Published in final edited form as:

Mol Biochem Parasitol. 2010 December ; 174(2): . doi:10.1016/j.molbiopara.2010.07.007.

Transduction of *Schistosoma japonicum* schistosomules with vesicular stomatitis virus glycoprotein pseudotyped murine leukemia retrovirus and expression of reporter human telomerase reverse transcriptase in the transgenic schistosomes

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Abstract

Although draft genome sequences of two of the major human schistosomes, *Schistosoma japonicum* and *S. mansoni* are available, the structures and characteristics of most genes and the influence of exogenous genes on the metabolism of schistosomes remain uncharacterized. Furthermore, which functional genomics approaches will be tractable for schistosomes are not yet apparent. Here, the vesicular stomatitis virus glycoprotein (VSVG)-pseudotyped pantropic retroviral vector pBABE-puro was modified to incorporate the human telomerase reverse transcriptase gene (*hTERT*) as a reporter, under the control of the retroviral long terminal repeat (LTR). Pseudotyped virions were employed to transduce *S. japonicum* to investigate the utility of retrovirus-mediated transgenesis of *S. japonicum* and the activity of human telomerase reverse transcriptase as a reporter transgene in schistosomes. Schistosomules perfused from experimentally infected rabbits were cultured for six days after exposure to the virions after which genomic DNAs from virus-exposed and control worms were extracted. Analysis of RNA from transduced parasites and immunohistochemistry of thin parasite sections revealed expression of

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hTERT in the transduced worms. Expression of *hTERT* was also confirmed by immunoblot analysis. These findings indicated that *S. japonicum* could be effectively transduced by VSVG pseudotyped retrovirus carrying the *hTERT* gene. Given the potential of *hTERT* to aid in derivation of immortalized cells, these findings suggest that this pantropic retroviral approach can be employed to transduce cells from specific tissues and organs of schistosomes to investigate the influence of transgene *hTERT* on growth and proliferation of schistosome cells.

Keywords

Schistosoma japonicum; glycoprotein of vesicular stomatitis virus (VSVG); pantropic retrovirus; murine leukemia virus; transgene; telomerase

1. Introduction

Schistosomiasis, which is caused mainly by three of the major human schistosomes, *Schistosoma mansoni*, *S. haematobium* and *S. japonicum*, is a debilitating, chronic and widespread disease. It is estimated that as many as 200 million people in 76 countries are infected, and that another 600 million live at risk of infection with schistosomes [1,2]. *S. japonicum*, a zoonotic species endemic in regions of East Asia including southern China and the Philippines, is considered the most pathogenic of the major human schistosomes.

Establishment of transgenic schistosomes can be expected to facilitate research on the biology, genetics and immunology of schistosomes and to enhance our understanding of the host-parasite relationship [3]. However, derivation of transgenic schistosome lines, as well as immortalized schistosome cell lines, may depend on transduction of an appropriate exogenous gene(s). Although the draft genome sequences for *S. japonicum* and *S. mansoni* are now available [4,5], the structure and characteristics of most schistosome genes, the value of their expression products as intervention targets, and the influence of exogenous genes on the metabolism of schistosomes remain to be established.

The development of molecular tools for functional genomics studies in schistosomes is still at an exploratory phase. Utilizing existing research resources and gene sequence information for schistosomes to explore and develop tools for effective transgenesis may be a key to solving many challenges in schistosome pathobiology. Thus far, transgenics technology has been developed for a number of medically and economically important pathogens [6-9] but the approach for studying parasitic helminths is not well advanced, although there are some reports of transgenesis in schistosomes. By utilization of biolistics, Davis et al [10] pioneered schistosome transgenesis; they bombarded adult *S. mansoni* parasites with mRNA encoding firefly luciferase and subsequently detected the enzyme activity in transformed worms. Thereafter, other investigators employed electroporation [11-13], biolistics (particle bombardment) [14-20] or lipofection [13] to transfer exogenous RNAs or plasmid DNAs into miracidia, sporocysts, schistosomules, adult worms and cultured cells; gene expression could be detected. Usually in these cases, the exogenous genes were episomal in location, and were expressed transiently or for only short terms [11-20]. It is unlikely that plasmid-based transgenes, introduced for example by biolistics or electroporation, would result in transgene-integration into the schistosome genome and produce a heritable transgenic schistosome line [3]. More recently, approaches employing mobile genetic elements including the *piggyBac* transposon and pseudotyped murine leukemia virus have been described that resulted in the integration of reporter transgenes into the chromosomes of *S. mansoni* [21-23].

