes pBluescript II SK+.
[b] Denotes F1 larvae co-expressing both reporter constructs.

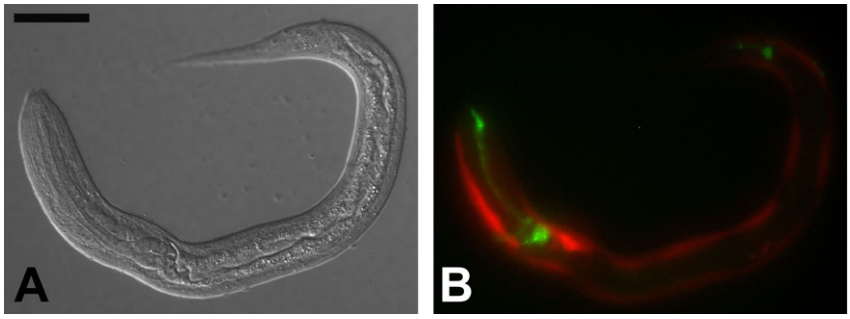

Fig. 5. Co-transformation of *S. stercoralis* with dual reporter constructs, pAJ09 and pAJ50 (Table 1). (A and B) DIC and merged red and green fluorescence images, respectively, of an F1 transgenic *S. stercoralis* L1 expressing *gfp* under the *Ss gpa-3* promoter (pAJ09) in amphidial neurons and nerve ring and *mRFPmars* under the *Ss act-2* promoter (pAJ50) in body wall muscles. Scale bar = 30 μM.

mated total of 1847 GFP-positive L3i were injected into gerbils over the course of 8 independent experiments (Table 5, trials 1–8). In addition, 1286 GFP-positive L3i were injected into a single dog (Table 5, trial 9). We observed continued expression of pAJ08 in 14 out of 35 F1 parasitic females recovered at necropsy from gerbils infected with pure populations of transgenic L3i (Table 5, trials 1–3 and 7; Fig. 6). Three of the gerbil infections (trials 3–5) yielded pools of F2 larvae in which transgene construct DNA could be detected by PCR. In trial 3, two PCR positive pools were detected from a total of 13 pools (2–90 larvae per pool) examined. In trial 4, two positive pools were detected among nine examined (125–1000 larvae per pool), and in trial 5 a single positive pool was observed among 8 examined (60–11,000 larvae per pool). The dog infection (trial 9) also yielded F2 progeny carrying the transgene construct (one of four pools positive; pool size ranging from 10–150 larvae per pool). Two of the necropsied gerbils (trials 3 and 4) found to harbor a total of 13 GFP-positive parasitic females yielded PCR-positive F2 larval pools. The yield of PCR-positive F2 larval pools indicate that a minimum of two or 15.4% of the F1 transgenics in these infections transmitted the transgene to their progeny. However, none of the 25,741 F2 larvae examined in all the host passage attempts expressed *gfp*. Control reactions with *gfp*-specific primers gave negative results with all pools of non-transformed larvae and positive results in every case with purified vector plasmid.

4. Discussion

The ability to generate F1 transgenic *S. stercoralis* will allow many studies of gene expression and gene function in this parasite. We have developed a vector "toolkit" to facilitate such transgenic studies. We showed that the *Ss era-1* 3′ UTR can act as a multipurpose terminator for

Table 5
Summary of host passage attempts using F1 pAJ08 transformant *S. stercoralis* L3i

Trial	Host species	Carrier DNA	Concentration (ng/μL; vector:carrier)	No. L3i inoculated	No. L3i GFP+	Parasitic females recovered at necropsy		F2 progeny		
						Total (% total L3i inoculated)	GFP+ (% GFP+ L3i inoculated)	L1 Screened	No. L1 GFP+	L1 pools PCR+
1	Gerbil[a]	SK+[b]	20:80	130	130	4 (3.1)	3 (2.3)	23	0	—
2	Gerbil[a]	SK+	20:80	125	125	1 (0.8)	1 (0.8)	15	0	—
3	Gerbil[a]	SK+	20:80	105	105	29 (27.6)	10 (9.5)	330	0	+
4	Gerbil	SK+	20:80	9000[c]	360[d]	500 (5.6)	3 (0.8)	4485	0	+
5	Gerbil	SK+	2:98	3200	160[d]	ND	ND	6130	0	+
6	Gerbil	None	200	2350	550[d]	4 (0.2)	3 (0.5)	2900	0	—
7	Gerbil	gDNA	20:80	180	180	1 (0.6)	0	101	0	—
8	Gerbil	pTG96_2	20:80	237	237	ND	ND	77	0	—
9	Dog	SK+	20:80	1286	1286	ND	ND	11,680	0	+
Total				16613	3133	539	20	25,741	0	

