REVIEW ARTICLE

Transgenesis of schistosomes: approaches employing mobile genetic elements

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SUMMARY

Draft genome sequences for *Schistosoma mansoni* and *Schistosoma japonicum* are now available. However, the identity and importance of most schistosome genes have yet to be determined. Recently, progress has been made towards the genetic manipulation and transgenesis of schistosomes. Both loss-of-function and gain-of-function approaches appear to be feasible in schistosomes based on findings described in the past 5 years. This review focuses on reports of schistosome transgenesis, specifically those dealing with the transformation of schistosomes with exogenous mobile genetic elements and/or their endogenous relatives for the genetic manipulation of schistosomes. Transgenesis mediated by mobile genetic elements offers a potentially tractable route to introduce foreign genes to schistosomes, a means to determine the importance of schistosome genes, including those that could be targeted in novel interventions and the potential to undertake large-scale forward genetics by insertional mutagenesis.

Key words: schistosome, transgenesis, electroporation, reporter gene, RNA interference, promoter, gene manipulation, functional genomics.

SCHISTOSOME TRANSGENESIS

Schistosomes have comparatively large genomes, estimated at ~300 megabase pairs (mb) for the haploid genome of *Schistosoma mansoni*, arrayed on 7 pairs of autosomes and 1 pair of sex chromosomes (Hirai *et al*. 2000; Haas *et al*. 2007; Wilson *et al*. 2007). This is approximately the same size as that of the malaria mosquito, *Anopheles gambiae*, 10-times the size of the *Plasmodium falciparum* genome, and a tenth the size of the human genome. *S. japonicum* and the other major schistosome species parasitizing humans probably have a genome size similar to that of *S. mansoni*, based on the similarity of their karyotypes (Hirai *et al*. 2000). The genome is AT-rich (60–70% AT in the euchromatin), replete with repetitive sequences. The ~13 000 protein-encoding genes include several to numerous introns ranging from small (<50 bp) to very large (>50 kb) size (Haas *et al*. 2007). Single nucleotide polymorphisms occur, trans-splicing of a subset of the transcriptome apparently takes place, and alternative splicing is known to occur for some genes, expanding the complexity of the proteome (reviewed by Brindley, 2005). Draft genome sequences for *S. mansoni* and *S. japonicum* are now available, although the identity of the majority of the parasite genes has not been determined (see Haas *et al*. 2007; Wilson *et al*. 2007).

Functional genomics and genetic manipulation tools would aid in the determination of the role of the so far unannotated genes and in the determination of their value as intervention targets. Loss-of-function procedures, employing RNA interference, are becoming available for schistosomes (reviewed by Skelly, 2006). In addition, gain-of-function approaches, involving introduction of exogenous nucleic acids into schistosomes, have also been reported; particle bombardment (biolistics) and electroporation have been used to transfer mRNAs and plasmid-based reporter genes to several developmental stages of *S. mansoni* and *S. japonicum*. The procedures and findings of these studies have been reviewed recently (Grevelding, 2006; Brindley and Pearce, 2007; Kalinna and Brindley, 2007). Here, we discuss reports focused on the transgenesis of schistosomes that involve approaches harnessing mobile genetic elements to facilitate transgene integration into schistosome chromosomes and the establishment of lines of transgenic parasites.

ENDOGENOUS MOBILE GENETIC ELEMENTS OF SCHISTOSOMES

Forty to 50% of the schistosome genome is comprised of repetitive sequences, much of which is

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Table 1. Representative mobile genetic elements characterized from schistosome genomes

Name of element/ Accession no.	Category of MGE	Size (kb)	Copy number (estimated)	Key citations
SM-α, SJ-α M27676, AF213692	SINE-like element	0·3	7000–10 000	Spotilla *et al*. (1989) Laha *et al*. (2000)
SR1 U66331	Non-LTR retrotransposon	4·5	200–2000	Drew and Brindley (1997) DeMarco *et al*. (2005)
SR2 AF025672	Non-LTR retrotransposon	3·9	1000–10 000	Drew *et al*. (1999)
SR3 DQ008121	Non-LTR retrotransposon	3·2	> 1000	Laha *et al*. (2005)
Perere-1 BK004067	Non-LTR retrotransposon	4·9	250–2500	DeMarco *et al*. (2004)
Boudicca AY662653	LTR retrotransposon	5·9	1000–10 000	Copeland *et al*. (2003)
fugitive BK005226	LTR retrotransposon	4·1	2000	Laha *et al*. (2004)
Sinbad AY506538	LTR retrotransposon	6·3	50	Copeland *et al*. (2005)
Saci-1 BK004068	LTR retrotransposon	6·0	70–700	DeMarco *et al*. (2004)
Merlin AY735396	Transposon	1·4	> 500	Feschotte (2004)
SmTRC1 AM268205	Transposon	4·5	30–300	DeMarco *et al*. (2006)

constituted of mobile genetic elements (Brindley *et al*. 2003; Haas *et al*. 2007). A growing number of mobile genetic elements have been characterized in detail, and associated sequences and structures described. Both Class I and Class II elements occur in the schistosome genome, although Class I elements are more numerous in both species present and copy number of the elements. The Class I mobile genetic elements are the retroviruses, long terminal repeat (LTR) retrotransposons, and the non-LTR retrotransposons. This Class also includes the retroposons, or SINE elements. Class I elements are transcribed and move and multiply by reverse transcription. Examples of schistosome LTR retrotransposons include *Boudicca* (Copeland *et al*. 2003), the *fugitive* (Laha *et al*. 2004), *Sinbad* (Copeland *et al*. 2005) and the *Saci* elements (DeMarco *et al*. 2005). Class II elements are the DNA transposons, which can move by direct 'cut-and-paste' mechanisms. Nine superfamilies of transposons occur in eukaryotes at large. Two transposons, *Merlin* and *SmTRC1*, have been reported from schistosomes (Feschotte, 2004; DeMarco *et al*. 2006). Table 1 provides a summary of several of the retrotransposons, SINE elements and transposons that have been described from schistosomes. Recently, Copeland *et al*. (2007) and Laha *et al*. (2007) have reviewed the literature on schistosome retrotransposons.

