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International Journal for Parasitology 36 (2006) 671–679
www.elsevier.com/locate/ijpara

Successful transgenesis of the parasitic nematode *Strongyloides stercoralis* requires endogenous non-coding control elements☆

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Received 14 October 2005; received in revised form 16 December 2005; accepted 22 December 2005

Abstract

Critical investigations into the cellular and molecular biology of parasitic nematodes have been hindered by a lack of modern molecular genetic techniques for these organisms. One such technique is transgenesis. To our knowledge, the findings reported here demonstrate the first heritable DNA transformation and transgene expression in the intestinal parasite *Strongyloides stercoralis*. When microinjected into the syncitial gonads of free-living *S. stercoralis* females, a construct fusing the *S. stercoralis era-1* promoter, the coding region for green fluorescent protein (*gfp*) and the *S. stercoralis era-1* 3′ untranslated region was expressed in intestinal cells of normally developing F1 transgenic larvae. The frequency of transformation and GFP expression among F1 larvae was 5.3%. By contrast, expression of several promoter::*gfp* fusions incorporating only *Caenorhabditis elegans* regulatory elements was restricted to abortively developing F1 embryos of *S. stercoralis*. Despite its lack of regulated expression, PCR revealed that one of these *C. elegans*-based vector constructs, the *sur-5*::*gfp* fusion, is incorporated into F1 larval progeny of microinjected female worms and then transmitted to the F2 through F5 generations during two host passages conducted without selection and punctuated by free-living generations reared in culture. Heritable DNA transformation and regulated transgene expression, as demonstrated here for *S. stercoralis*, constitute the essential components of a practical system for transgenesis in this parasite. This system has the potential to significantly advance the molecular and cellular biological study of *S. stercoralis* and of parasitic nematodes generally.

Keywords: *Strongyloides stercoralis*; Transgenesis; Microinjection; Green fluorescent protein

1. Introduction

Parasitic nematodes profoundly impact human health, causing disease, debilitation or retarded cognitive development in over one billion people worldwide (Chan et al., 1994; Awasthi et al., 2003). A significant body of descriptive genomic information for parasitic nematodes, including a number of large databases of expressed sequence tags (Parkinson et al., 2003, 2004; Aboobaker and Blaxter, 2004), has been gathered over the past decade. However, due to their complex and protracted life histories, parasitic nematodes have resisted the application of functional genomic and other contemporary experimental methods that could elucidate gene function, thereby facilitating the discovery of new drug or vaccine targets as well as studies of host parasite interactions at the molecular level. One such method, transgenesis, would allow gene function to be rigorously investigated by assessing the effects of strategic transgene expression or of various forms of targeted gene ablation. The current status and future prospects for transgenesis and other functional genomic methods in parasitic helminths are reviewed by Aboobaker and Blaxter (2004). Briefly, transient gene transfer and expression have been achieved by particle bombardment in *Litomosoides carinii*, *Brugia malayi* and *Ascaris suum* (Davis et al., 1999; Jackstadt et al., 1999; Higazi et al., 2002) and by microinjection in *B. malayi* and *Strongyloides stercoralis* (Higazi et al., 2002; Lok and Massey, 2002). Some of these transient transfection systems have already found application in studies of the mechanisms of transcript expression and processing (Shu et al., 2003; Cohen et al., 2004; Higazi and Unnasch, 2004; Lall et al., 2004).

☆ Note: Nucleotide sequence data for the *S. stercoralis era-1* promoter and 3′UTR are available under the following accession numbers in the EMBL, Genbank, and DDJB databases: DQ333398, and DQ333399, respectively.

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0020-7519/$30.00
doi:10.1016/j.ijpara.2005.12.007

Strongyloides spp. and their relatives are viewed as potential models for the study of parasitic nematode genetics (Viney, 1999; Dorris et al., 2002; Grant et al., 2005). Unlike the majority of parasitic nematodes, members of this group alternate parasitic generations with one or more generations of free-living development. Species such as *S. stercoralis* and *Strongyloides ratti* undergo a single generation of free-living males and females with all progeny of these re-entering the parasitic cycle via the infective third-stage larva (L3i). Other species, such as *Strongyloides planiceps*, have been observed to go through up to eight sequential generations of free-living development before all development proceeds to the L3i (Yamada et al., 1991). *Parastrongyloides trichosuri*, a related parasite of the Australian brush tailed possum, appears able to cycle indefinitely as a free-living organism and then revert to parasitic development under defined culture conditions (Crook et al., 2005; Grant et al., 2005). The free-living microbivorous stages of these parasites may be cultured on laboratory plates with bacterial lawns identical to or only slightly modified from those used for standard culture of the widely used model organism *Caenorhabditis elegans* (Lewis and Fleming, 1995), making them potentially tractable to genetic manipulation (Viney et al., 1993; Viney, 1999).

