Title
Gene expression patterns during adaptation of a helminth parasite to different environmental niches.

Permalink
https://escholarship.org/uc/item/7rf262p7

Journal
Genome biology, 8(4)

ISSN
1474-7596

Authors
Jolly, Emmitt R
Chin, Chen-Shan
Miller, Steve
et al.

Publication Date
2007

DOI
10.1186/gb-2007-8-4-r65

Peer reviewed
Gene expression patterns during adaptation of a helminth parasite to different environmental niches

Emmitt R Jolly*, Chen-Shan Chin*, Steve Miller*, Mahmoud M Bahgat†, KC Lim*, Joseph DeRisi* and James H McKerrow*

Addresses: *California Institute for Quantitative Biomedical Research (QB3) of the University of California, San Francisco, 4th Street, San Francisco, CA 94158 USA. †Theraputic Chemistry Department, Infectious Diseases and Immunology Laboratory, the Road to Nobel Project, the National Research Center, Dokki, 12311 Cairo, Egypt.

Correspondence: James H McKerrow. Email: jmck@cgl.ucsf.edu

© 2007 Jolly et al.; licensee BioMed Central Ltd.
This is an open access article distributed under the terms of the Creative Commons Attribution License (http://creativecommons.org/licenses/by/2.0), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Abstract

**Background:** Schistosome bloodflukes are complex trematodes responsible for 200 million cases of schistosomiasis worldwide. Their life cycle is characterized by a series of remarkable morphological and biochemical transitions between an invertebrate host, an aquatic environment, and a mammalian host. We report a global transcriptional analysis of how this parasite alters gene regulation to adapt to three distinct environments.

**Results:** Utilizing a genomic microarray made of 12,000 45-50-mer oligonucleotides based on expressed sequence tags, three different developmental stages of the schistosome parasite were analyzed by pair-wise comparisons of transcript hybridization signals. This analysis resulted in the identification of 1,154 developmentally enriched transcripts.

**Conclusion:** This study expands the repertoire of schistosome genes analyzed for stage-specific expression to over 70% of the predicted genome. Among the new associations identified are the roles of robust protein synthesis and programmed cell death in development of cercariae in the sporocyst stages, the relative paucity of cercarial gene expression outside of energy production, and the remarkable diversity of adult gene expression programs that reflect adaptation to the host bloodstream and an average lifespan that may approach 10 years.

**Background**
Schistosomiasis is a chronic debilitating parasitic disease affecting some 200 million people across 74 countries within Africa, Asia, the Middle East and South America. In terms of public health and socio-economic impact, it ranks second only to malaria among parasitic diseases [1,2]. The causative agents of schistosomiasis are schistosome bloodflukes, multicellular trematodes whose life cycle is characterized by a series of striking morphological and biochemical transitions between an intermediate host snail in an aquatic environment, two free-swimming aquatic larval forms, and a warm-blooded mammalian host (Figure 1). As such, the schistosome represents an ideal but challenging biological system in which to identify programs of gene regulation that have evolved to facilitate adaptation of metazoa to different biological microenvironments.
In the intermediate host aquatic snail, sac-like clusters of differentiating larvae called daughter sporocysts give rise to large numbers of free-swimming aquatic cercariae. Cercariae navigate their environment by a side to side motion of their tails and find the mammalian host through chemical and environmental signals [3-6]. Upon entering the mammalian host, the tail is detached, and an early vascular stage called the schistosomulum rapidly readjusts to the new host environment. During this time, the schistosomulum undergoes changes in basic metabolism and surface properties [7]. The schistosomulum navigates the vascular system, including transiting through at least two capillaryplexuses. Between 14 and 28 days, the organisms undergo rapid growth and differentiation to adult male and female schistosomes (c), which pair and produce eggs (d). Eggs are excreted from the host. In fresh water, the eggs hatch into miracidia (e), which infect a snail host and develop into sporocysts (f). Daughter sporocysts generate infectious cercariae, completing the life cycle.