Mobile genetic elements (MGEs) (Class I) as tools for functional genomics

MGEs from other organisms have been harnessed as gene therapy vectors. These include the transposons *mariner*, *piggyBac* and *Sleeping Beauty* (Plasterk *et al*. 1999). Endogenous retroviruses and retrotransposons from other species have also been developed as vectors for gene transfer. Examples include the human L1 element, a non-LTR retrotransposon (Kazazian, 2004), the *Ty1* retrotransposon of yeast (Garraway *et al*. 1997), murine leukemia virus (Blesch, 2004) and the endogenous retrovirus HERV-K_{CON} reconstituted from the human genome (Lee and Bieniasz, 2007). MGEs generally are inactive, having accumulated disabling mutations during their evolutionary history, because they are not subjected to positive selection (Miskey *et al*. 2005). However, experience with *Sleeping Beauty* and other elements has shown that these elements can be 'back-mutated' in the laboratory to ostensibly active sequences and thereby resurrected (Ivics *et al*. 1997). It is feasible that the endogenous MGEs of schistosomes, none of which has been shown to be transpositionally active so far, could be back-mutated to an active form that could then be used in genetic manipulation of schistosomes (or other target species). The LTR retrotransposons *Boudicca* and *fugitive* from *S. mansoni* are candidates for consideration, given that the presence of numerous transcripts spanning their structural genes indicates that they are transcriptionally active (Copeland *et al*. 2004; Laha *et al*. 2004). An obvious advantage of harnessing the endogenous MGEs of schistosomes is that they may have evolved strategies to subvert natural blocks and inhibition activities of the host cells of the schistosome. An examination of the sequence and structure of some of the schistosome MGEs readily identifies candidates in which only minimal back mutation(s) should be required to derive an ostensibly active sequence; the *fugitive* retrotransposon is a good candidate in that only two or so nucleotide residues might need to be replaced to derive the intact open reading frames (Laha *et al*. 2004).

TRANSPOSON-BASED TRANSGENESIS

Transposons (Class II)

About 9 superfamilies of transposons have been described, they are known from numerous phyla, and they occur in both prokaryotes and eukaryotes

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(Feschotte, 2004; Pritham *et al.* 2005; DeMarco *et al.* 2006). Most of them share a similar structure, a single open reading frame (ORF) encoding the transposase flanked by a pair of terminal inverted repeat sequences. After transcription and translation, the transposase *in trans* cleaves the cognate transposon from its chromosomal site, and transfers to and inserts it at a new position into the chromosomes of the host cell. Transposons are known to spread among unrelated species by horizontal transfer between/among species, a characteristic which has been exploited to develop transformation and transgenesis tools (Plasterk *et al.* 1999). Transposons, such as *piggyBac* (isolated originally from the cabbage looper caterpillar *Trichoplusia ni*), *mariner* (isolated originally from *Drosophila mauritiana*) and *Sleeping Beauty* (from salmonid fishes), have been used to derive lines of transgenic insects, including *Anopheles stephensi* and *Aedes aegypti*, and for gene therapy applications (O'Brochta *et al.* 2003; Miskey *et al.* 2005). It is feasible that transposons might be active in schistosomes because (i) of the ability of some of these elements to mobilize in species phylogenetically distant from those from which they were first isolated, (ii) endogenous transposons have been described from the genomes of *S. mansoni* and *S. japonicum* (see Feschotte, 2004; DeMarco *et al.* 2006), and (iii) several exogenous transposons have been shown to 'jump' in planarians which, though not being parasites, are members of the same phylum (i.e. Platyhelminthes) as schistosomes.

Planaria

Gonzales-Estevez *et al.* (2003) introduced the transposons *Hermes*, *mariner* and *piggyBac* into the planarian *Girardia tigrina*, and reported transposition activity, integration into planarian chromosomes, transmission of the transposon transgenes in the germline, and activity of an eyespot specific promoter (3xP3) driving reporter green fluorescent protein (GFP) expression. More specifically, donor plasmids were constructed, comprising the reporter gene 3xP3-EGFP within *mariner*, *piggyBac* or *Hermes* transposons. Helper plasmids encoding *mariner* and *Hermes* transposase, under the control of the *Drosophila hsp82* promoter or *piggyBac* transposase, under the control of the *Drosophila hsp70* promoter, were also constructed. Cognate donor and helper plasmid pairs were injected into the intestinal cavity and parenchyma of adult or regenerating *G. tigrina*. Subsequently, the planarians were subjected to electroporation using a single square wave pulse of 15 V for 30 ms. All three transposons showed high transformation efficiency. Following long-term maintenance of the transformed worms and their progeny, *Hermes* and *piggyBac* were found to have stably integrated and to be transmitted through subsequent generations by sexual reproduction. The transposon *mariner* was less stable; Gonzales-Estevez *et al.* (2003) proposed that it was frequently lost because of the presence of active *mariner*-type transposons in the genome of the *G. tigrina* (although these authors provided no evidence supporting this hypothesis). One of the cell types transformed in this planarian was the neoblast, a totipotent stem cell present in the adults, representing 30% of total cells. The neoblast represents a unique planarian cell type, with the capacity to proliferate and to differentiate into all somatic cell types as well as into germ cells. These findings demonstrated the activity of 3 transposons of insect origin within the tissues and chromosomes of a flatworm. Furthermore, they suggested that *Hermes*, *mariner* and *piggyBac* might also be active in parasitic flatworms, such as schistosomes and tapeworms (=cestodes), although the stem cell-like neoblasts of planarians have not been characterized from trematodes or cestodes.