Persistent, stable and heritable expression of transgenes in worms is an important step for the study of long-term transgene model expression, gene function in schistosomes, the exploration of the influence of schistosome gene expression and regulation, investigation of the effect on schistosome cells by exogenous genes, building a transgenic worm line, and developing insertional mutagenesis approaches [3]. The pantropic retroviral vector pseudotyped with vesicular stomatitis virus glycoprotein (VSVG) is widely used to transduce exogenous genes into a broad range of host cell types. As it is able to infect not only mammalian cells but also non-mammalian vertebrate and invertebrate cells [24] which are difficult to infect with amphotropic retroviral vectors, the pantropic vector approach has significant potential as a tool in transgenic research [6,7,24-31]. However, to date, the reporter genes inserted as cargo into retroviral vectors consist of only short sequences, such as genes encoding jellyfish green fluorescent protein (<800 bp) [32-34] or firefly luciferase (<560 bp) [35]. To test whether the pantropic retroviral vector, inserted with a long fragment (3500 bp in length) of a functional and exogenous gene encoding human telomerase reverse transcriptase (hTERT), was able to transduce *S. japonicum*, with the transgene transcribed and expressed under the control of the viral long terminal repeat (LTR) promoter, a protocol was designed and is described in this study. VSVG pseudotyped, replication-defective retroviral particles were produced in mammalian GP2-293 cells co-transfected with the plasmids pBABE-puro-hTERT and pVSV-G. Thereafter, *in vitro* cultured schistosomules were transduced with these virions. Active transcription and successful protein expression of the reporter transgenes *hTERT* and *puro'* were evident within tissues of the schistosomes.

2. Materials and Methods

2.1 Schistosomes, snails, rabbits

A Chinese mainland strain of *S. japonicum* was used in this study. Schistosome cercariae were shed from *Oncomelania hupensis* snails provided by the Institute of Parasitic Diseases, Yueyang, Hunan Province, People's Republic (P.R.) of China. White hybrid rabbits were purchased from the Central South University Animal Unit, Changsha, Hunan Province, P.R. China.

2.2 Cell lines

GP2-293 packaging cells were purchased from BD Biosciences (Clontech, Palo Alto, USA), and cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco, Carlsbad, CA, USA) supplemented with 100 units/ml penicillin G, 100 µg/ml streptomycin (Sigma, St. Louis, MO, USA), 4 mM L-glutamine (Amresco, Solon, OH, USA), 1 mM sodium pyruvate (Sigma) and 10% (v/v) fetal bovine serum (FBS, Gibco) at 37 °C under 5% CO₂ in air according to the manufacturer's recommendations. The NIH3T3 mouse fibroblast cell line was kindly provided by the Cell Center of the Central South University, P.R. China, and cultured in DMEM (Gibco) with 4 mM L-glutamine, 1.5 g/L sodium bicarbonate, 4.5 g/L glucose, and 10% bovine calf serum (BCS, Gibco).

2.3 Preparation of schistosomules

S. japonicum schistosomules were recovered by perfusion of mesenteric blood vessels of rabbits twelve days after percutaneous exposure to ~4,000 cercariae. The schistosomules were thoroughly washed three times with RPMI 1640 medium (Gibco) containing 1,000 U/ml penicillin G, 1,000 µg/ml streptomycin (Sigma) and heparin (10 U/ml, Sigma) to remove contaminating host tissues, then cultured in modified Basch's medium at 37°C in a humidified chamber under 5% CO₂ in air [36].

2.4 Constructs - retroviral vector and pVSV-G plasmids

The retroviral vector (plasmid) pBABE-puro-hTERT was kindly provided by Dr. Dongming Zhou, the Wistar Institute, Philadelphia, PA. It was assembled from the pBABE-puro plasmid (Supplementary Figure 1A) (Fig. S1A), which is derived from Moloney murine leukemia virus (MMLV), designed for retroviral gene delivery and expression [37,38]. Based on transfection of viral packaging cell lines including PA317 or GP2-293 cells, the pBABE-puro-hTERT retrovirus vector can transiently express, or integrate and stably express a transcript encoding the viral packaging signal ψ , the puromycin resistance selection marker (*puro*^r) and the reporter gene encoding human telomerase reverse transcriptase (hTERT). The 5'-retrovirus long terminal repeat (LTR) drives expression of the *hTERT* gene (Fig. S1B), and a puromycin-resistant gene (*puro*^r), which allows for the antibiotic selection of transduced eukaryotic cells in puromycin-containing culture media, is expressed from the simian virus 40 immediate early promoter (SV40 IEP) (Fig. S1B) [37-39]. The pVSV-G plasmid (Clontech) expresses the glycoprotein of the vesicular stomatitis virus (VSV-G) under the control of the cytomegalovirus (CMV) immediate-early promoter (Fig. S2). Restriction endonuclease cleavages with *SalI* or *EcoRI* were performed to identify the plasmids and authenticate insert fragments; digestion products were separated by electrophoresis through 1% (w/v) agarose gels containing ethidium bromide, visualized under UV light, and digital images recorded (Upland, Newport, WA, USA).