Efforts to reduce schistosome prevalence have included combinations of sanitation, health education, snail control, better diagnosis and chemotherapy [8]. Control of parasitemia has relied primarily on praziquantel, an effective chemotherapeutic drug that has been in use for over 20 years. Recurring morbidity and evidence of emerging resistance to praziquantel in endemic countries emphasizes the need for both an effective vaccine and new chemotherapy [9-11]. Several potential vaccine candidates have been identified [12,13], but to date none is sufficiently effective for practical use. Given the complexity of the schistosome lifecycle and its ability to evade the immune system, a more comprehensive approach to understanding the molecular biology and biochemistry of schistosomes is essential to rationally identify and develop potential vaccine candidates or new drugs.

The approximately 270 Mb genome of Schistosoma mansoni [14] is organized into 8 chromosomes [15], including the...
heterologous female pair WZ [16]. It is estimated to have 30% GC content [17]. Currently, the *S. mansoni* genome is thought to contain some 14,000 predicted genes [18,19]. Until recently, there have been relatively few attempts to analyze the functional genomics of schistosomes due to limited genomic sequence information. Identification of genes expressed in a sex-specific manner by adult worms was made using either a 576, 7,335, or 4,608 oligonucleotide cDNA based microarray system [20-22] and an analysis of genes associated with pairing of adult male and female worms was addressed [23]. The transition between miracidium and mother sporocyst was also analyzed using the same 7,335 component oligonucleotide array noted above [24]. Comparisons of adult transcripts of two schistosome species, and comparison of lung stage versus experimentally produced schistosomula, were carried out using a larger array [7,25]. A comparative study between the *S. japonicum* proteome and transcriptome was also undertaken [26].

Now that the genome is nearing completion, we present a comprehensive analysis of gene expression during three of the major stages of *S. mansoni* development: daughter sporocyst, cercaria, and adult. These stages represent key transitions from intermediate host snail to aquatic environment to mammalian host. We designed a 12,000 oligonucleotide microarray chip made of 45-50-mer oligonucleotides and we analyzed expression of a majority of the predicted 14,000 *S. mansoni* genes under very stringent conditions. While the present annotation of the genome does not distinguish gene insert orientation, we found that over 9,700 of the oligonucleotides printed yielded clear transcriptional signals in at least one parasite stage. We have examined pair-wise differential expression at each stage and identify those genes whose RNA expression profile is conserved or differentially expressed across developmental stages. Noteworthy gene clusters that support previous hypotheses or provide new insights into the responses of the parasite to environmental transitions during the lifecycle are identified. Highly expressed transcripts in sporocysts include those involved in general protein synthesis and quality control, consistent with the function of sporocysts in production of large numbers of cercariae. Cercarial transcripts are dominated by genes involved in mitochondrial function, supporting the energy production necessary for cercarial swimming. Adult worms express a diverse pattern of transcripts necessary for egg production, energy metabolism, immune evasion and physiological maintenance of a relatively long-lived organism.

**Results**

**The 12,000 element schistosome array**

The microarray used in this study contained 12,000 individual 45-50-mer oligonucleotides based on 11,998 tentative consensus sequences (TCs), as documented by the *Schistosoma mansoni* Genome Index maintained at The Institute for Genomic Research (TIGR) [27]. TCs are created by virtual assemblage of full or partial cDNA sequences into transcripts. Spliced forms of genes are listed separately. Of the 12,912 sequences provided by TIGR, 11,998 TCs were chosen based on the maximum size of the TC available. Thus, this microarray contains a majority of known sequences available in the *S. mansoni* genome. There have been concerns raised about the orientation of inserts in the current annotation of the TIGR assemblage; however, we found that >9,700 of the 12,000 oligonucleotides printed gave some transcript signals in one or another parasite stage by type II analysis (see Materials and methods) and, as detailed below, many key gene programs could be identified and correlated with environmental transitions.

**The microarray chip is specific for *S. mansoni* transcripts**

Extraction of RNA from schistosome daughter sporocysts involves excision of the entire snail hepatopancreas, and analysis of the snail and schistosome material. To control for any background hybridization of snail-specific material (to our *S. mansoni* cDNA-based 12,000-oligonucleotide microarray), we hybridized hepatopancreas RNA from infected snails containing daughter sporocysts versus uninfected snail hepatopancreas RNA across four different chips. With the exception of two genes, we found that intermediate host snail RNA was not detected (data not shown). Even for these two genes (TC8129, similar to beta-1 4-galactosyltransferase, and TC6896, similar to Unknown), hybridization signals were not seen on every run and were detected only by type II analysis. In summary, no significant contribution of snail RNA to the hybridization analysis was found. Daughter sporocysts contain cercariae at various stages of differentiation. Nevertheless, the transcriptome profile of this stage in the snail differed significantly from that of mature aquatic cercariae.

