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Quantitative retrotransposon anchored PCR confirms transduction efficiency of transgenes in adult *Schistosoma mansoni*

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Abstract

A quantitative retrotransposon anchored PCR (qRAP) that utilizes endogenous retrotransposons as a chromosomal anchor was developed to investigate integration of transgenes in *Schistosoma mansoni*. The qRAP technique, which builds on earlier techniques, (i) *Alu*-PCR which has been used to quantify lentiviral (HIV-1) proviral insertions in human chromosomes and (ii) a non-quantitative retrotransposon anchored PCR known to detect the presence of transgenes in the *S. mansoni* genome, was tested here in a model comparison of retrovirus-transduced adult schistosomes in which one group included intact worms the other included fragments of adult worms. At the outset, after transducing intact and viable fragments of schistosomes with reporter RNAs, we observed enhanced reporter activity in fragments of worms than in intact worms. We considered this simply reflects the increased surface area in fragments compared to intact worms exposed to the exogenous reporter genes. Subsequently, intact worms and worm fragments were transduced with pseudotyped virions. Transgene integration events in genomic DNA extracted from the virion-exposed worms and worm fragments were quantified by the qRAP, which revealed that fragmenting adult schistosomes resulted in increased density of proviral integrations. The qRAP findings confirmed the likely value of this qRAP technique for quantification of transgenes integrated in schistosome chromosomes. Last, considering the absence of schistosome cell or tissue lines, primary culture of fragmented worms offers an opportunity to optimize transgenesis, and other functional genomic approaches.

Keywords

Schistosome; pseudotyped retrovirus; transgene; anchored PCR; integration; retrotransposon; luciferase

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1. Introduction

The genome sequences of two of the three major species of schistosomes are now available [1,2], and transcriptomic data on other trematodes are rapidly increasing [3,4]. The identification and validation of putative gene function requires functional genomics methods and tools that are now in development, e.g., [5–10]. RNA interference (RNAi) has been employed to determine the function and importance of a number of schistosome genes (e.g. [11–13]), and its deployment is expected to expand [14]). Whereas RNAi provides insights, transgenesis approaches may provide a flexible framework for both reverse and forward genetics in schistosomes and other trematodes. Recently, it has been demonstrated that vesicular stomatitis virus glycoprotein pseudotyped murine leukemia virus virions (VSVG-MLV) can be employed to transduce the surface of several developmental stages of both *Schistosoma mansoni* [15–17] and *S. japonicum* [18], including eggs, sporocysts, schistosomules and adult worms. Furthermore, vector based RNAi was employed recently to investigate the importance of schistosome genes; in vitro transcription of short hairpin RNAs specific for the gene encoding a papain-like cathepsin B of *S. mansoni* rapidly depleted adult worms of target protease [19].

Because of the nature of the gammaretroviral life cycle, the proviral genome of MLV integrates into the genome of the transduced cell [20]. Studies with VSVG-MLV have confirmed that proviral sequences integrate in the chromosomes of *S. mansoni* [16]. A potential functional genomics application of this ability of MLV to integrate in the schistosome genome is for insertional mutagenesis screen of schistosomes, now feasible given that draft genome sequences for *S. mansoni* and *S. japonicum* are available. Langridge and colleagues [21] recently demonstrated that power of insertional mutagenesis using a bacterium-transposon model, and indeed were able to characterize *Salmonella* Typhi genes that were essential for this enteric pathogen. Whereas each developmental stage of the schistosome life cycle might provide particular advantages to be targeted for introduction of transgenes (see [8,17,22]), the adult worms are readily and reliably obtained from experimentally infected rodents, can be easily maintained in culture, and the females continue to release viable eggs for several days after perfusion from mice [23]. These characteristics make the adult developmental stage an attractive target for transgenesis. However, since replication-deficient retroviral systems can only integrate into the chromosomes of the cell to which the provirus attaches, only the surface and gut are readily targeted in cultured schistosomes.

In this study we analyzed whether fragmenting the adult worm and maintaining the fragments in culture (a procedure that reduces the large ratio of surface area to body mass in schistosome adults) might result in improved transduction rates. A quantitative retrotransposon anchored PCR (qRAP) that utilizes endogenous retrotransposons as a chromosomal anchor was developed to investigate integration of the transgenes. Using the qRAP, we confirmed increased density of transgenes in cultured fragments of adult schistosomes, and more importantly confirmed the utility of this anchored PCR to quantify transgene transduction.

2. Materials and Methods

2.1 Parasites

Mixed sex adults of *S. mansoni* were perfused from experimentally infected mice six to nine weeks after infection and maintained in culture [22].