2.5 Production and titer determination of VSVG-pseudotyped retrovirus

GP2-293 packaging cells modified to express the MMLV *gag* and *pol* gene products (Clontech) were transfected with the pBABE-puro-hTERT and pVSV-G plasmids aided by LipofectamineTM 2000 (Invitrogen, Carlsbad, CA, USA) and chloroquine (25 μ M) (Sigma). Transfected GP2-293 cells were cultured in DMEM supplemented with 10% (v/v) fetal bovine serum (FBS) (Gibco), 100 units/ml penicillin G, 100 μ g/ml streptomycin (Sigma), 4 mM L-Glutamine (Sigma) and 1mM sodium pyruvate (Sigma) at 37 °C under 5% CO₂ in air. Culture media were replaced 8 h after transfection to remove the LipofectamineTM 2000, chloroquine, and residual plasmids, and following a further 36 h culture, supernatants containing the replication incompetent virions were harvested, centrifuged at 500g for 10 min to remove cellular debris and filtered through 0.45 μ m pore size membranes (Pall Life Sciences, Port Washington, NY, USA). Subsequently, virus-containing supernatants were concentrated by high speed centrifugation (Beckman, Fullerton, CA, USA), 50,000 $\times$ g, 90 min, 4°C. The pellet of concentrated virions was resuspended to 1% of the original volume in 50 mM Tris, 130 mM NaCl, 1 mM EDTA, pH 7.8 (TNE) at 4°C overnight, after which the virions in TNE were aliquoted, and stored at -80 °C. Functional titers of concentrated virions were determined as described [40].

2.6 Infection with pantropic retrovirus

Both schistosomules recovered from rabbits, and NIH-3T3 cells, were exposed to the concentrated virions in the presence of the cation polybrene (Sigma), which can enhance the effectiveness of retroviral transduction [41]. After washing twice with serum-free RPMI 1640 (Gibco), a total mixture of 200 μ l of concentrated retrovirus virions, 1.8 ml modified Basch's medium [36] and polybrene (8 μ g/ml) was added to cultures of 50-100 schistosomules per well in 6-well plates (Corning, Lowell, MA, USA). Subsequently, the schistosomules were incubated for 3 h at 37 °C, mixed gently by rocking the plate back and forth for 30 min, and then 2 ml modified Basch's medium was added. Following a further 24 h incubation, media were replaced with 2 ml modified Basch's medium, after which the schistosomules exposed to virions were cultured for 6 days. During cultivation, media were replaced daily.

Infection of NIH3T3 cells was undertaken as a positive control to determine the infectivity of the virions. Target NIH3T3 cells were plated to a density of 2×10^5 cells in 6-well plates and cultured in the complete growth medium, as above. When cells reached ~70% confluence, media were removed and the cells washed twice with serum-free DMEM. In the presence of polybrene (8 $\mu\text{g/ml}$), a mixture of 200 μl concentrated retrovirus and 800 μl serum-free DMEM was added and the cells incubated for 3 h at 37 °C, after which 2 ml complete medium was added. The media were replaced 24 h later with 2 ml conditioned medium supplemented with puromycin (3 $\mu\text{g/ml}$). After 10-12 days of puromycin selection, puromycin-resistant clones were obtained, and one of these was randomly selected to culture for up to three subsequent generations in conditioned media containing puromycin (3 $\mu\text{g/ml}$). Negative controls included schistosomules and NIH3T3 cells, not exposed to virions, cultured in modified Basch's medium and complete growth medium, respectively.

2.7 PCR based detection of reporter *hTERT* and *puro*^r genes in schistosomules and NIH3T3 cells

Transduced and negative control schistosomules were harvested after 6-day cultivation, and washed three times with centrifugation, 800g $\times$ 2 min, in ice cold PBS to remove unbound virus. At the time of harvest of the cultured schistosomules, an aliquot of culture media was also collected for analysis to investigate the presence of residual, contaminating pBABE-puro-hTERT plasmids carried over in virion inocula. Puromycin-resistant and negative control NIH3T3 cells after passage for three generations were trypsinized and collected, washed three times in ice cold PBS (500g $\times$ 5 min). Total genomic DNAs (gDNAs) were extracted from schistosomules and NIH3T3 cells using the illustra tissue and cells genomicPrep Mini Spin Kit (GE Healthcare, Piscataway, NJ, USA). Primers specific for the *hTERT* gene, forward-CGGAAGAGTGTCT GGAGCAA and reverse-GGATGAAGCGGAGTCTGGA, and primers for the *puro*^r gene, which were designed by Primer 3 (v.0.4.0) program (<http://frodo.wi.mit.edu/>) according to the coding sequences of the *puro*^r gene (GenBank accession DQ322642), forward-GTCACCGAGCTGCA AGAACT and reverse-CAGGAGGCCTTCCATCTGT, were employed to amplify proviral transgenes. PCRs were carried out using Go Taq Green Master Mix (Promega, Madison, WI, USA), and amplification for the *hTERT* gene was one cycle at 94 °C for 5 min, 30 cycles at 94 °C, 30s, 58 °C, 45s, 72 °C, 40s, followed by a 5-min extension at 72 °C, as well as 30 thermal cycles for the *puro*^r gene of 94 °C for 30s, 61.7 °C for 45s, and 72 °C for 40s. Negative controls included DNAs isolated from schistosomules and NIH3T3 cells not exposed to virions. The pBABE-puro-hTERT plasmid was employed as the positive control template. PCR products were separated by electrophoresis through 2% (w/v) agarose gels, stained with ethidium bromide, visualized under UV illumination and digital images captured (UpLand). Predicted sizes for PCR products from the *hTERT* and *puro*^r genes were 145 bp [42] and 204 bp, respectively.