We also found that microarray samples and chips were reproducible and correlative. There was an average Pierson correlation coefficient (log ratio of medians) of 0.7 for biological replicates and 0.96 for technical replicates. Dye switch experiments showed that data differences were not due to dye-labeling bias (Additional data file 1).

**S. mansoni transcriptome overview**

This clustering analysis includes 431 genes analyzed by pair-wise comparison with at Examination of all developmental stages showed a transcript pattern specific to each. Figure 2 (Table 1) is a clustering analysis of the major transcripts identified in which duplicates of sporocyst (Cy5) were compared to cercariae (Cy3). For example, cluster 1 represents transcripts that are upregulated in the daughter sporocyst stage but not significantly in adults or cercariae. Cluster 2 shows transcripts up-regulated in both sporocysts and adults relative to cercariae. Clusters 3 and 4 show transcripts enriched in cercariae relative to adults. Clusters 5 and 6 show transcripts that are upregulated in adult worms but are either relatively low or not enriched in cercariae or sporocysts. least a
3-fold difference in transcript level between developmental stages. Overall, we identified enrichment of 1,154 genes during the schistosome developmental life cycle, the largest proportion of which is expressed in adult worms (type II analysis). Of the 1,154 genes, 406 represent genes of unknown function (Figure 3).

**Validation of microarray data by real time PCR**

To test the validity of the microarray results, we performed a quantitative analysis study on the expression levels of several parasite genes by real-time PCR. Among the transcripts analyzed were actin, cathepsin B, cytochrome C, eggshell protein precursor, Sm23 and cathepsin L. Gene-specific primers were designed to validate the gene expression profile for sporocyst, cercariae, and adult developmental stages (Additional data file 2). To ascertain gene expression transitions between cercariae and adults, we also analyzed transcript levels in 24-hour schistosomula, the stage of schistosomal development between cercarial penetration and adult worms. We found...
Table 1

Some of the major transcripts from clustering analysis of the microarray data

| Transcript | Description |
|------------|-------------|
| Cluster 1: highly enriched in sporocysts |
| TC16551    | Similar to 90 kDa heat shock protein |
| TC13362    | Homologue to 40S ribosomal protein S14 |
| TC16641    | Homologue to elongation factor 1-alpha |
| TC13505    | Homologue to AUT1 |
| TC10547    | Homologue to ribosomal protein S8 |
| TC17388    | Similar to ribosomal protein S15a |
| TC7475     | Similar to 60S ribosomal protein L8 |
| TC11590    | Ubiquitin/ribosomal fusion protein |
| TC7403     | Similar to 60S ribosomal protein L30 |
| TC9217     | Weakly similar to cathepsin L-like cysteine proteinase |
| TC17720    | Similar to putative high mobility group-like nuclear protein 2 |
| TC11170    | Similar to Usmg5 protein |

Cluster 2: highly enriched in sporocysts and enriched in adults |
| TC10486    | Glutathione S-transferase 28 kDa (SM28 antigen) |
| TC11169    | Similar to Usmg5 protein |
| TC13573    | 23 kDa integral membrane protein (SM23) |
| TC7377     | Similar to Y-box binding protein |
| TC16738    | 14-3-3 protein homolog 1 |
| TC6863     | Homologue to 14-3-3 protein homolog 1 |
| TC8189     | Similar to dynein 8 kDa light chain flagellar outer arm |
| TC13511    | Homologue to AUT1 |
| TC13698    | 40S rRNA protein homolog |
| TC16537    | Heat shock 70 kDa homolog protein (HSP70) (major surface antigen |
| TC10528    | Probable dynein light chain (T-cell-stimulating antigen SM10) |
| TC11430    | Thioredoxin |
| TC10691    | Thioredoxin peroxidase |
| TC16806    | Similar to NADH dehydrogenase 6 |
| TC7615     | Similar to ATP synthase lipid-binding protein-like protein |
| TC11552    | Homologue to cytochrome b |