2.2 Synthesis and delivery of luciferase RNA

To synthesize firefly luciferase mRNAs (mLuc), DNA templates were amplified by PCR from plasmid pGL3-Basic (Promega, Madison, WI) templates, as described [5]. *In vitro* transcriptions of capped RNAs from template PCR products were accomplished using the mMessage mMachine T7 Ultra kit (Ambion, Austin, TX). mRNAs were precipitated with ammonium acetate, dissolved in nuclease-free water and concentration determined with a spectrophotometer (ND-1000, NanoDrop Technologies, Wilmington, DE). Mixed sex adult worms were removed from culture [22], washed five times in schistosomule wash medium (Dulbecco's modified Eagle's medium (DMEM) with 200 U/ml penicillin G sulfate, 200 mg/ml streptomycin sulfate, 500 ng/ml amphotericin B, 10 mM HEPES) and sliced in two, three or more pieces, as indicated, using a sterile scalpel blade. Thereafter, the intact worms and the worm fragments were subjected to square wave electroporation (250 V, 30 ms) in 4 mm gap BTX cuvettes containing 6 µg of firefly luciferase mRNA in 100 µl of schistosomule wash medium. After electroporation, intact worms and worm fragments were transferred to DMEM supplemented with 10% fetal bovine serum (FBS) and 100 U of penicillin and streptomycin (Invitrogen, Carlsbad, CA), pre-warmed to 37°C [22]. Three hours after electroporation, the worms were washed well with schistosomule wash medium, and then stored as wet pellets at -80°C.

2.3 Luciferase activity assay

Luciferase activity in extracts of these schistosomes was determined using Promega's luciferase assay reagent system in a Sirius luminometer (Berthold, Pforzheim, Germany), as described [7]. In brief, pellets of parasites were subjected to sonication (3 X 5s bursts, output cycle 4, Misonix Sonicator 3000, Newtown, CT 06470) in 300 µl 1X CCLR lysis buffer (Promega). The sonicate was cleared by centrifugation at 14,000 rpm at 4°C for 15 min (Eppendorf model 5810 centrifuge), and activity in the supernatant containing the soluble fraction determined. Aliquots of 100 µl of the soluble fraction were dispensed into 100 µl luciferin substrate (Promega) at 23°C, mixed, and the relative light units (RLUs) were determined in the luminometer 10 s later. Duplicate samples were measured, with results presented as the average of the duplicate readings per mg of soluble fluke protein. Protein concentrations were determined using the bicinchoninic acid assay (Pierce, Rockford, IL). Recombinant luciferase (Promega) was included as a positive control.

2.4 Exposure of fragmented worms to Cy3-labeled siRNA

S. mansoni mixed sex adults were fragmented and subjected to square wave electroporation in the presence of Cy3-labeled siRNAs (Silencer Cy3-Labeled Negative Control siRNA, catalog AM4621, Ambion, Austin, TX) at 3.6 µM (50 ng/µl) following the conditions described above. Immediately after electroporation parasites were transferred into complete DMEM at 37°C. Four hours after exposure to Cy3-siRNA, worms were washed in culture medium five times in order to remove the unincorporated Cy3-labeled siRNAs. Thereafter, they were observed under bright and fluorescent light (see below) using a Zeiss Axio Observer A.1 inverted microscope fitted with a digital camera (AxioCam ICc3, Zeiss). Manipulation of digital images was undertaken with the AxioVision release 4.6.3 software (Zeiss).

2.5 Transduction of schistosomes with pseudotyped murine leukemia retrovirus (VSVG-MLV)

VSVG-pseudotyped virions were produced in GP2-293 cells transfected with plasmid constructs pLNHX-SmAct-Luc and pVSVG [15]. Viral titers were determined using two complementary approaches; a functional (biological) assay using target NIH-3T3 mouse fibroblast cells cultured in the presence of geneticin [15] and second by real time PCR

(Retro-X™ qRT-PCR Titration Kit, Clontech). Intact or fragmented worms were cultured in 24 well plates, in 200 µl of medium plus 200 µl of virions (VSVG-MLV) at 4×10^5 colony forming units (cfu)/ml in the presence of 8 µg/ml polybrene (Sigma-Aldrich, St. Louis, MO). The same preparation of virus was used to estimate the viral titer by real time PCR resulting in a viral titer of 7.03×10^7 copies/ml. The worms were washed 18 hours later and cultured for a further 24 hours, after which genomic DNAs (gDNAs) were extracted from the worms. The density of integrated proviral luciferase transgenes investigated in the gDNAs was investigated by quantitative, anchored PCR (below).