2.8 Reverse transcription-polymerase chain reaction (RT-PCR)

Transduced and non-transduced control schistosomules were obtained, as above. Total RNA was isolated from the worms using Trizol Reagent (Gibco-BRL) according to the manufacturer's protocol, and the RNA incubated with RNase-free DNase I in the presence of Ribonuclease inhibitor (Fermentas, Burlington, Canada) to remove any residual, contaminating genomic DNA (gDNA). cDNA synthesis was accomplished using 5 μg DNase I-treated RNA, 1 μl random primer, 2 μl dNTPs, and 200 U M-MuLV Reverse Transcriptase using the First Strand cDNA Synthesis Kit (Fermentas), and the *hTERT* and *puro*^r genes amplified using the primers described above. Simultaneously, the control *S. japonicum* housekeeping gene α -tubulin was amplified using the primers 5'-CTGGAGTTCAAATGGGCAAT-3' and 5'-TACCTCCACCGAA AGAATGG-3'; this produced a 399 bp amplicon [43], and this was taken as an internal control to confirm the

integrity of the schistosomule RNAs and the presence of amplified cDNAs in all samples. Thermal cycling conditions involved denaturation at 94 °C for 5 min followed by 25 cycles of amplification at 94 °C for 30 s, 55°C for 45 s, 72 °C for 40 s, and a final extension at 72 °C for 5 min. Amplification products were separated by electrophoresis through 2% (w/v) agarose containing ethidium bromide, visualized under UV illumination, and digital images recorded. Negative controls included cDNA from non-virus exposed schistosomes, RNA, which was not reverse transcribed into cDNA, from virus-exposed schistosomes and reactions in which water replaced the template nucleic acids. The plasmid pBABE-puro-hTERT plasmid was included as a positive control.

2.9 *In situ* staining of hTERT protein in schistosomes

To confirm the expression of hTERT protein in *S. japonicum* and NIH3T3 cells transduced by pBABE-puro-hTERT retrovirus, immunohistochemical staining was performed. Transduced and control schistosomules were harvested 6 days following exposure to virions, rinsed and fixed with 4% (v/v) paraformaldehyde (Sigma) for 3-5 h at room temperature. Following rinsing in PBS, worms were embedded with 3% (w/v) agarose and dehydrated through an ethanol series. Subsequently, agarose-embedded worms were embedded with paraffin, cut in serial sections of 5 µm, deparaffinized in xylene and rehydrated. In parallel, puromycin-resistant and control NIH3T3 cells were mounted on slides and fixed with 4% (v/v) paraformaldehyde for 3-5 h at room temperature. Both the sections of worms and slides of NIH3T3 cells were immersed in 10 mM citrate (pH 6.0), and incubated at 80 °C for 20 min to retrieve antigenicity. Endogenous peroxidase was quenched with 3% (v/v) hydrogen peroxide (H₂O₂) and 0.3% (v/v) Triton X-100 in PBS for 10 minutes at 37 °C. After incubation in 10% normal goat serum and 0.3% (v/v) Triton X-100 in PBS for 60 min to block nonspecific immunoglobulin (Ig) binding, the slides were incubated in polyclonal rabbit anti-hTERT serum (Boster Biotechnology, Wuhan, China) at a 1:200 dilution overnight at 4 °C. After rinsing three times in PBS, a SABC detection kit (Boster Biotechnology, Wuhan, China) comprising biotinylated secondary antibody and avidin-conjugated horseradish peroxidase, was employed to detect antibody binding. Following washing in PBS, the slides were incubated with 0.05% (w/v) 3,3-diaminobenzidine (DAB) for 5 min at room temperature, washed with distilled water, counterstained with hematoxylin (Sigma), dehydrated through an ethanol series, and coverslipped. Worm sections were examined microscopically, and schistosomes and schistosome tissues exhibiting positive, brown colored staining were counted and photographed.