Cluster 3: highly enriched in sporocysts and enriched in cercariae |
| TC13775    | Antigen SM21.7 |
| TC13518    | Homologue to NADH dehydrogenase subunit |
| TC17477    | Homologue to NADH dehydrogenase subunit 2 |
| TC17193    | Similar to NADH dehydrogenase subunit 4 |
### Table 1 (Continued)

| TC   | Description                                                   |
|------|---------------------------------------------------------------|
| TC16807 | Homologue to NADH dehydrogenase 6                           |
| TC7310 | Homologue to cytochrome c oxidase subunit 1                   |
| TC14303 | Homologue to ATPase subunit 6                               |
| TC16782 | Enolase                                                      |
| TC16557 | Myosin heavy chain - fluke                                   |
| TC10480 | Elastase                                                     |
| TC8910  | Tubulin beta-2 chain.                                        |
| TC71016 | Stathmin-like protein                                         |
| TC7850 | Similar to actin-filament fragmenting protein                 |
| TC7158 | Similar to fibrillarin                                        |

**Cluster 4: highly enriched in sporocysts and cercariae**

| TC   | Description                                                   |
|------|---------------------------------------------------------------|
| TC13565 | Myosin light chain                                            |
| TC16878 | Similar to glyceral-3-phosphate dehydrogenase [NAD+] cytoplasmic |
| TC17004 | Homologue to cytochrome c                                     |
| TC16870 | ATP:guanidino kinase SMC7-4 (EC 2.7.3.7; ATP:guanidino phosphotransferase) |
| TC17192 | Homologue to NADH dehydrogenase subunit 4                     |

**Cluster 5: enriched in sporocysts and highly enriched in adults**

| TC   | Description                                                   |
|------|---------------------------------------------------------------|
| TC16575 | Major egg antigen                                             |
| TC8654 | Similar to anti-Mullerian hormone type II receptor precursor  |
| TC13882 | Similar to 26S proteasome regulatory subunit                   |

**Cluster 6: highly enriched in adults**

| TC   | Description                                                   |
|------|---------------------------------------------------------------|
| TC10493 | Cathepsin B1 isotype 1                                       |
| TC13457 | Eggshe1 protein precursor                                     |
| TC13487 | Homologue to eggshell protein precursor                       |
| TC13492 | Homologue to eggshell precursor protein                       |
| TC8804  | Weakly similar to retinoid X receptor RXR-2                   |
| TC12198 | Similar to mucin-like protein                                 |
| TC7011  | Similar to putative retroelement                              |
| TC16781 | Extracellular superoxide dismutase [Cu-Zn] precursor (EC 1.15.1.1; EC-SOD) |
| TC10637 | Weakly similar to aquaporin 9 (small solute channel 1)       |
| TC9163  | Similar to serine/threonine-protein kinase unc-51 (EC 2.7.1.-; uncoordinated protein 5) |
| TC7005  | Similar to putative senescence-associated protein              |

Transcripts enriched in specific schistosome stages are organized into six major clusters. For an updated list of the latest version of TC sequences see Additional data file 8.
that the expression of genes analyzed by RT-PCR correlates with our microarray analysis in 10 of 11 cases (Figure 4).

**Genes expressed in daughter sporocysts reflect production of cercariae**
The daughter sporocyst stage resides in the intermediate host snail. The key biological function of this stage is to support the differentiation and development of large numbers of cercariae, the aquatic larval stage that will initiate invasion of the mammalian host. The function of this stage in generating cercariae is underscored by the fact that many of the most highly expressed transcripts are gene products that function in general protein synthesis (40S and 60S ribosomal subunits, elongation factor) and post-translational protein folding and fidelity (chaperones, ubiquitin). Furthermore, many of the proteins that will be utilized by cercariae in the initial stages of host skin invasion are also produced in this stage, including cercarial elastase (aka cercarial protease), cercarial muscle proteins (actin, dynein light chain isoforms), and calcium binding proteins that are also abundant in the proteome of cercarial secretions [28].