Schistosomes

Recently, it has been shown that transposon-mediated somatic transgenesis was feasible. Morales *et al.* (2007) examined whether the *piggyBac* transposon could deliver reporter transgenes into the genomes of schistosomes. Linearized *piggyBac* donor plasmid modified to encode *Photinus pyralis* (firefly) luciferase under the control of schistosome gene promoters – actin (pXL-BacII-SmAct-Luc) or HSP70 (pXL-BacII-SmHSP70-Luc) – was introduced together with 7-methylguanosine capped RNAs encoding *piggyBac* transposase (transcribed *in vitro* from pBS-IE1-orf) into cultured schistosomula by square wave electroporation (125 V, 20 ms, 4 mm). The activity of the helper transposase mRNA was confirmed by hybridization of genomic DNA from the transformed schistosomes to a luciferase gene probe. The hybridization signals indicated that the *piggyBac* transposon had integrated into numerous sites within the parasite chromosomes. Fig. 1 provides a schematic representation of the process of excision of the donor cassette and transposition of the *piggyBac* constructs into target host cell chromosomes. A novel PCR technique, termed retrotransposon anchored PCR (RAP), which uses direct PCR targeting non-digested template DNA (Morales *et al.* 2007), was developed to retrieve the *piggyBac* integrations. RAP is reminiscent of the *Alu*-PCR procedure that has been employed to locate infectious proviral integrations, such as by HIV-1, within human chromosomes (Brussel *et al.* 2005). Of the 5 endogenous elements harnessed, *piggyBac* integrations were recovered from RAPs anchored with the retrotransposons *SR1*, *SR2* and *Boudicca* (Fig. 2; Table 1). Subsequently, *piggyBac* integrations, recovered by

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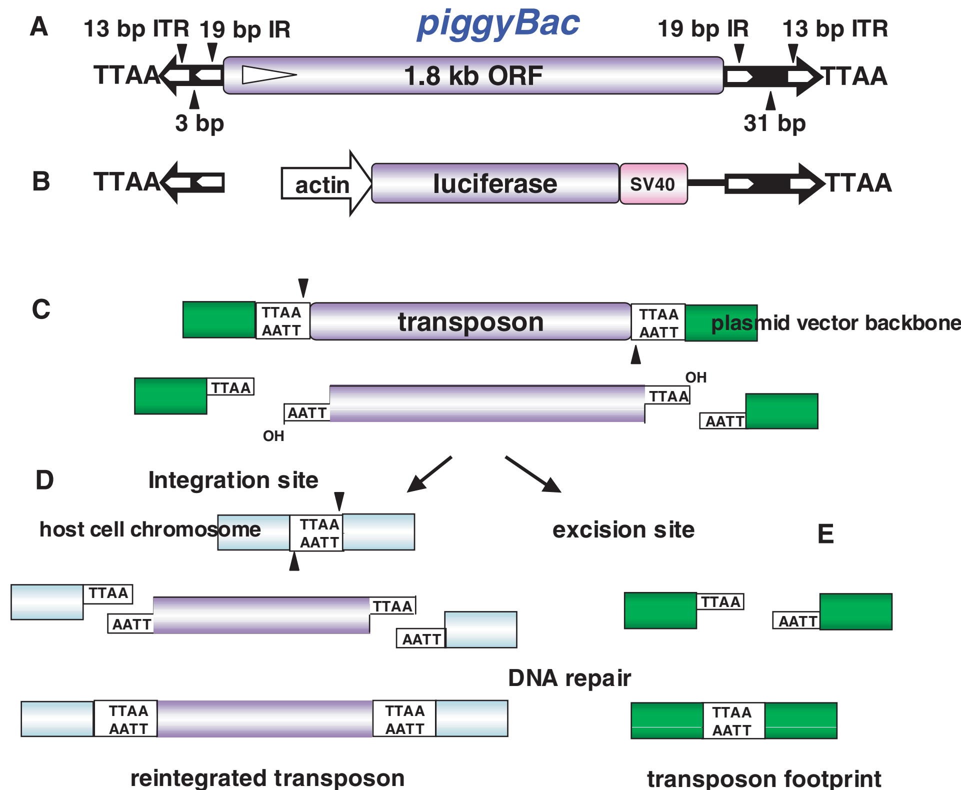

Fig. 1. Structure and mechanism of transposition of the *piggyBac* transposon. (A) Schematic representation of *piggyBac*. The terminal inverted repeats (IR, black arrows) contain binding sites for the transposase (white arrows). The 13 bp ITR and 19 bp IR regions of the terminal inverted repeats (TIRs) are repeat motifs within the TIRs. The 3 bp and 31 bp regions are located at a non-identical site of the left- and right-hand side TIRs, respectively. The element contains a single gene encoding the transposase. The NH_2-terminal part of the transposase contains a DNA binding domain, followed by a nuclear localization signal. The COOH-terminal part of the protein is responsible for catalysis, including the DNA cleavage and rejoining reactions (not shown). (B) Schematic of the donor *piggyBac* cassette from construct pXL-BacII-SmAct-Luc in which the transposase open reading frame has been replaced with a reporter transgene, the promoter from the *Schistosoma mansoni* actin 1.1 gene driving the firefly luciferase gene. (C) Cut and paste mechanism of transposition. The transposase initiates the excision of the transposon donor cassette with staggered cuts and reintegrates it at a TTAA target integration site. (D) The single-stranded gaps at the integration site as well as the double-strand DNA breaks in the donor DNA are repaired by the host DNA repair machinery. After repair, the target TTAA is regenerated at the integration site in the host cell chromosome, and (E) also at the site of excision from the donor plasmid. Adapted from Handler (2002), Miskey *et al*. (2005), with permission, and Morales *et al*. (2007).

retrotransposon-anchored PCR, revealed characteristic *piggyBac* TTAA footprints in the vicinity of protein encoding genes, including adenylosuccinate lyase, glutathione peroxidase 1 and glutathione *S* transferase as well as loci near the endogenous mobile genetic elements *Boudicca*, *SR1*, *SR2* and *Merlin*.