2.6 Quantitative-retrotransposon anchored PCR (qRAP)

Based on the *Alu*-PCR approach used to quantify the copy number of integrated HIV-1 provirus in the genome of human cells [24], and on a chromosomal anchored PCR technique we have used previously to identify transposon and proviral transgenes in the genome of *S. mansoni* [6,16], we developed a quantitative anchored PCR-based approach (qRAP), to investigate retrovirus integrations into the schistosome genome. In brief, qRAP includes two consecutive PCRs (Fig. 1). The first, retrotransposon anchored PCR (RAP), consists in a multiplex PCR using a specific primer for the luciferase (*luc*) transgene from the donor pLNHX construct in tandem with primers specific for endogenous retrotransposons present at high copy number and interspersed throughout the genome of natural populations of *S. mansoni* [1]. Second, RAP products are used as template in a quantitative PCR, targeting the *luc* transgene [17]. The first RAP amplification was performed using 100 ng template gDNA from populations of MLV-transduced schistosomes or control gDNA from untreated worms, Platinum *Taq* DNA Polymerase High Fidelity (Invitrogen) and primers specific for the retrotransposons *SR1*, *SR2*, *fugitive* and *Boudicca* in combination with the luciferase-specific primer in a 50 µl reaction. Four primer mixes were used: mix #1, *SR1F* (200 nM), *SR1R* (200 nM) and *luc* (400 nM); mix #2, *SR2F* (200 nM), *SR2R* (200 nM) and *luc* (400 nM); mix #3, *fugitive F* (200 nM), *fugitive R* (200 nM) and *luc* (400 nM); mix #4, *Boudicca F* (200 nM), *SMa* (200nM), *luc* (400 nM) (Table S1). Linear, one-way amplification was also evaluated by performing the preamplification PCR with the *luc* primer alone. RAP cycling conditions were 94°C for 2 min followed by 20 cycles of 94°C for 30 s, 57°C for 30 s and 68°C for 10 min, with a final extension at 68°C for 10 min. RAP products were employed as template in a quantitative PCR targeting *luc* was performed as described [17]. Briefly, *luc* specific primers and TaqMan probe were designed with the assistance of Beacon Designer (Premier Biosoft International, Palo Alto, CA); sequences of the primers and probe: *luc* forward primer, 5'-TGC TCC AAC ACC CCA ACA TC- 3'; reverse primer, 5'- ACT TGA CTG GCG ACG TAA TCC- 3'; probe, 5'-/56-FAM/ACG CAG GTG TCG CAG GTC TTC C/3IABlk_FQ/-3'. The PCR efficiency for the luciferase primers/probe set was estimated by titration analysis [26] to be 97% (not shown).

Quantitative PCRs were performed in triplicate, using 96-well plates (Bio-Rad), with a denaturation step at 95 °C of 3 min followed by 40 cycles of 30 sec at 95 °C and 30 sec at 55 °C, using a real-time thermal cycler (iCycler, Bio-Rad) fitted with the Bio-Rad iQ5 detector. Reactions were carried out in 20 µl volumes with *luc* primer-probe sets, Perfecta qPCR FastMix, UNG (Quanta Bioscience, Gaithersburg, MD), and using as template, 5 µl of a 0.1 dilution of the RAP products (preamplification dilutions) or matched dilutions of non-preamplified samples, i.e., gDNA dilutions that were not amplified by RAP. Quantification was undertaken using copy number standards, i.e. 10-fold serial dilutions of the firefly luciferase encoding plasmid pGL3 Basic (Promega), from 1.93×10^3 copies to 1.93×10^{10} copies. Luciferase transgene copy number was estimated by interpolation of the sample PCR signals from a standard curve [26]. Transgene copy numbers from schistosomes exposed to virions are presented as fold-increase of RAP-preamplified copy number compared to the non-preamplified copy number.

3. Results

3.1 Reporter RNAs introduced into schistosomes

Fragmented and intact worms were transduced with mRNA encoding firefly luciferase, and luciferase activity measured three hours later (Fig. 2A). Luciferase activity was readily detected in extracts of intact adult worms. As illustrated (Fig. 2B), significantly higher luciferase activity was seen in extracts of worms that had been cut into two pieces and even higher activity seen in extracts of the worms chopped into three or more fragments (646, 1,066 and 2,039 RLUs/sec/mg protein, respectively) ($P \leq 0.05$). In summary, the more fragmented the worms, the higher the luciferase activity.