As would be expected from the large-scale differentiation of cercariae within the daughter sporocyst, transcription factors such as the Y-box binding protein and stathmin, a phosphoryl protein involved in vertebrate growth and regulation of differentiation [29] are highly expressed. Sporocysts express a cathepsin L-like cysteine protease homolog (TC9217) that is not significantly enriched in adults (Figure 4). In adults a cathepsin L is gut localized and functions in digestion [30]. The lack of significant enrichment of other gut-specific genes in sporocysts, such as those encoding cathepsins B, B1, and C, and the unique profile of the cathepsin-like protease homolog, suggests that this protease isoform may function differently in the daughter sporocyst, possibly in the generation of cercarial progeny. In Caenorhabditis elegans, a cathepsin L is essential for embryogenesis and development [31].

**Genes expressed in cercariae reflect energy production and motility**
Cercariae are a relatively short-lived ‘transitional’ stage that are released from snails into fresh water and must swim to find, and ultimately invade, a mammalian host. Cercariae will not survive if they fail to enter the mammalian host before energy sources are exhausted. Consistent with this concept of the biological function of cercariae, transcripts that are upregulated are primarily factors necessary to sustain swimming behavior and invasion (Figure 2, clusters 3 and 4). The highest expressed transcripts are genes involved in mitochondrial function or energy metabolism. These include NADH dehydrogenase and its various subunits, cytochrome C and its homologues, and ATP/ADP carrier proteins. Other transcripts highly expressed in cercariae include structural and motility genes like actin and fibrillin and transcripts coding for a protease that plays a role in host invasion, cercarial elastase [32]. In general, fewer transcripts are found in cercariae relative to other stages.

**Genes expressed in adult worms**
Compared to sporocysts and cercariae, the most abundant transcripts in adult worms (male and female worm pairs) are significantly more diverse. This reflects the fact that adult worms must evade the mammalian host immune system, maintain motility, acquire and metabolize a variety of nutrients, form mating pairs, and produce large numbers of eggs. In parallel with these functions, highly abundant transcripts in adult worms include a group of genes involved in protein degradation in the gut (cathepsin B, cathepsin L), egg production, and oxidative stress responses [33-36]. Genes involved in gonadal differentiation for both male and female worms, as well as genes coding for proteins for egg-associated proteins are readily identified. These include the anti-mullerian hormone receptor for males, and members of the RXR family and Smad family, which are cell signaling pathways previously associated with function of female gonads [37,38]. Highly expressed egg transcripts include eggshell protein precursor, major egg antigen, and several homologues of these proteins. Perhaps most striking is the abundance of transcripts in adult worms from genes coding for surface proteins, including nutrient transporters such as the glucose transporter and a number of surface proteins of unknown function previously identified in research projects aimed at subunit vaccine development. These include Sm23 [39-45], integral membrane protein 25 [46], Sm14 [47,48], and 26 and 28 glutathione S-transferases [49-52].

**Discussion**
The stage-specific transcriptome of *S. mansoni* provides an informatics foundation for the study of parasite gene regulation and a correlative for proteomic studies. This microarray study validates and extends observations made with a 7,335-oligonucleotide array from previously available expressed sequence tags (ESTs) [53], and correlates well with comparative analyses between the transcriptome and proteome of adult *Schistosoma japonicum* and *S. mansoni* [26]. With 12,000 oligonucleotides, a more complete picture of gene programs that mark transitions between key schistosome stages in distinct environmental niches is now apparent. The entire schistosome genome is estimated to have 14,000 functional genes. While it has been noted that the TIGR annotation to date has not distinguished the orientation of the inserts sequenced, we found that 9,700 of the 12,000 genes analyzed on this array gave clear positive signals for at least one stage. More importantly, many genes could be functionally annotated and gene programs correlating with environmental transitions of the helminth parasite discovered or validated.
One of the most remarkable aspects of the schistosome parasite is the dramatic morphological change that takes place between life cycle stages (Figure 1). These morphological changes parallel transition of the parasite between three markedly different environments. The daughter sporocyst stage is the late developmental stage in the intermediate host snail - a mollusk of fresh water habitats. Motile cercariae emerge from the snail and must navigate an aquatic environment to find the mammalian host. Following entry into the skin of the mammalian host, the parasite must now adapt to a different osmotic environment and a warm-blooded host. Finally, within the mammalian host, two distinct sexual stages develop and eggs are subsequently produced.

