Biolistic Gene Transfer to Generate Transgenic Schistosomes

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Introduction

Schistosomes are parasitic helminths that cause bilharzia (schistosomiasis) in humans, a tropical disease of worldwide significance (Savioli et al. 1997). One focus of international research on such pathogens is to increase the understanding of their molecular biology and to identify and characterize genes with key cellular functions. These genes could be new targets for the development of drugs and vaccines (Johnston et al. 1999).

Gene transfer in homologous systems has been shown to be a powerful tool for the functional analysis of genes and their regulatory elements (Buono and Linser 1992). However, there is comparatively little documentation of attempts to transform multicellular parasites. The goal of our study was to establish a gene delivery system for *Schistosoma mansoni*, a digenean trematode with a complex life cycle. Specifically, we used biolistic gene transfer techniques to transiently transform adult and larval stages of the schistosome.

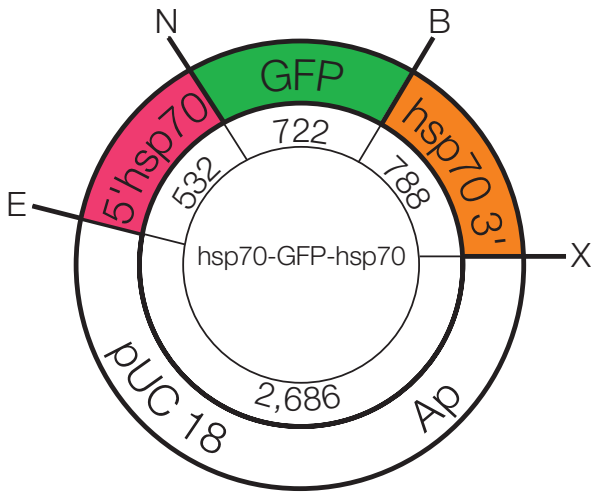

Fig. 1. Diagram of the hsp70-GFP-hsp70 vector used for transformation. The vector has a pUC18 plasmid backbone and harbors an ampicillin resistance gene (Ap). The insert contains the promoter (5' hsp70, red background), GFP gene (GFP, green background) and terminator (hsp70 3', red background). The following restriction sites are indicated: E, *Eco*RI; N, NcoI; B, *Bam*HI; X, *Xba*I. Sizes shown in the inner circle are given in base pairs; not drawn to scale.

Biolistic gene transfer, also known as particle bombardment, was initially designed to transform plants; however, several other types of organisms have been successfully transformed using the Helios® gene gun and the PDS-1000/He™ particle delivery system, both from Bio-Rad (Williams et al. 1991, Schiedlmeier et al. 1994, Davis et al. 1999, Jackstadt et al. 1999, Hara et al. 2002). The principle of biolistics is based on the acceleration of DNA- or RNA-coated microparticles by high-pressure helium. These particles are able to penetrate cell layers of the target and introduce genetic material into living cells. The microprojectiles we used were gold particles, which are uniform in size and do not catalytically attack DNA. In addition, gold is biologically inert and is not toxic to most cells (Sanford et al. 1993).

Methods

Transformation Vector

Figure 1 shows the transformation vector used in this study. The vector contained the inducible regulatory elements of the heat shock protein 70 (HSP70) gene from *S. mansoni* and the Green Fluorescent Protein (GFP) gene as the reporter gene.

Parasite Stock and Culture Conditions

A Liberian strain of *S. mansoni* was maintained in snails, *Biomphalaria glabrata*, and in Syrian hamsters, *Mesocricetus auratus*. Adult worms were obtained by perfusion at day 42 post-infection, and washed 3 times with RPMI 1640 containing 10% fetal calf serum, before they were kept in 60 mm petri dishes at 37°C, 5% CO_2 (Grevelding et al. 1997). Isolation of miracidia and in vitro transformation to sporocysts were done as described elsewhere (Wippersteg et al. 2002).

Coating of Microparticles

Gold particles (0.6–1.6 µm) were coated with vector DNA purified by chromatography on a Macherey-Nagel column. For experiments using the PDS-1000/He system, microprojectiles for five bombardments were prepared by mixing 50 µl of gold (in 60 mg/ml 50% glycerol), 5–25 µg of DNA (0.2–1 µg/µl), 40 µl of spermidine (0.05 M, free base), and 50 µl of $CaCl_2$ (2.5 M). After 3 min of vortexing, the gold

particles were pelleted by centrifugation, and the supernatant was removed. The pellet was washed 2 times with ethanol (100%) by pipetting up and down, and then concentrated by centrifugation. Finally, the pellet was resuspended in 50 µl of ethanol (100%). For each bombardment, 10 µl of this DNA-gold suspension were loaded onto a macrocarrier.

For experiments with the gene gun, DNA-coating and loading of gold projectiles into Tefzel tubing were done according to Jackstadt et al. (bulletin 2433).