These findings provided the first direct evidence of somatic transgenesis of schistosomes, or indeed of any parasitic helminth. They demonstrated the transpositional activity of *piggyBac* in schistosomal tissues, expanding the host range of *piggyBac* to the Digenea. Morales *et al*. (2007) indicated that future studies with *piggyBac*-mediated transgenesis of schistosomes will include a focus on germline transgenesis and enhanced reporter gene activity. Moreover, these authors predicted, by utilizing chimeric transposase, that it should be possible to direct *piggyBac* integration into specific sites of schistosome chromosomes (Maragathavally *et al*. 2006). In the longer term, *piggyBac* transposon-mediated transgenesis can be expected to facilitate functional genomic investigations of schistosomes, including the forward genetics techniques of insertional mutagenesis, promoter-trapping and exon-trapping (Miskey *et al*. 2005).

RETROVIRAL TRANSDUCTION OF SCHISTOSOMES

Retroviruses

The Retroviridae represents a large family of viruses primarily of vertebrates, although they have also been found in other organisms, including molluscs and insects (Fields *et al*. 1996; Leblanc *et al*. 2000; Syomin *et al*. 2001). Retroviruses may have evolved from LTR retrotransposons (Malik *et al*. 2000;

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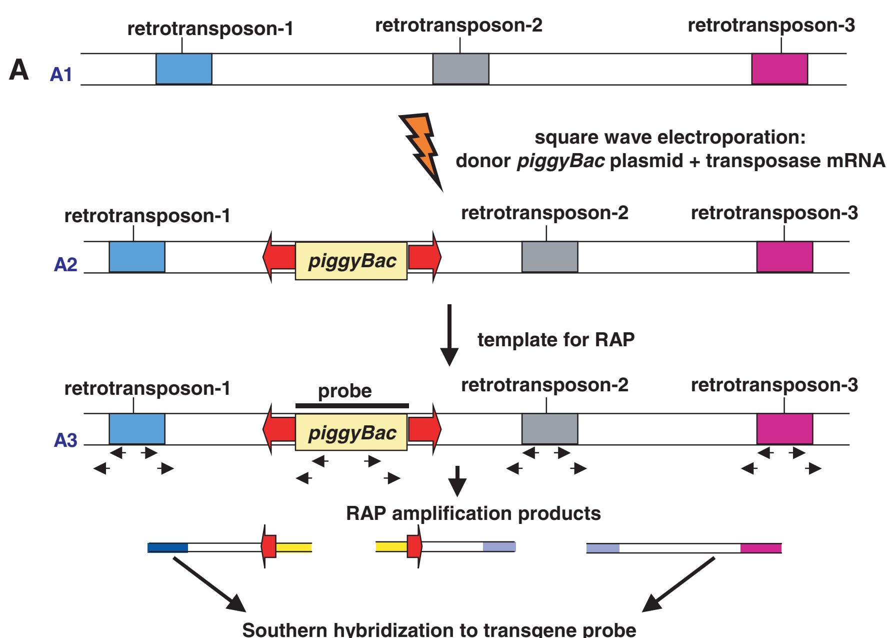

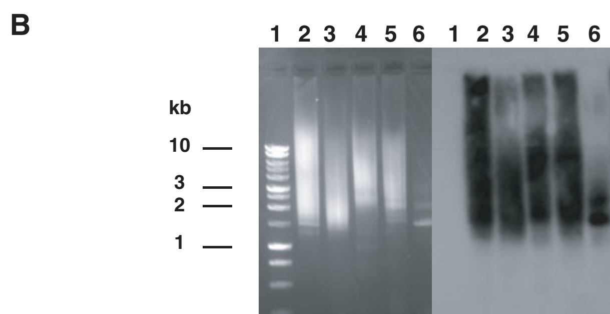

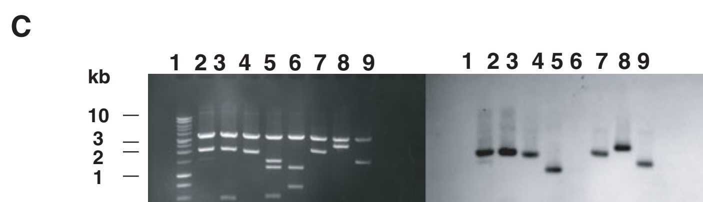

Fig. 2. (A) Schematic representation of the RAP (retrotransposon anchored PCR) technique designed to recover integration junctions between integrated transposons and endogenous retrotransposons and other mobile genetic elements resident within the *Schistosoma mansoni* genome. (A1) Cartoon of endogenous retrotransposons within the schistosome chromosomes. Numerous copies of *SR1*, *SR2*, *Boudicca*, etc. have been described in the *S. mansoni* genome (Table 1). (A2) Schematic representation of the integration of the *piggyBac* transposon into schistosome chromosomes after electroporation of linearized donor cassette and *in vitro*-transcribed transposase mRNA. (A3) Schematic depiction of the RAP technique used to investigate transgene integrations. The position of the probe used in Southern hybridizations is indicated. The small arrowheads indicate positions of the PCR primers used in the RAP. (B) Representative RAP results in the analysis of genomic DNA from schistosomules transformed with the linearized pXL-BacII-SmHSP70-Luc *piggyBac* transposon. Left side: ethidium-stained PCR products amplified using primers specific for the endogenous schistosome retrotransposons and the luciferase transgene. Right side: Southern hybridization of labelled luciferase gene probe to these PCR products. Lane 1, molecular size standards in kilobases (kb); lane 2, nested RAP products amplified with luciferase left specific primer and retrotransposon *SR2* 3′-directed primer; lane 3, nested RAP products from luciferase left specific primer and retrotransposon *SR2* 5′-directed primer; lane 4, nested RAP products from luciferase left specific primer and *SR1* 3′-directed primer; lane 5, nested RAP products from luciferase left specific primer and *Boudicca*-specific primer; lane 6, nested RAP products from luciferase left specific primer and *fugitive*-specific primer. (C) RAP products such as those presented in panel B, lane 4 above were cloned into a plasmid pCR-XL-TOPO, after which the inserts of 8 randomly selected clones were released by digestion with *Eco*R I. Left side, ethidium-stained fragments of pCR-XL-TOPO clones from RAP products amplified with a pair of *SR2*- and luciferase-specific primers. Right side, Southern hybridization of *Eco*R I products to a labelled luciferase gene probe. The hybridizing band likely included the integration junction. The inserts of positive clones (lanes 2, 3, 4, 5, 7, 8 and 9 in this experiment) were sequenced, which revealed integration of *piggyBac* into schistosome chromosomes in the vicinity of copies of the *SR2* retrotransposon. Adapted from Morales *et al.* (2007), with permission.