2.10 Immunoblot analysis of reporter *hTERT* transgene expression

To investigate whether the exogenous *hTERT* transgene was expressed in schistosomes exposed to the VSVG pseudotyped virions, virus-treated and non-treated control worms were harvested after cultivation for 6 days, as described above. Protein lysates were prepared by ultrasonication (300W, 5 min) in the lysis buffer (50 mM Tris-HCl, pH 8.0; 1 mM EDTA, 2% (w/v) sodium dodecyl sulfate (SDS); 5 mM dithiothreitol (DTT); 10 mM phenylmethyl sulfonylfluoride (PMSF)), and then centrifuged at 10,000g for 10 min. The supernatant was collected as whole-worm lysate for western blot analysis. Protein concentration was determined using the BCA Protein Assay Reagent (Pierce, Rockford, USA). Protein extracts (50 µg/sample) were separated by 10% (w/v) SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and electrotransferred to nitrocellulose (NC, Pall Corporation, USA). The membranes were blocked with 5% (w/v) nonfat milk in PBS with 0.05% (v/v) Tween-20 (PBST) for 2 h at room temperature. Subsequently, membranes were probed with polyclonal rabbit anti-hTERT serum (above) and monoclonal mouse anti-human beta actin (Abcam, San Francisco, CA, USA) as primary antibody overnight at 4°C. After rinsing in PBST, the membranes were incubated with HRP-conjugated goat anti-rabbit IgG (H+L) and goat anti-mouse IgG (H+L) (Santa Cruz, CA, USA) for 1 h at room

temperature. Signals were detected with an enhanced chemiluminescence assay (Pierce). Both PA317/hTERT strain cells, which are derived from PA317 cells transfected with pBABE-puro-hTERT plasmid and which stably produce amphotropic retrovirus virions, and a puromycin-resistant NIH3T3 cell strain, following 30 passages after infection with pantropic pBABE-puro-hTERT retrovirus, were included as positive controls.

3. Results

3.1 Identification of infectious VSVG-pseudotyped pantropic retroviral particles

To test the viability of the retroviral particles, which were concentrated from culture supernatants of GP2-293 packaging cell line cotransfected with vector pBABE-puro-hTERT and pVSVG plasmid, target NIH3T3 murine fibroblasts were infected with retroviral particles and the titer of concentrated virions was determined. Following screening, puromycin-resistant clones were obtained and counted (Fig. S3); the titer was 3.2×10^8 colony forming units (cfu)/ml. Direct PCR analysis showed that two fragments of the predicted sizes of 145 and 204 bp were generated from the gDNAs of puromycin-resistant NIH3T3 cells (Fig. S4A, lane 3; S4B, lane 5), respectively, but not from uninfected control NIH3T3 cells (Fig. S4A, B lanes 4), indicating the presence of heterologous *hTERT* and *puro*^r gene in the genomes of the puromycin-resistant NIH3T3 cells. In addition, immunohistochemical staining revealed intense staining of the hTERT protein, localized to nuclei of puromycin-resistant NIH3T3 cells, demonstrating protein expression of transgene *hTERT* in transduced NIH3T3 cells (Fig. S5B), although a few control NIH3T3 cells showed faint staining for hTERT in nuclear regions (Fig. S5A), possibly as a consequence of cross-reaction between human and murine telomerase reverse transcriptase [44]. These findings verified that the virion preparation was active and therefore suitable for investigation of retroviral transduction of the schistosomules.

3.2 Detection of *puro*^r and *hTERT* reporter genes in schistosomes

To determine whether VSVG-pseudotyped pantropic retrovirus could transduce *S. japonicum* and mediate transgene into schistosome cells, cultured schistosomules were exposed to pBABE-puro-hTERT virions. After 6 days, gDNA was extracted from the parasites and targeted by PCR with primers specific for the *puro*^r and *hTERT* genes. Amplicons of the predicted sizes of 204 bp and 145 bp were obtained from virion-treated schistosome gDNAs, respectively; the products stained intensely with ethidium (Fig. 1A, B, lanes 4). By contrast, products of 204 bp and 145 bp were not present in untreated schistosome gDNAs (Fig. 1A, B, lanes 3). The culture supernatants from schistosomules exposed to virions before harvesting also were investigated by PCR for the presence of contaminating pBABE-puro-hTERT plasmids; no *puro*^r or *hTERT* gene specific targets were amplified, indicating the absence of residual plasmid carried over in the virion inoculum (Fig. 1A, B, lanes 2). Based on these data, we infer that attachment, uncoating and entry into schistosome worms of VSVG-pseudotyped pantropic retrovirus can be supported in *S. japonicum* schistosomules, and that *puro*^r and *hTERT* reporter transgenes were present within the cells of these virus-exposed schistosomules.