ND, not determined.
[a] In these trials gerbils were immunosuppressed by treatment with methylprednisolone acetate as described (Nolan et al., 1993).
[b] Denotes pBluescript II SK+.
[c] Consists of three gerbils inoculated with 3000 non-selected L3i each.
[d] Estimates derived by counting GFP+ individuals in 3–5 measured aliquots of an unselected population of transgenic and nontransgenic L3i.

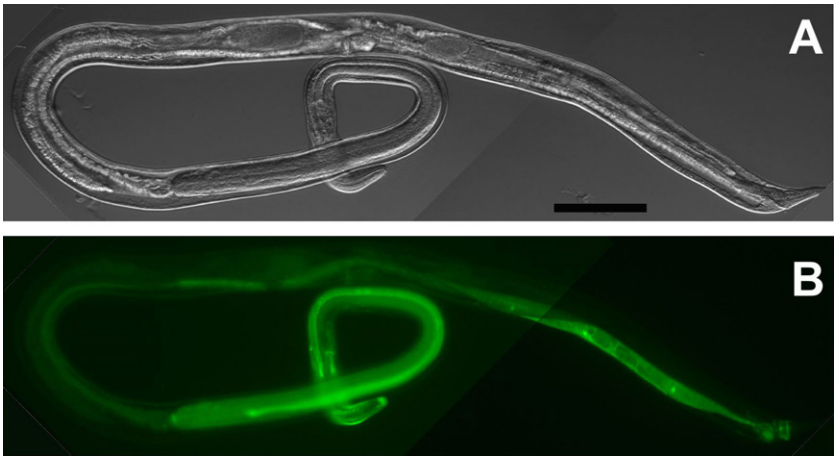

Fig. 6. Photomontage depicting expression of *gfp* under the *Ss act-2* promoter in an F1 parasitic female transformed with construct pAJ08 (Table 1) and recovered at necropsy from an experimentally infected gerbil. (A and B) Composite DIC and fluorescence images, respectively. Scale bar = 100 μM.

other reporter transgenes. This is analogous to the function of the relatively small collection of *C. elegans* 3′ UTRs (eg. *unc-54* and *let 858*) used in the collection of modular vectors developed by A. Fire and colleagues (Fire et al., 1990a,b). By the same token, the fact that several different *C. elegans* 3′ UTRs can act as multipurpose terminators in vector constructs suggests that the *Ss era-1* 3′ UTR is probably not unique in this capability in *S. stercoralis* and that as 3′ flanking sequences are captured from more genes in this nematode, other 3′ regulatory elements will be obtained. Finally, we used these modular vectors to characterize three new *S. stercoralis* gene promoters that drive tissue-specific (*gpa-3, act-2*) or ubiquitous (*rps-21*) patterns of reporter expression in the parasite. These constructs could easily be modified to drive expression of any gene sequence of interest. All of the promoterless and promoter-containing vectors described in this and our previous paper (Li et al., 2006), have been organized into a documented vector collection that is available to the research community through AddGene, Inc. (http://www.addgene.org).