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Malik and Eickbush, 2001). Retroviral particles are enveloped, and are ~100 nm in diameter. The retroviral genome consists of 2 copies of single-stranded RNA, of approximately 10 kb in length, which contain genes (*gag*, *pol* and *env*) encoding the structural and enzymatic proteins. Retroviruses containing additional regulatory genes are referred to as being 'complex retroviruses', whereas those lacking these genes are called 'simple retroviruses'. The internal nucleocapsid or core is composed of products of the *gag* gene in addition to the catalytic enzyme products of *pol*. The viral membrane that contains the single product of the *env* gene surrounds the core; this envelope protein endows the retrovirus with its host specificity. The Retroviridae are so named because retroviruses possess a unique enzyme, reverse transcriptase (RT). RT uses the viral RNA as a template for making a DNA copy, which then integrates into the genome of the host cell, where it is termed the provirus. The provirus serves as the platform for viral replication. Some retroviruses also carry an additional ORF encoding a host-derived oncogene. This ability of retroviruses to carry and express host genes makes them natural choices for the construction of recombinant vectors for transgenesis (Blesch, 2004).

Retroviruses enter host cells *via* attachment of the viral envelope protein to a specific cell-surface receptor. Most retroviruses enter *via* a single receptor; if the receptor is missing from target cells, the replication cycle cannot proceed. Receptor-mediated binding is followed by fusion of the envelope to the cell membrane. This fusion results in the penetration of the virion core through the plasma membrane. Once the viral core enters the cytoplasm, the RT packaged within the virion catalyses the production of a double-stranded DNA copy of the viral RNA genome. The DNA copy, which remains associated with the incoming viral proteins, localizes to the nucleus. Once inside the nucleus, the viral integrase catalyses the insertion of the viral DNA into the host genome to form the provirus. Subsequently, cellular enzymes catalyse viral RNA genome replication, transcription and the production of viral catalytic and structural proteins required for capsid assembly, budding and maturation. In most cases, retroviral infection has no adverse effects, as the host cell continues to divide normally, replicating the integrated provirus with each cell division.

Pseudotyped Moloney Murine Leukemia Virus (MMLV) vectors

MMLV is an exogenous murine C-type retrovirus. The genomes of C-type retroviruses are considered simple, as they only encode *gag*, *pol* and *env*, the genes for the structural and enzymatic proteins. MMLV vectors have been used to facilitate gene transfer in studies of gene therapy in humans (reviewed by Blesch, 2004). In MMLV vectors, viral protein coding sequences have been replaced by exogenous genes, whereas *cis*-acting sequences for priming reverse transcription and packaging signals are retained. Packaging cell lines have been constructed that express the structural genes, but lack the *cis*-acting elements, rendering them unable to produce infectious virus (Blesch, 2004). Transfection of the packaging cell line with bacterial plasmids containing the foreign gene flanked by the viral LTRs yields infectious but replication-defective virions.

MMLV vectors are ecotropic (will only infect mouse cells) or amphotropic (will infect vertebrate cells, primarily cells of mammalian origin) (Fields *et al*. 1996). Since the host range of MMLV vectors is dictated by the specificity of the viral envelope protein, their use in the transduction of invertebrate cells may be limited. To extend their host range towards pantropism, it is possible to exchange the viral envelope (env) with an envelope protein from another virus, not necessarily another retrovirus, with the desired host range. This process is called pseudotyping. Techniques for pseudotyping retroviruses involve the expression of an envelope protein with the desired host range in packaging cell lines where retroviruses, for example MMLV vectors, are being produced (Yee *et al*. 1994; Ory *et al*. 1996). MMLV vectors have been pseudotyped with the envelope protein of the rhabdovirus and vesicular stomatitis virus (VSV) (Coll, 1995). As the virions bud from the cell membrane, the desired envelope protein is incorporated in place of the MMLV env. Both VSV-G (vesicular stomatitis virus glycoprotein) and the MMLV env bind to specific protein receptors. The difference is that VSV-G binds to an evolutionarily conserved receptor found in almost all eukaryotes, whereas the MMLV receptor is exclusively found on mouse cells (for ecotropic *env*) or mammalian cells (for amphotropic *env*) (Fields *et al*. 1996). Both of them probably share the same mechanism of fusion, where receptor binding triggers a conformational change that results in protrusion of fusion peptides that draw the two membranes together. Thus, VSV-G-pseudotyped retroviruses have a much broader host range than non-pseudotyped MMLV vectors. Additionally, since VSV-G is expressed as a single unit (the MMLV envelope protein has 2 non-covalently linked subunits), it does not fall apart during virus stock preparation, endowing VSV-G pseudotyped viruses with enhanced stability that facilitates concentration by centrifugation and long-term storage without loss of infectivity (Burns *et al*. 1993).