3.3 Transcription activity for *puro*^r and *hTERT* gene in transduced schistosomes

RT-PCR analysis was performed to investigate the transcriptional activity of the *puro*^r and *hTERT* transgenes within the schistosomules exposed to pBABE-puro-hTERT retrovirus. Products of the anticipated sizes of 204 bp and 145 bp, respectively, were amplified from virus-exposed worms (Fig. 2A, B, lanes 4) and the control plasmid pBABE-puro-hTERT (Fig. 2A, B, lanes P), but not from control, non-virus exposed parasites (Fig. 2A, B, lanes 3). The band at 204 bp (Fig. 2A, lane 4) was not intense, suggesting that although transcription of the *puro*^r transgene in schistosomules transduced by pBABE-puro-hTERT virions had

taken place, the transcription level may not have been as high as observed in mammalian cells. Nonetheless, presence of the 145 bp product (Fig. 2B, lane 4) indicated that the transcription of the *hTERT* transgene was occurring in schistosomes exposed to pBABE-puro-hTERT virions, and that the transcription level was as high as observed within *S. mansoni* [22,23]. The product of predicted size of 399 bp of the housekeeping gene - tubulin was amplified from all the cDNA preparations (Fig. 2A, B, lanes 3, 4), confirming the integrity of the schistosome RNA in all samples. A negative control of water as template yielded no product (Fig. 2A, B, lanes 1). Additional negative control reactions were included, utilizing RNA from virus-exposed schistosomes rather than cDNA as the template, to confirm the absence of gDNAs. No specific band was amplified, indicating that the RNA preparations were free of contaminating DNA (Fig. 2A, B, lanes 2). These findings indicated that the *puro*^r and *hTERT* transgenes in schistosome worm cells could be transcribed under the control of the MMLV LTR and SV40 IEP promoters respectively, although the transcription level of the *puro*^r transgene was modest compared to levels reported in mammalian cells [45,46].

3.4 Human telomerase reverse transcriptase expressed in transduced schistosomes

To determine whether the presence of the *hTERT* gene transcript led to hTERT protein expression, we analyzed the hTERT protein levels in virus-transduced schistosomes and in corresponding controls, using immunohistochemical approaches. The labeling revealed brown staining for the hTERT protein in 44% (15/34) of the worm sections that we examined from parasites exposed to pBABE-puro-hTERT retrovirus. Furthermore, intense staining was located in sub-tegumental regions at the anterior of the worms, especially in the oral (head) sucker (hs) and ventral sucker (vs), as well in tissues as at the posterior of the schistosomes (Fig. 3C, D, E, F, G). By contrast, control non-transduced schistosomes were negative for the hTERT protein (Fig. 3A, B). These findings confirmed that protein expression of the proviral *hTERT* transgene had taken place in virus-transduced worms, a result consistent with the RT-PCR analysis (Fig. 2).

3.5 Protein expression of *hTERT* gene in schistosomes

To confirm the expression of hTERT shown by immunohistochemistry (Fig. 3), we undertook an immunoblot analysis of proteins extracted from retrovirus-exposed schistosomes. When membranes were probed with polyclonal rabbit anti-hTERT antibody, a moderately intense band of ~120 kDa was observed in worms exposed to virions (Fig. 4, lane B). By contrast, no signal was seen with proteins from control, non-virus exposed worms (Fig. 4, lane A). The positive controls of PA317/hTERT and puromycin-resistant NIH3T3 cells showed intense bands at ~120 kDa (Fig. 4, lanes P1, P2). As a loading control, a specific band of ~42 kDa (α -actin) was present in all samples, indicating similar quantities in each lane. These results demonstrated that the *hTERT* transgene was active in the transduced schistosomes, and supported the findings from the RT-PCR and immunohistochemical analysis.

4. Discussion

Approaches for transgenesis that involve the pantropic murine leukemia virus (MLV) have provided a tractable and effective means to introduce foreign genes into mammalian cells. With this vector, the coding sequences for the retrovirus structural gene (*gag*), the reverse transcriptase gene (*pol*) and envelope protein gene (*env*) are removed and replaced with exogenous genes under the control of the retroviral long terminal repeat (LTR) or endogenous promoters. Replication-defective retroviral vector particles that can transduce target cells can be produced by transfection into specific retroviral packaging cells, which provide the virus envelope protein, structural protein and reverse transcriptase needed for

formation of infectious virions, and retroviral particles can be recovered by collection of culture supernatants from packaging cells. The retroviral provirus genome, along with reporter transgenes, can integrate into the chromosomes of target cells. Subsequently, the integrated transgenes replicate, during mitosis of target cells, resulting in heritable changes in the phenotypes of target cells [39,47,48]. The pantropic retroviral vector is a gene transfer vector based on the Moloney murine leukemia virus, which has been pseudotyped with the envelope glycoprotein of vesicular stomatitis virus (VSVG) [24] substituted for the retroviral envelope protein. VSVG is able to bind to phospholipids on the target cell membrane to meet the requisition of virus for specific protein receptors on the membrane of target cells and mediate virus entry [49,50]. Furthermore, VSVG pseudotyped pantropic retroviral particles are more stable than amphotropic retrovirus, and the titer of virus can be concentrated to 10^9 CFU/ml after ultracentrifugation. Compared with normal amphotropic retroviral particles, which require binding of virus to specific protein receptors on the membrane of target cells, VSVG pseudotyped retroviral vectors possess a broad host cell range, are termed pantropic retroviruses, and have been widely employed for transgenesis of non-mammalian cells [24]. Pantropic retroviruses have been successively applied to transgenic research on numerous target species of non-mammalian vertebrates or invertebrates, including zebra fish [6], *Xenopus* [26], newts [25], oysters [27], surf clams [7], shrimp [28,29], mosquitoes [30], *Drosophila* [51] and amoebae [31].