To investigate whether higher luciferase activity measured in fragmented parasites was caused by increasing the surface area exposed to the reporter, we transduced fragmented parasites with Cy3-labeled siRNA, a fluorescent probe employed previously to demonstrate entry of macromolecules into other trematode developmental stages [7,17]. Four hours after electroporation, the worm pieces were washed and examined. As is shown in Fig. 3 bright foci of fluorescence were evident at or proximal to the cut surfaces of the worms indicating that Cy3-labeled siRNA was introduced into the worms not only through the intact tegument but apparently also through the lesions.

3.2 Retrotransposon anchored PCR quantifies transgene integrations

Increasing the surface area of transduced worms facilitated entry of reporter RNAs. Accordingly, we proceeded to transduce intact and fragmented schistosomes with VSVG-MLV virions. Integration events in gDNAs extracted from the worms were estimated by qRAP, using primer pairs targeting the luciferase transgene and endogenous mobile genetic elements that occur at high density in the *S. mansoni* genome [1,6,16]. With each of four separate anchored primer sets, targeting the *SR1*, *SR2*, *fugitive* or *Boudicca* (plus S α) retrotransposons, similar trends were seen - fragmenting the schistosomes resulted in increased density of proviral integrations. As shown in Fig. 4A, left panel, the qPCR signal detected from fragmented parasites amplified with the *SR1* primer mix crossed the threshold cycle about four cycles earlier than for intact parasites. The signal from control, untreated worms did not reach the threshold before 40 cycles. Similar outcomes were seen with the *SR2*, *fugitive* and *Boudicca*/S α primer mixes (not shown). Significant difference in threshold cycle was observed for RAP-preamplified compared with non-preamplified templates, i.e. gDNA that was amplified only by qPCR (Fig. 4A, centre panel). When one-way amplification was monitored by performing the first PCR with only the *luc* primer, again a significant difference in threshold cycle was detected for RAP-preamplified compared with either preamplified with only the *luc* primer or with the non-preamplified templates (Fig. 4A, right panel).

Given the lack of an accurate quantitative standard curve in our model, as in the *Alu*-PCR of O'Doherty et al [24] who developed an integration standard CD4⁺ T-lymphoblastoid cell line in which the copy number of integrated HIV-1 provirus was known, with our qRAP we cannot predict the absolute transgene copy number per nanogram of virion-transduced gDNA. However, we were able to predict the absolute transgene copy number from samples that were not RAP-preamplified. Consequently, we have estimated fold-increase of transgene copies between RAP-preamplified and non-preamplified samples. As shown in Fig. 4B, the transgene copy number fold-increase estimated from parasites that had been cut into pieces was significantly higher than in intact worms. Specifically, the transgene copy number fold-increase between the RAP-preamplified samples and matched non-preamplified samples was 5 to 7 fold (depending on the anchoring primer - *SR1*, *SR2*, etc.) in intact worms and ~16–24 fold in the fragmented worms.

4. Discussion

We observed increased incorporation of exogenous reporter RNA into fragmented compared to intact schistosomes. Further, we estimated the density of transgene chromosomal integrations in the treated worm population using a quantitative retrotransposon anchored PCR approach. This procedure that we termed qRAP is an adaptation of previous methods [6,16,24], and involves the amplification of integration events by a multiplex PCR using a transgene primer and a set of primers directed to retrotransposons widely dispersed in the schistosome genome, followed by a quantitative PCR determination of the amount of transgene copies against a standard. We anticipate qRAP can now be employed to quantify transgene copy number in transgenic schistosomes and other helminths.

Although the qRAP technique does not provide absolute copy number per genome, it is useful for comparing integration events between treatment conditions. Here, significant differences were observed between the transgene copy number fold-increase in parasites that had been diced into pieces than in the intact worms: ~6-fold in intact worms and ~20-fold for diced worms (Fig. 4B). Depending on the anchoring primer - *SR1*, *SR2*, *fugitive* or *Boudicca*/SMA the transgene copy number fold-increase between the RAP-preamplified samples and matched non-preamplified samples range from 5 to 7 in intact worms and from 16 to 24 in sliced parasites, e.g. the highest fold-increase was observed in diced parasites when *SR2* was used. This could reflect the variation in abundance among the target retrotransposons in the *Schistosoma mansoni* genome [1]. The fold increases between RAP-preamplified and non-preamplified samples were lower than those reported by O'Doherty and colleagues for HIV-1 using quantitative *Alu*-PCR [24]. However, whereas they observed a ~4,000-fold difference with a chronic HIV-infected cell line, they reported a much smaller difference (~200-fold) between pre-amplified and non-preamplified gDNA from an acute *in vitro* infection. In the present study, we harvested worms one day after virion exposure, conditions more similar to the acute rather than chronically infected cells of O'Doherty et al [24]. In addition, there are additional reasons why the qRAP may not be as efficient as *Alu*-PCR including (1) differences in biological characteristics of the target retrovirus, HIV-1 versus MLV, (2) densities of *Alu* in human chromosomes (>1.1 million copies) and the endogenous schistosome retrotransposons (<10,000 copies), (3) relative frequency of truncation phenomena of MLV proviral genome in the schistosome genome [16], and (4) distance of transgene primer from the 3'-LTR (Fig. 1).