We are working towards a system for heritable genetic transformation of *S. stercoralis*. Previously, we reported that constructs fusing the promoters for *S. stercoralis act-2* and *era-1* to the coding sequence for green fluorescent protein (*gfp*) are expressed in embryonic progeny of free-living female worms microinjected intragonadally with the plasmids. No expression of these constructs was observed in larval progeny, and so no host passage of transgenic parasites was attempted (Lok and Massey, 2002). Similar findings were made in the present study when we investigated the expression and inheritance patterns of constructs fusing several strong *C. elegans* promoters, *myo-2*, *sur-5* (Gu et al., 1998) and *mec-7*, to the *gfp* coding sequence in the progeny of microinjected free-living female *S. stercoralis*. Detailed study of one of these constructs, *sur-5::gfp*, revealed that, although expression occurs only during abortive embryonic development in the F1 generation, the construct itself is inherited through multiple generations in the host and is thus presumably incorporated into the germ line. More importantly, we describe a modification of a construct previously observed to express only in abortively developing embryos (Lok and Massey, 2002) that has resulted in strong GFP expression in intestinal cells of normally developing F1 transgenic larvae. These findings constitute the crucial steps in building a practical system for transgenesis in *S. stercoralis*.

2. Materials and methods

2.1. Parasite maintenance and culture

The UPD strain of *S. stercoralis* was maintained in immunosuppressed dogs and cultured as described (Schad et al., 1984).

2.2. Preparation and analysis of RNA and DNA

Genomic DNA (gDNA) was prepared from *S. stercoralis* L3i, which were harvested and cleaned by migration through low melt agarose as described (Massey et al., 2001). Cleaned L3i were pelleted by brief centrifugation, and the supernatant was removed. Fifty microliters of packed larvae were resuspended in 450 µl Lysis Buffer (0.1 M Tris–Cl, pH 8.5; 0.1 M NaCl; 50 mM EDTA; 1% SDS). Five microliters of 2-mercaptoethanol and 400 µg proteinase K were added and the suspension was frozen at −80 °C. Preparations were then thawed and incubated at 60 °C for 60 min with periodic vortexing. Eighty microliters of 5 M NaCl were added and mixed thoroughly. Eighty microliters of 10% CTAB in 0.7 M NaCl was added, mixed and incubated 10 min at 37 °C. Seven hundred microliters of chloroform was added, mixed and briefly spun. Seven hundred microliters of phenol/chloroform (1:1) was added to the recovered aqueous phase, mixed, briefly spun and the aqueous phase was recovered. Six tenths volume (about 400 µl) of −20 °C isopropanol was added, and the precipitated DNA was recovered by centrifugation, washed, dried and resuspended in 300 µl TE pH 7.4. Five microliters of RNase A was added and incubated at 42 °C for 2 h. This DNA was digested with *Pvu* II restriction endonuclease and purified using QIAquick PCR Purification columns (Qiagen) before being used for co-injection with plasmid constructs. RNA was purified after proteinase K digestion as above using the Trizol reagent (Invitrogen) according to the manufacturer's protocol. Template DNA for single-worm PCR was isolated from individual L3i using the method described by (Williams et al., 1992) for *C. elegans*.

In studies of construct inheritance patterns, primers for amplification of *gfp*-specific construct DNA and cDNA were: CeGFP-476F, 5′-CCCTTGTTAATAGAATCGAGTT and CeGFPnoTerR, 5′-TTTGTATAGTTCATCCATGCC. Primers specific for the constitutively expressed ribosomal small subunit protein gene, *rps-21* (SsRPS-21F2, 5′-CGACAAAGGAGAACTTGTTGAA and SsRPS-21R2, 5′ATTTCGTTGACGTCGATC) were used in control reactions. These control primers were designed to span a 53 bp intron in the *rps-21* gene, thus allowing products amplified from genomic DNA and cDNA to be distinguished based on their differing molecular sizes of 166 and 113 bp, respectively. PCR amplification of *gfp*-specific and control DNA was conducted with Taq polymerase (Invitrogen) using the following protocol: denaturation for 2 min at 94 °C; 35 cycles of 94 °C for 30 s followed by 55 °C for 30 s and extension at 72 °C for 6 min. For reverse transcriptase (RT)-PCR, first-strand cDNA was synthesised from total RNA from L3i using the SuperScript First Strand Synthesis System® (Invitrogen) according to the manufacturer's protocol. *gfp*-specific and control cDNAs were amplified in separate reactions using the primers and conditions described above. Plasmid constructs for microinjection included *myo-2::gfp* (pPD118.33) and *mec-7::gfp* (pPD117.01) were kindly provided by A. Fire (Stanford University) and *sur-5::gfp*