Like that of its *C. elegans* ortholog, *gpa-3* (Jansen et al., 1999), the *Ss gpa-3* promoter drives *gfp* expression predominantly in a cluster of amphidial neurons and in two pairs of phasmidial neurons in L1 (Fig. 1A–D). *C. elegans gpa-3* is expressed in eight pairs of chemosensory neurons in the amphidial complex (ADF, ADL, ASE, ASG, ASH, ASI, ASJ and ASK), two pairs of sensory neurons in the phasmids (PHA and PHB) and two other non-sensory cells (Jansen et al., 1999). The amphidial neurons of *S. stercoralis* have been described and their homologies with amphidial neurons of *C. elegans* have been proposed based on morphology, position of cell bodies within the lateral ganglion and, in several cases, function inferred from microlaser ablation studies (Ashton et al., 1995, 1998, 2007; Lopez et al., 2000; Forbes et al., 2004; Nolan et al., 2004). We have not yet determined the specific identities of the GFP-expressing neurons in *S. stercoralis* transformed with pAJ09. However our imaging thus far (Fig. 1C and D) indicates that the transgene is expressed in eight amphidial neuron pairs along with two phasmidial neuron pairs in some specimens. Thus, *Ss gpa-3* expression appears to be localized in a pattern that is strikingly similar to that of its ortholog in the distantly related *C. elegans*. The expression of reporter constructs like pAJ09 in neurons may provide a fourth criterion, in addition to morphology, position and function, for assessing homologies between amphidial and other neurons of *S. stercoralis* and *C. elegans* (Ashton et al., 1995). In addition, transformation constructs that are expressed in such small, defined groupings of cells in *S. stercoralis* may provide the basis for genetically targeted cell ablation using modified channel forming proteins as described for *C. elegans* (Driscoll and Chalfie, 1991; Harbinder et al., 1997). In some instances such an approach could provide an alternative to microlaser surgical techniques currently in use for *S. stercoralis* (Lopez et al., 2000; Forbes et al., 2004; Nolan et al., 2004; Ashton et al., 2007).

The *Ss act-2* promoter drives expression of both *gfp* and mRFPmars such that the fluorescent reporters are localized in the body muscle of *S. stercoralis* L1 (Fig. 2). The expression pattern indicated by this finding is consistent with the natural expression pattern of *act-1*, the presumed ortholog of *Ss act-2*, in *C. elegans* (Seydoux and Fire, 1994). Because of its relatively high frequency of F1 transformation, pAJ08 was selected as the construct for use in attempts to establish a stable transgenic line by host passage. A high level of *gfp* expression persisted in L3i transformed with pAJ08, facilitating both manual recovery of tranformants and automated selection with the COPAS BioSorter. Automated sorting with this instrument dramatically increased the number of transgenic worms that could be recovered for inoculation into the host, due to both an increase in the number of F1 progeny analyzed and an increase in the number of weaker GFP expressing individuals recovered, which are not routinely isolated using manual sorting. Contrasting with the body wall-specific pattern of reporter expression under the *Ss act-2* promoter in L1 (Fig. 2) and L3i, the localization of GFP in the pharynges and intestines of parasitic females transformed with pAJ08 (Table 1 and Fig. 6) more closely resembles the pharyngeal expression pattern of this construct in the majority of *C. elegans* larvae (Fig 4A, B and D).

As expected of an element driving expression of a gene encoding a protein required for basic cellular function, the promoter for the ribosomal small subunit protein gene *Ss rps-21* drives *gfp* expression in virtually all body tissues of transformant *S. stercoralis* larvae. This finding is consistent with the broad pattern of *C. elegans rps-21* expression (unpublished, WormBase Release WS177 http://www.wormbase.org, expression pattern ID Expr5980) and makes available a promoter to drive ubiquitous transgene expression in *S. stercoralis*. One potential application for such a promoter would be the *in situ* expression of inhibitory RNA hairpins in *S. stercoralis* as described recently as a high throughput RNAi screening system for *C. elegans* (Johnson et al., 2005).

Confirmation that the mRFPmars coding sequence can give expression of red fluorescent protein in *S. stercoralis* makes an alternate *in vivo* reporter transgene available for this parasite. This will be important for future studies requiring expression of multiple reporters such as experiments designed to examine the relative localization patterns of two molecules at the tissue, cellular or sub-cellular levels. Results of the present study in which we co-transformed *S. stercoralis* with constructs *gfp* and mRFPmars and observed simultaneous expression of both reporters in individual F1 transformants (Fig. 5) demonstrate the feasibility of such an approach. Moreover, the expression frequencies of the two reporters in co-transformed *S. stercoralis* (Table 4) suggest that transgene sequences delivered to this nematode on separate plasmid constructs are incorporated together into transgenic F1 progeny. This finding is consistent with the hypothesis that in *S. stercoralis* microinjected transgene sequences are assembled in tandem extrachromo-

somal arrays as they are in *C. elegans* (Stinchcomb et al., 1985; Mello and Fire, 1995). Our results with co-expression of transgenes in *S. stercoralis* also suggest that fluorescent reporters such as *gfp* or mRFPmars could be used as visual co-transformation markers in the same way that *rol-6* is in *C. elegans* (Kramer et al., 1990; Mello et al., 1991).