Despite these potential impediments, the present findings confirmed that qRAP facilitates comparison of integration outcomes between treatment conditions. Although an accurate standard curve would enhance estimation of absolute transgene copy number in transgenic schistosomes, qRAP represents the first approach that allows relative quantification of integration events in schistosomes. We anticipate that qRAP will find utility in evaluating transduction/integration efficiency of retroviruses or other integrative vectors, such as transposons or retrotransposons. We predict that functional genomics for schistosome will be enhanced by transgenesis studies utilizing VSVG-MLV, as several recent reports have indicated [16,17,19]. As methods both to quantify and to increase the genomic density of integrations, the techniques described here should enhance the prospects of progress with functional genomics with MLV-based transgenesis of schistosomes. In addition, increasing the density of retrovirus integration events not only would allow to identify preferential target sites in schistosome chromosomes, but also would facilitate insertional mutagenesis screens and in development of gain-of-function studies, for example with selection for drug resistance, reporter gene activity, and so forth.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Illustration of the quantitative Retrotransposon Anchored PCR (qRAP) approach
 First PCR: 20 cycles of end-point PCR preamplification with primers that target endogenous mobile genetic elements and luciferase transgene sequences. Heterogeneous amplicons of variable length are expected. Second PCR: quantitative PCR to estimate the copy number of luciferase-specific sequences within the transduced schistosome genome. Quantification was undertaken using copy number standards, i.e. 10-fold serial dilutions of the luciferase encoding plasmid pGL3, after which copy number of luciferase transgene in schistosome genomic DNAs was calculated by interpolation from a standard curve. The qRAP was adapted from reference [24].

Figure 2. Luciferase activity in adult *Schistosoma mansoni* worms and worm pieces following transduction with firefly luciferase mRNA

Panel A: Representative images of an intact female adult (left panel), a female adult cut in two pieces (middle panel) and worms diced into three or more pieces (left panel). Scale bar, 500 μ m. Panel B: Luciferase activity measured in extracts of adult worms 3 hours after electroporation, (a) mock control adult worms treated with no molecule, (b) intact worms treated with mRNA, (c) adult worms cut in two pieces and treated with mRNA and (d) worms diced into three or more pieces and treated with mRNA. *, $P \leq 0.05$, **, $P \leq 0.01$.

Figure 3. Fluorescent Cy3-labeled short interfering RNA enters fragmented adults of *Schistosoma mansoni*

Fragmented schistosome adults four hours after electroporation in 50 ng/μl Cy3-siRNA; (A, B, C) bright and (D, E, F) fluorescent fields of representative images of fragmented worms, (G) high magnification image of a representative fragmented worm with fluorescent foci in sliced region, (H) representative image of an intact worm showing fluorescent foci in the tegument, (I) bright and (J) fluorescent fields showing control, untreated worms. White arrows indicate fluorescent foci. Scale bar 500 μm in A–F and H–J, and 200 μm in G.

Figure 4. Relative transgene copy number estimated by qRAP in the genome of virus exposed adults of *Schistosoma mansoni*

A: Left panel. Amplification plots observed in preamplified samples using *SR1* primer mixes. Arrows point threshold cycle of the indicate sample, red arrow: MLV-transduced fragmented worms, blue arrow: MLV-transduced intact worms, black arrow: control untreated worms. Centre panel. Amplification plots observed in preamplified (P) and non-preamplified (N) MLV-transduced fragmented worms using all primer mixes. Arrows point threshold cycle of the indicate sample. Right panel. Amplification plots observed in MLV-transduced fragmented worms in preamplified template using *SR2* primer mix (red arrow), in preamplified template using only the *luc* primer (blue arrow), and in non-preamplified template (black arrow). Arrows indicate the threshold cycle. B: Transgene copy numbers in virions exposed schistosomes shown as fold-increase of preamplified copy number compared to the non-preamplified copy number in each sample. #1: *SR1* primer mix, #2: *SR2* primer mix, #3: *fugitive* primer mix, #4: *Boudicca/SMα*. ANOVA **P≤0.01.