Bombardment and Heat Shock Procedures

For gene delivery experiments with adult schistosomes, approximately 25 males were cultured at 37°C in 60 mm petri dishes for 1 day after collecting them from the final host. Immediately before bombardment, the medium was carefully removed, and the worms were placed in the middle of the dish. Biolistics were performed using the following parameters: 1.350–2.000 psi helium pressure, target distance stage 1 (3 cm), and 15–20"Hg chamber vacuum. After bombardment, medium was added, and the worms were cultivated for an additional 24 hr. Heat shock was applied for 4 hr at 42°C.

For biolistic experiments with sporocysts, groups of 2,000–4,000 individuals were pooled and transferred to 35 mm petri dishes. Pressures of 900 and 1,350 psi were applied at a distance of 3 cm and a vacuum of 15–20"Hg. After bombardment, 2 ml of culture medium were added, and the larvae were cultured for an additional 48 hr without heat shock.

Molecular and Microscopic Analyses

DNA or RNA for PCR or RT-PCR analyses was isolated from bombarded worms that had been frozen in liquid nitrogen 6 hr after heat shock (Neumann et al. 1993). Protein from bombarded adults was extracted 24 hr after heat shock (Wippersteg et al. 2002). Protein from larval stages was extracted 48 hr after bombardment. SDS-PAGE and western blots were done according to standard protocols.

Microscopic analyses of bombarded worms were done 24 hr after heat shock. Adults were individually placed onto glass slides and carefully fixed with cover slips. Larval stages were analyzed by microscopy 48 hr after bombardment. Microscopy was performed with a Leica TCS NT confocal laser scanning microscope using the GFP-defined wavelength of 488 nm for excitation. Laser activity was adjusted to 50–60%.

Results

To investigate whether biolistic gene transfer can be used to generate transgenic schistosomes, bombardment experiments were performed with two different systems, the Helios gene gun and the PDS-1000/He. In both cases the hsp70-GFP vector was used for transformation.

With the gene gun, a total of 328 worms were bombarded in seven independent experiments. Due to air pressure conditions, nearly 1/3 of the worms were lost during bombardment. The remaining schistosomes were kept under standard culture conditions until heat shock to induce reporter gene activity. After this treatment, the survival rate decreased below 50%. Light microscopy showed gold particles within the

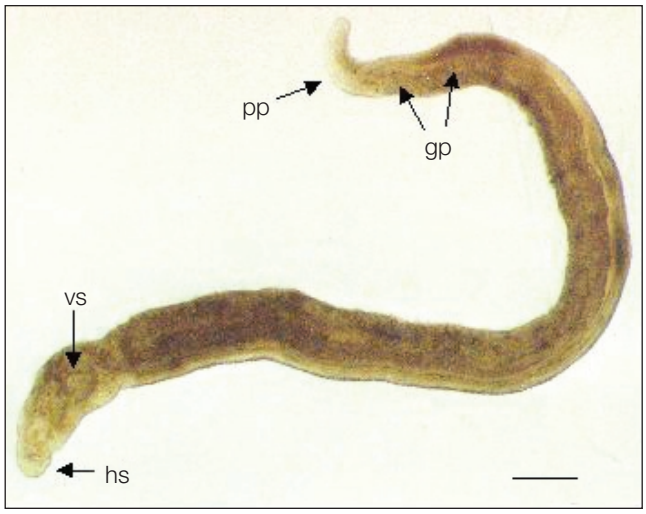

Fig. 2. Male schistosome after bombardment. Gold particles can be observed in the tegumental area. gp = gold particles; hs = head sucker; pp = posterior part; vs = ventral sucker (scale bar: 0.5 mm).

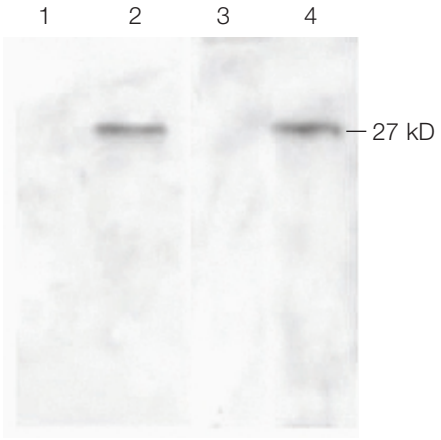

Fig. 3. Western blot with protein from adult worms and sporocysts using a GFP-specific antibody. Lane 1, protein from worms that were bombarded but not heat shocked; lane 2, protein from bombarded and heat-shocked worms; lane 3, protein from sporocysts that were not bombarded; lane 4, protein from bombarded sporocysts.

remaining worms, and also on the tegument. The presence and transcription of the transgene in bombarded worms were confirmed by PCR/RT-PCR with extracted DNA/RNA and GFP-specific primers. Fluorescence microscopy showed green areas in the worms indicating GFP activity (results not shown).

In contrast to the air-pressure conditions of gene gun treatment, the PDS-1000/He system works with a vacuum chamber. After bombardment, the worms were still present in the middle of the petri dish, and nearly all worms survived the procedure. Even after heat shock, the number of worms remained constant, and they recovered within a short period (Figure 2). PCR and RT-PCR analyses confirmed the presence and transcription of the transgene (results not shown). Western blot analysis showed that translation of the GFP occurred only in worms that had been stressed by heat shock, indicating that the regulatory elements worked as expected (Figure 3).