Transfection systems using retroviruses

Viral transfection systems are more efficient at transducing cells than mechanical or chemical

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methods by at least an order of magnitude – viral vectors can often transduce ⩾90% of the cells in a population, whereas non-viral systems are rarely able to transduce more than a small percentage of cells. Retroviruses have been observed to infect, integrate and express reporter genes in various vertebrate and invertebrate organisms, including zebra fish, *Xenopus* (oocytes), newts, several molluscs, an amoeba and insects, such as mosquitoes and species of *Drosophila*. In addition, retroviral vectors have been used to establish transgenic fish and clams (Burns *et al*. 1994, 1996; Lin *et al*. 1994; Lu *et al*. 1996; Matsubara *et al*. 1996; Franco *et al*. 1998; Que *et al*. 1999; Boulo *et al*. 2000).

Transduction of Schistosoma mansoni *with VSVG-pseudotyped MMLV*

Recently, Kines *et al*. (2006) reported an approach using a pseudotyped retrovirus to accomplish the transformation of *S. mansoni*. Retroviral constructs were assembled from the pLNHX plasmid, which is derived from MMLV and Moloney murine sarcoma virus, designed for retroviral gene delivery and expression (BD Biosciences Clontech, USA) (Miller and Rosman, 1989; Emi *et al*. 1991; Burns *et al*. 1993; Matusbara *et al*. 1996; Coffin *et al*. 1997). Upon transfection into a packaging cell line, pLNHX can transiently express, or integrate, and stably express a transcript containing the viral packaging signal Ψ^+, the neomycin selection marker (*neo*^{r}) and a reporter transgene. The 5′-viral LTR of pLNHX contains promoter/enhancer sequences that control expression of the neomycin phosphotransferase II gene to allow antibiotic selection with geneticin (G418) in eukaryotic cells.

Kines *et al*. (2006) modified pLNHX to include endogenous schistosome gene promoters from the *hsp70* gene of *S. mansoni* (see Heyers *et al*. 2003) and the spliced leader (SL) RNA gene of the same species (see Davis *et al*. 1999) as well as reporter genes encoding firefly luciferase and *Aequorea victoria* (jellyfish) enhanced green fluorescent protein (EGFP). Infectious, replication incompetent virions pseudotyped with VSVG were produced by GP2-293 cells transfected with the pLNHX-based constructs and the VSVG pseudotype encoding gene (pVSVG). Virions were harvested from the producer cell culture supernatant and concentrated by high speed centrifugation. Sporocysts, schistosomules and adults of *S. mansoni* were exposed to these virions at a multiplicity of infection (MOI) of 10^4 to 10^7 colony forming units/ml. These replication incompetent retroviruses were able to transduce the cultured schistosomes, leading to apparent integration of proviral forms of the retrovirus into the schistosome chromosomes and to transcription of the reporter transgenes. In particular, two-colour immunofluorescence indicated that the VSVG envelope interacted with the schistosome surface and that the retroviral capsid and RNA genome was released within the surface (subtegumental) cells of the transduced worms. This was a remarkable result because of the unusual double membrane on the surface of blood-stage schistosomes (McLaren and Hockley, 1977) and the potential impediment for the transit of the virions across such a membrane. Hybridization analysis indicated the presence of proviral forms of the retrovirus within the schistosome genome, while expression from the transgenes was indicated by the presence of transcripts encoding neomycin phosphotransferase and firefly luciferase. The evidence obtained using immunofluorescence, hybridization and RT-PCR appeared to confirm successful transduction of these schistosome developmental stages by the VSVG-pseudotyped MMLV virions and integration of the retroviral transgenes into schistosome chromosomes. Figure 3 presents a pictorial summary of the methods and key findings from this recent study (Kines *et al*. 2006).

Targeting appropriate developmental stages for the introduction of transgenes is considered critical to enhance the prospects for routine transgenesis in schistosomes. Although it is probable that most cells transduced were somatic (probably tegumental) cells, the transduction of the germline would be required for heritable transmission of the transgenes. In this regard, the transduction of mother or daughter sporocysts holds considerable promise for introducing transgenes into germ cells, because of their accessibility and comparative abundance in these developmental stages (see Ivanchenko *et al*. 1999) and to eventually obtain cercariae to establish transgenic lines of the parasite. However, transformed sporocysts may have to be introduced surgically into snails in order to obtain cercariae (Cohen and Eveland, 1984), since routine culture of intramolluscan stages of schistosomes from sporocysts through to the release of cercariae remains a technical challenge (Ivanchenko *et al*. 1999).

The transduction of miracidia is another approach worthy of consideration because of the small numbers of cells present in miracidia, because of the accessibility and comparative abundance of germline cells in this developmental stage, and because genetically manipulated miracidia should remain capable of accomplishing natural infection of snails. Heyers *et al*. (2003) demonstrated that miracidia transformed by particle bombardment retained their ability to naturally infect the host snail, *Biomphalaria glabrata*. Another consideration for the selection of a suitable developmental stage of schistosome relates to the comparative efficiency of transduction. The findings obtained using immunofluorescence suggested greater uptake of virions *via* the surface of schistosomules and adult males than by sporocysts (Kines *et al*. 2006). As