Kines and co-workers [22,23] reported transduction of *S. mansoni* sporocysts, juvenile and adult worms using VSVG-pseudotyped retroviral vectors and that both EGFP (enhanced green fluorescence protein, EGFP) and firefly luciferase reporters could be detected in the genome of virus treated worms. These findings also demonstrated integration of transgenes into schistosome chromosomes, and they provided the impetus for this investigation into whether *S. japonicum* likewise could be transduced with VSVG pseudotyped MMLV virions. Here the MMLV vector pBABE-puro-hTERT, which includes an exogenous *hTERT* gene, was used as the gene transfer vector. A foreign (non-schistosome) gene *hTERT* was employed as the reporter or functional transgene under the control of the MLV viral long terminal repeat (LTR) sequence within the construct [37,38], and was expressed in some telomerase-negative cells to recover telomerase activity, extend cellular life span and, further, lead to cellular immortalization. This approach has been used to establish immortalized cell lines from a wide variety of cell types [38,52-54].

Although Kines et al [22,23] transduced several developmental stages of *S. mansoni* with pantropic retroviral vector containing reporter genes encoding EGFP and luciferase, the reporter genes were relatively short in length (<800 bp and <560 bp, respectively). We wondered whether similar results could be obtained using pantropic retrovirus containing a longer gene cargo. Here schistosomules of *S. japonicum* were selected as the developmental stage target and transduction carried out using a retroviral vector carrying the *hTERT* gene. PCR, targeting genomic DNAs isolated from worms cultured for 6 days after exposure to the retrovirus, indicated the presence of the transgenes *hTERT* and *puro* within the schistosome cells. Our findings indicated that VSVG pseudotyped retrovirus could adhere to and enter cells of *S. japonicum* schistosomules and that the successful infection of this schistosome had taken place. Although we have not provided direct evidence of retroviral integration into schistosome genomes, for instance by sequence analysis of integration junctions, preliminary Southern blot analysis (not shown) suggested that genome integration occurred although additional analysis is needed to confirm this phenomenon.

The contact interface between the schistosome and its host is the parasite surface which is the tegument composed of a syncytium of fused cells surrounding the entire worm with a single continuous double-bilayer membrane [55]. Consistent with other eukaryotic species, the lipid structure of the membrane is also composed of phospholipids [55-57]. It seems

likely that transduction of *S. japonicum* schistosomules by pantropic retrovirus is also mediated by interaction between the VSVG envelope protein and phospholipid components of the worm surface [49,50].

Our analysis of RNA showed that there were transcripts of *hTERT* and *puro^r* in the transduced *S. japonicum* schistosomules. Immunohistochemical staining indicated expression of hTERT in regions below the tegument of the virus-exposed worms. Furthermore, hTERT protein expression was confirmed by immunoblot analysis. These findings demonstrated that there was mRNA transcription of the transgenes *hTERT* and *puro^r* under the control of the MMLV LTR or SV40 IEP promoter of Simian virus, protein expression of the *hTERT* gene in *S. japonicum* schistosomules, and that transgene silencing was not apparent. (Whether the transgenes in the schistosome cells are expressed continuously will be a key point in future studies. In some contexts, MMLV transgenes are silenced after integration into host chromosomes [58,59].) It has been widely reported that the LTR promoter of MMLV can drive strong transgene expression in diverse invertebrate species [7,22,23,28,30,31] and that the SV40 IEP promoter mediates high levels of expression in mammalian cells [45,46] and in the filarial nematode *Litomosoides sigmodontis* [60]. However, transcription levels from the SV40 IEP promoter were apparently not strong in these *S. japonicum* schistosomules. Intense expression of hTERT was localized to sub-tegumental sites at the anterior of the transduced worms, especially in the oral and ventral suckers, as well as at the posterior of the worms.

Dirks and Miller [61] reported that when VSVG pseudotyped MMLV virus infected cell lines of non-mammalian origin, including cells of both vertebrate and invertebrate species, there was frequent post-entry blocking whereby all or most viruses failed to complete reverse transcription and/or integration. They speculated that the possible reason for this phenomenon resulted from evolutionary divergence between mammals and non-mammals in cellular co-factors of requisite developmental steps in the retroviral life cycle. By contrast, Brindley and coworkers did not observe incapacitating blocks within *S. mansoni* [22,23], confirming that MLV was active, to at least some extent in invertebrates and in platyhelminths in particular. The low transduction efficiency in the current study may relate to adhesion of the virions to the cultured schistosomules or other factors. In any event, we propose to optimize the performance of the virions in future studies, including the use of repeated infection [62,63] and centrifugation infection protocols [64], aiming to enhance transduction efficiency. The retroviral vector also could be modified by introducing a powerful endogenous promoter, such as an actin gene promoter [12,23,65], to enhance the level of transcription and expression of the *puro^r* transgene.