In general, the promoters used in this and our previous study (Li et al., 2006) drove reporter gene expression in an anatomical pattern approximating that of their orthologs in *C. elegans*. However, when expressed in *C. elegans*, constructs incorporating some of these *S. stercoralis* promoters gave unexpected and inconsistent patterns of expression. In previous studies where reporter constructs incorporating promoters from genes in parasitic nematodes were expressed in *C. elegans*, observed spatial expression patterns have, with a single exception, been consistent with putative gene function, with expression patterns of orthologous genes in *C. elegans* and, where available, with immunolocalization data from the parasite (Qin et al., 1998; Britton et al., 1999; Winter et al., 2003). Each of these studies has concluded that *C. elegans* represents a reliable surrogate system in which to study temporal and spatial expression patterns of genes from parasitic nematodes. By contrast however, a construct fusing the promoter for *Ov*-GST1a, which encodes a glutathione S transferase in *Onchocerca volvulus*, is expressed in both the pharynx and hypodermis of *C. elegans* while immunolocalization studies indicate that the gene product is present exclusively in the hypodermis of the parasite (Wildenburg et al., 1998). This discrepancy is strikingly similar to the present observation of differential expression patterns of the construct pAJ08 in *C. elegans* and *S. stercoralis*. Along with the highly disparate expression patterns of the *Ss rps-21* promoter in transgenic *S. stercoralis* and *C. elegans*, these results argue for caution in interpreting spatial expression data obtained from heterologous expression of parasite regulatory sequences in *C. elegans*. Such caution in interpreting data on reporter construct expression patterns is standard in *C. elegans* science even where homologous promoters are used. Mello and Fire (1995), for example, stress that although such data may be useful they must be confirmed by more definitive studies involving *in situ* hybridization or immunohistochemistry using antibody probes specific for the gene product in question. We considered the possibility that the disparity between transgene expression patterns in *S. stercoralis* and *C. elegans* could be due to the pairing of non-cognate promoters and 3′UTRs. However, we now consider this unlikely, having observed that the *gfp* reporter construct pPV230.3, which incorporates both 5′ and 3′ regulatory sequences from the same gene, *Ss era-1,* is expressed efficiently in intestinal cells of *S. stercoralis* (Li et al., 2006) but not at all in *C. elegans* (data not shown).

Because it can undergo only one generation of free-living development, all progeny of free-living males and females are fated to become parasitic L3i. Thus, establishment of continuous transgenic lines of *S. stercoralis* from F1 transformants will require passage through a susceptible host. Recovery of parasitic females expressing pAJ08 from experimentally infected gerbils is significant because it confirms that transgenic *S. stercoralis* L3i expressing the *gfp* marker on a strong promoter are viable and can undergo the extensive tissue migration necessary to establish infection in the host intestine. Our current estimate that approximately 15% of F1 *S. stercoralis* transformants transmit transgenes to their progeny compares favorably with the frequency of stable transformation with extrachromosomal transgene arrays following microinjection in *C. elegans* (Mello et al., 1991). However, none of the F2 *S. stercoralis* transformants to date has been observed to express *gfp*, leaving open the possibility that active silencing of transgenes in their present configuration is occurring. Evidence of such transgene silencing has recently been presented for *P. trichosuri*. In this case, when F2 transgenics derived by *in vitro* passage (possible in *P. trichosuri*, which undergoes multiple free-living generations) were subjected to a single host passage, only 10–20% of the resulting F3 worms shown by PCR to harbor the transgene expressed the β galactosidase reporter (Grant et al., 2006). We are currently investigating modifications to our basic vector backbone that may prevent apparent silencing in *S. stercoralis*.

Although stable transgenesis in *S. stercoralis* remains a challenge, transgenic F1s are readily obtainable and can be used to address many biological questions relating to this parasite's free-living and infectious larval stages in the environment and, most significantly, to the parasitic larval and adult stages in the tissues and gasterointestinal tract of the host. Many of these have been discussed at length in our previous paper (Li et al., 2006). It is hoped that the system of modular vectors described here will facilitate these and other investigations in the future.

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