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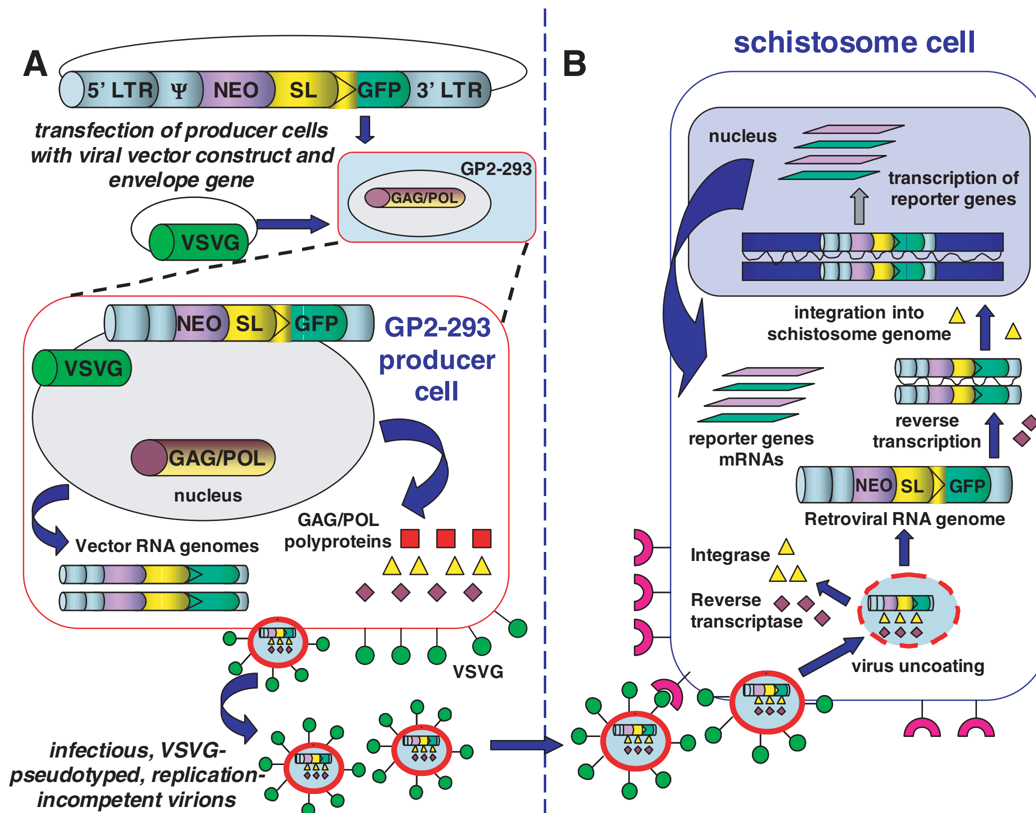

Fig. 3. Schematic representation of transduction of a schistosome cell by vesicular stomatitis virus glycoprotein (VSVG)-pseudotyped Moloney murine leukemia virus (MMLV) virions. (A) The viral vector construct containing the MMLV long terminal repeats (LTR) and psi packaging signal was modified to contain an endogenous schistosome gene promoter and the reporter gene encoding green fluorescent protein (GFP) or firefly luciferase. GP2-293 producer cells containing the *gag/pol* genes were transfected with the viral vector construct and a plasmid encoding the VSVG envelope gene. Infectious, replication incompetent, VSVG-pseudotyped virions budded from the 293 cells and were collected in the supernatant. (B) Schistosomes were transduced by the VSVG pseudotyped virions. The retrovirus RNA genome was apparently reverse transcribed into a DNA copy which integrated into the schistosome genome as the provirus. Reporter gene mRNAs were transcribed from proviral DNA by schistosome host enzymes. Adapted from Kines *et al.* (2006), with permission.

with other target organisms, the transduction of schistosomes may be mediated by the interaction of the VSVG envelope with phosphatidylserine in schistosome tegumental cell membranes (Coil and Miller, 2005). Finally, reporter gene silencing of proviral MMLV transgenes occurs in some contexts, which could impede transgenesis studies (Nabekura *et al.* 2006). Nonetheless, the formidable progress using pseudotyped MMLV in studying the functional genomics of the zebra fish (Amsterdam and Hopkins, 2006), for example, augurs well for its use in functional genomics in schistosomes.

Lentiviruses

Lentiviral vectors, another class of retroviruses, have been modified for *in vitro* and *in vivo* gene transfer (Blesch, 2004). The ability of lentiviruses to transduce non-dividing cells makes them attractive for *in vivo* gene transfer into differentiated, non-dividing tissues. Commercial lentiviruses based on HIV-1 are available; the HIV-1-based system has been modified to increase safety margins, including provision of the genetic components on several plasmids (e.g., ViraPower system, Invitrogen) (Dull *et al.* 1998). In addition, systems based on other lentiviruses, including the feline immunodeficiency viruses (FIVs) have been developed (Poeschla *et al.* 1998). Lentiviral vectors, employing VSVG as the pseudotype, hold the potential to transduce schistosomes.

FUTURE DIRECTIONS AND CONCLUDING REMARKS

Grevelding and colleagues reported that after miracidia were transformed with an ER60-GFP plasmid construct in circular or linearized forms by particle bombardment, the plasmid and reporter GFP were detectable in cercariae released from snails infected with the bombarded miracidia (Grevelding, 2006; Beckmann *et al.* 2007). Indeed, they were detected in F2 generations of cercariae. It is likely that the transgenes were transmitted as episomes, given that

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they included short schistosome sequences, which were unlikely to have been of sufficient length to facilitate homologous recombination and integration into the chromosomes. Deployment of gene therapy-type approaches utilizing, for example, retroviruses, lentiviruses, retrotransposons or transposons, offers the potential to markedly enhance the likelihood of establishment of heritable, transgenic lines of schistosomes. This is particularly likely if germline cells, such as those within the germ cells of miracidia or sporocysts can be targeted for transduction by retroviruses. Approximately 20 germinal cells are present in the posterior part of each miracidium, each with a diameter of about 5 μm and each with a large nucleus of $\sim$3 μm diameter (Grevelding, 2006). After infection of the snail, each miracidium of *S. mansoni* produces several hundred germ balls by asexual reproduction, and these germ balls differentiate into the daughter sporocyst, which in turn generates the cercariae.