This present study demonstrated that VSVG pseudotyped pantropic retroviral vectors can transduce long fragments of the exogenous *hTERT* gene into tissues of *S. japonicum* schistosomules and that this reporter transgene can be transcribed and expressed in schistosomes under the control of the MMLV retroviral LTR promoter. In overview, these findings confirmed that VSVG-MLV represents an effective gene transfer vector and tool for studying the functional genomics of schistosomes. We consider that VSVG-MLV will also be potentially useful for large-scale genetic analysis through insertional mutagenesis and for the establishment of lines of transgenic schistosome lines and, indeed, immortalized schistosome cell lines. In future studies, we plan to employ this pantropic retroviral vector containing the *hTERT* transgene to transduce cells from specific tissues and organs of schistosomes to investigate transgene integration into their chromosomes, the influence of the transgene *hTERT* on growth and proliferation of schistosome cells, and the vertical transmission and longevity of transgene activity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We sincerely thank Dr. Dongming Zhou of the Wistar Institute, Philadelphia for the gift of retroviral plasmids, pBABE-puro and pBABE-puro-hTERT, and technical support. We also acknowledge Dr. Robert Weinberg for generously allowing us to use the pBABE-puro-hTERT construct. These studies were supported by Grant No. 30570952 from the Nature Science Foundation of China (NSFC), and in part by award number R01AI072773 (to PJB) from the NIAID-NIH, USA and the National Health and Medical Research Council of Australia (DPM).

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Research highlights

Schistosoma japonicum could be transduced by VSVG-pseudotyped pantropic retrovirus.

Transcription of transgene *hTERT* was evident within schistosomes post transduction.

Protein expression of transgene *hTERT* was detected in schistosome post infection.

Fig. 1. Proviral sequences detected in transduced *Schistosoma japonicum* by direct PCR
(A) Identification of the *puroF* transgene in schistosomules transduced with pseudotyped pBAGE-puro-hTERT virions; **(B)** Identification of the *hTERT* transgene in schistosomules transduced with pseudotyped pBAGE-puro-hTERT virions. Lanes: M, molecular size standards; P, positive control, pBAGE-puro-hTERT plasmid; 1, negative control (water); 2, culture supernatants from schistosomules exposed to pBAGE-puro-hTERT virions; 3, gDNA from non-transduced schistosomules; 4, gDNA from schistosomules exposed to pBAGE-puro-hTERT virions.

Fig. 2. Assessment by RT-PCR of *puro*^r and *hTERT* transgene expression following transduction of *Schistosoma japonicum* schistosomules by pBABE-puro-*hTERT* retrovirus
 Total RNA samples were extracted from schistosomules and treated with DNase I. End point PCR was performed using reverse transcribed RNA/cDNA. The α -tubulin gene, amplified as an internal control to verify the integrity of the schistosome RNA and equivalent loadings of cDNAs, produced a 399 bp amplicon of the expected size. (A) Amplification using primers specific for the *puro*^r gene; (B) Amplification using primers specific for the *hTERT* gene. Lanes: M, molecular size markers; 1, water, negative control; 2, RNA not reverse transcribed into cDNA from schistosomules transduced by virions; 3, cDNA from non-virus exposed schistosomules; 4, cDNA from schistosomules transduced by virions; P, positive control, pBABE- puro-*hTERT* plasmid.

Fig. 3. Immunohistochemical staining for human telomerase reverse transcriptase in *Schistosoma japonicum* schistosomules exposed to VSVG-pseudotyped pBABE-puro- hTERT virions

(A, B) Control, non-virion exposed schistosomules (showing head sucker, ventral sucker and posterior parts of the worms): absence of reaction for hTERT in sub-tegumental regions. (C, D, E, F, G) Schistosomules exposed to virions: intense staining for hTERT mainly located in sub-tegumental regions of the head sucker, ventral sucker and posterior tissues of the parasites as indicated by the arrows (red). Scale bar, 100 µm. hs, head (oral) sucker; vs, ventral sucker.

Fig. 4. Western blot analysis for expression of human telomerase reverse transcriptase in virion-transduced *Schistosoma japonicum* schistosomules

Lane A, non-transduced worms; B, virus transduced schistosomules; P1, PA317/hTERT cell strain, derived from PA317 cells transfected with the pBABE-puro-hTERT plasmid, and which can stably produce amphotropic retrovirus virions; P2, puromycin-resistant NIH3T3 cell strain following 30 passages after infection with pantropic pBABE-puro-hTERT retrovirus.