Bright-field microscopy demonstrated that pressures of 1,550 and 1,800 psi worked best, resulting in a high number and an equal distribution of microprojectiles in the worms (Figure 4A). Confocal laser scanning microscopy revealed green fluorescing signals occurring at the dorsal tegument

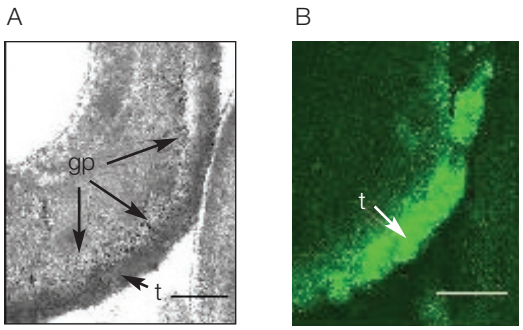

Fig. 4. Male schistosomes bombarded at 1,550 psi. A, gold particles in the tegumental area; B, confocal laser scanning microscopy of the same area showing bright fluorescence; C, worm showing fluorescence in the posterior region. gp = gold particles, gc = gynecophoric canal, pp = posterior part, t = dorsal tegument (scale bar: 0.5 mm).

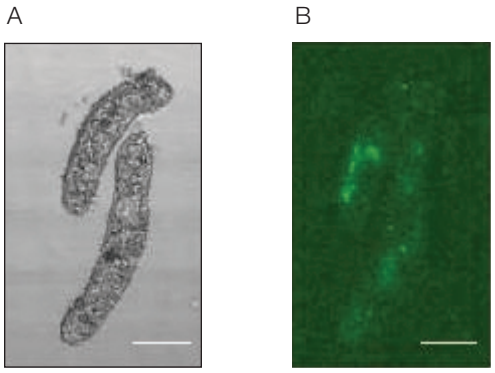

Fig. 5. Microscopy of sporocysts bombarded at 900 psi. A, bright-field microscopy; B, confocal laser scanning microscopy (scale bar: 50 µm).

(Figure 4B) as well as in the anterior and posterior portions of the worms (Figure 4C). Bombarded schistosomes that were not stressed by heat shock did not show any fluorescence (results not shown).

To determine whether the PDS-1000/He system is also suitable for transformation of larval schistosomes, we performed experiments with sporocysts generated in vitro. Here, lower pressures (900 and 1,350 psi) were applied. According to the constitutive expression of HSP70 in larval stages (Neumann et al. 1993), sporocysts were not heat shocked after bombardment. In a first series of experiments, up to 10,000 sporocysts were bombarded. The expression of GFP was verified by RT-PCR (results not shown) and immunoblotting (Figure 3). Confocal laser scanning microscopy revealed GFP expression within the larvae after biolistic treatment at 900 psi (Figure 5) as well as 1,350 psi. In control sporocysts (not bombarded), no fluorescence was detected (results not shown).

Discussion

Transformation protocols for parasitic helminths have not been established or reproducibly applied. Recently, the first experiments were reported to introduce DNA or RNA into the parasitic nematode, *Ascaris lumbricoides*, by particle bombardment (Davis et al. 1999). In the same study, the first evidence was obtained that this method could also be applicable to adult schistosomes. However, no molecular results on transgene expression were presented, and no microscopic analyses were performed.

The results demonstrated here show that biolistic gene transfer can be used to transiently transform adult schistosomes as well as larval stages. In our study, two different systems for biolistic gene transfer were used. Results with the Helios gene gun showed that this approach was practicable, but has disadvantages. The rate of worm survival was low and the conditions during bombardment were unfavorable. Worms that survived the procedure showed significant tissue damage. The Helios gene gun has been used successfully to transform the nematode, *Caenorhabditis elegans*, and the filarial parasite, *Litomosoides sigmodontis* (Jackstadt et al. 1999). In contrast to schistosomes, filariae are bigger and seem to be more resistant to mechanical stress.

Results obtained with the PDS–1000/He system demonstrate that this system is more suitable than the Helios gene gun to transiently transform *S. mansoni*. In spite of the vacuum, nearly all worms survived the procedure without obvious damage. Likewise, transformation of larval stages was achieved using lower pressures. Molecular analyses showed that the plasmid vector was successfully introduced into both developmental stages; the transgene was transcribed and translated. Microscopic analyses demonstrated that transgene expression can be monitored easily in living schistosomes. GFP activity was localized in the tegumental area of adult worms after heat shock. Interestingly, fluorescence was detected inside the larvae, indicating a different mechanism of HSP70 regulation in this life stage.

With the particle bombardment protocol presented here, we show a practical way to study genes in a homologous system. This method makes it possible to characterize regulatory elements of genes (Finken and Kunz 1997), to elucidate tissue-specific gene expression (Quijada et al. 2000), and to investigate the consequences of overexpression (Gillingham and Munro 2000). Since gold particles can be coated with RNA (Davis et al. 1999), it is feasible to design experiments for the silencing of genes using RNA interference (Bass 2000, Tavernarakis et al. 2000). Furthermore, it should be possible to use this transformation method not only for schistosomes but also for other multicellular parasites.

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