New approaches using mobile genetic elements are likely to supersede the episome-based approaches because they facilitate chromosomal integration of the transgene. Whereas heritable transgenesis – rather than the demonstration of somatic transgenesis using transposons or MLV (Kines *et al*. 2006; Morales *et al*. 2007) – has yet to be reported, it can be expected to be developed given the advances accomplished with transposons in mosquitoes and other species (e.g., Maragathavally *et al*. 2006; Balu *et al*. 2005). Transposon- or retrovirus-based transgenesis has the potential to derive lines of stable transgenic schistosomes. Moreover, the technique should also facilitate stable heritable RNAi (Brown *et al*. 2003), insertional mutagenesis and other informative functional genomics approaches. The demonstration of movement of the *piggyBac* transposon into schistosome chromosomes confirmed that transposon-mediated transgenesis in schistosomes is feasible. This progress provides exciting opportunities for functional and reverse genetics studies in these parasites.

Although some progress has been made with somatic transgenesis of larval *S. mansoni* using MLV and *piggyBac* (Kines *et al*. 2006; Morales *et al*. 2007), this field is in its infancy. In particular, the constructs presently available could be improved with more efficient promoters and reporter genes, including selectable markers. Promoters, such as those from viral CMV, SV40 and HSV genes, which are 'strong promoters' in mammalian tissues, do not seem to be active in schistosome tissues (see Brindley and Pearce, 2007). Promoters from the endogenous schistosome gene HSP70 and the spliced leader RNA gene have been used to drive reporter gene expression in transiently transformed schistosomes (Davis *et al*. 1999; Wippersteg *et al*. 2002; Heyers *et al*. 2003), but stronger promoters such as that from the actin 1.1 gene may be more useful (Beckmann *et al*. 2007; Correnti *et al*. 2007). Accordingly, more information on promoters for transgene expression in schistosomes would be useful, particularly in relation to developmental and conditional expression. Recent reports, employing promoters driving several schistosome proteases, have demonstrated the utility of the approach for the investigation of tissue-specific and stage-specific expression of schistosome antigens (see Grevelding, 2006). Better reporters will likely help, since GFP is not optimal in schistosomes because of substantial autofluorescene in adult females, and because firefly luciferase may not be as active in schistosome tissue as in mammalian tissues. The poor performance of these reporters may also reflect the activity of stronger promoters in mammalian cells, such as the CMV immediate early promoter, which is probably not active in *S. mansoni*. In addition, several other reporter genes, including *Renilla* (sea pansy) luciferase and red fluorescent protein from the anthozoan *Heteractis crispa*, are active in schistosome tissues (Osman *et al*. 2006). One way forward may be to employ the luciferase from *Gaussia princeps* (a marine copepod) as a novel reporter protein. It is substantially more robust (delivers manifold more relative light units [RLUs] per μg host protein) than firefly and *Renilla* luciferases, is encoded by only a short ORF and is secreted (Tannous *et al*. 2005). Cheng and Davis (2007) delivered mRNA encoding *Gaussia* luciferase (GLuc) to cultured *S. mansoni* worms by both electroporation and biolistics, and observed that GLuc was secreted from transformed schistosomes. In general, electroporation was efficient for the treatment of schistosome sporocysts or schistosomules with mRNA encoding GLuc. Since GLuc is secreted, it should facilitate analysis for transgenic schistosomes without having to kill the parasites; in this regard, the use of GLuc as a reporter could facilitate the establishment of lines of transgenic schistosomes.

In addition to the use of (retro)transposons for schistosome transgenesis, other related strategies might be considered including site-specific transgene integration systems. Mobile genetic elements, such as *piggyBac* and MMLV, utilize their integrase-like domains to insert themselves into chromosomes of host cells. Similarly, related integrase sequences are utilized by bacteriophages and other mobile sequences. The deployment of site-specific integrases, such as the CRE-*lox* recombinase from bacteriophage P1, the FLP-FRT from the 2 micron plasmid of *Saccharomyces cerevisiae* and the serine integrase of mycobacteriophage Bxb1 from *Mycobacterium smegmatis* may find utility in gene delivery and manipulation protocols for schistosomes as has been the case in *Plasmodium falciparum*, mosquitoes and other model systems (Jasinskiene *et al*. 2003; Nkrumah *et al*. 2006; Thomson and Ow, 2006).

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In any event, using these systems, even random low frequency integration could be useful if coupled to a powerful selection protocol. Schistosomes are susceptible to the widely used culture selection agent hygromycin B (see Brindley and Pearce, 2007), as are many parasitic nematodes (Kamps-Holtzapple *et al*. 1994). Conversely, a variety of obstacles may militate against 'drug pressure selection' to facilitate the development of transgenic schistosomes. Not least among these are the relatively small numbers of parasites from which it might be feasible to select from. Also, there is the requirement for the drug resistance gene to be expressed not only in the germline but also in the multiple other hygromycin-sensitive tissues within the parasite. Nonetheless, transgenic lines of planaria (free-living relatives of schistosomes) and mosquitoes have been developed using transposon-based technologies (Gonzalez-Estevez *et al*. 2003; Catteruccia *et al*. 2005). Moreover, transposons or retroviruses can be engineered to carry gene cassettes for the production of siRNAs and thereby offer the possibility for the heritable, targeted suppression of specific genes through RNAi (Tavernarakis *et al*. 2000; Brown *et al*. 2003; Paddison *et al*. 2004).

Transgenesis mediated by retroviruses, transposons or other mobile genetic elements offers a tractable method for functional genomic studies of schistosomes. With the imminent availability of the draft genome sequences of *S. japonicum* and *S. mansoni* (see Haas *et al*. 2007), and also the genomes of numerous other parasitic helminths (Mitreva *et al*. 2006), the availability of routine transgenesis approaches and other gene manipulation procedures can be expected to facilitate the important public health goal of identifying and characterizing novel anti-schistosomal targets. These approaches have been validated in the characterization of the recently available genome sequences in other complex models including medaka, zebra fish and mice (Miskey *et al*. 2005). Finally, although developmental cycle constraints make it more difficult to study *S. haematobium* and *S. japonicum* in the laboratory, we anticipate that the transgenesis approaches being developed in *S. mansoni* will be applicable to a range of schistosomes.

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