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Short technical report

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 more 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.

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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 *Schistosoma 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]),

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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 mRNA

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 × 5 s bursts, output cycle 4, Misonix Sonicator 3000, Newtown, CT 06470) in 300 μl 1× 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 into100 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^{TM} 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 h later and cultured for a further 24 h, 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* [1,25]. 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 combina-

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