Array analysis comparing daughter sporocysts to the cercarial stage highlighted gene programs responsible for supporting larval development. The sporocyst stage is a very efficient protein synthesis factory. This correlates with the need for mass production of cercariae and the availability of nutrients from an intimate relationship with the snail host. In keeping with the complex morphological differentiation of cercariae with distinct body parts and 'organs' from an embryonic cell mass, daughter sporocyst transcripts included genes involved not only in general protein synthesis such as ribosomal and heat shock genes, but also transcripts annotated to function in programmed cell death (TC18024) and ubiquitination, a process key to morphological differentiation.

In Figure 2, cluster 2, daughter sporocysts share with adult worms a previously unrecognized requirement for transcriptional upregulation of antioxidant genes such as glutathione-S-transferase, thioredoxin, and thioredoxin peroxidase. This may reflect oxidative stress generated within the immediate snail host, or storage of these proteins in developing cercariae, so that they are available in the earliest stages of mammalian host invasion. Utilizing a 7,335 oligonucleotide array, Vermeire et al. [24] documented gene expression patterns between miracidia, the aquatic stage that invades the snail host, and the mother sporocyst stage, which is the initial stage following invasion of the snail host. As is the case for daughter sporocysts analyzed in our study, they also found upregulation of several genes involved in protein synthesis, the redox pathway, and proteolysis. This suggests that these gene programs are initiated in mother sporocysts following entry of miracidia into the snail, and sustained throughout the daughter sporocyst stage until mature cercariae leave the snail.

Transition to the cercarial stage is marked by a reduction in transcript level of a large number of genes as seen in clusters 1 and 2 of Figure 2. We found 116 genes whose abundance is reduced in cercariae by 3-fold relative to sporocysts (type II analysis where sporocyst intensity units are set >3,000). In comparison to other stages, the cercariae are less transcriptionally active. Fewer than 7 genes are upregulated more than 2-fold relative to adults and only 34 are upregulated when compared pair-wise to adult transcripts.
The cercariae express a cluster of genes consistent with the energy required to move rapidly through water in search of a mammalian host. These include genes functioning in ATP production and utilization, presumably for muscle function and swimming behavior. Consistent with previous Northern blot analyses and biochemical studies, much of the repertoire of proteins that cercariae use to invade the skin of their host, as well as structural proteins, have already been produced during cercarial development in the daughter sporocyst stage within the nutrient rich intermediate host snail (Figure 2a-d). Cercariae do express caspases and related cell death programs, which may be required for morphological remodeling during transition into the schistosomulum stage. Cercariae detach their motile tail and surface glyocalyx shortly after entry into the mammalian host. Large gland structures (acetabular glands) producing invasive proteases involute within 48–72 hours of invasion.

Residence of adult schistosomes in the mammalian host bloodstream is supported by expression of gene families that respond to oxidative stress and genes involved in adjustment and adaptation to a new osmotic environment (aquaporin) [54-56]. Adult worms have a major requirement to digest blood-proteins and acquire other nutrients (cathepsin B, glucose transporter) [33,35,57]. Schistosomes differentiate into male and female worms and expression of an anti-mullerian hormone receptor [58,59] is likely related to sexual differentiation. Female parasites produce hundreds to thousands of eggs per day as reflected in the upregulation of egg shell proteins. Recent analysis of gene expression in earlier intravascular stages can now be compared with the adult (versus cercariae) transcriptome presented here. Using a cDNA array, Dillon et al. [60] equated seven-day cultured schistosomula with lung schistosomula and compared gene expression across life stages. Chai et al. [7] utilized schistosomula directly obtained from the lungs of infected mice, and compared transcripts expressed to adult worms, cercariae, and newly transformed schistosomula. By and large, the genes we found expressed in adult parasites versus cercariae were similar to those observed in the comparison of lung worms versus cercariae. These included nutrient acquisition genes, such as those encoding the glucose transporter and the proteolytic cathepsins. The genes down-regulated in adult worms relative to lung schistosomula include several we found expressed in cercariae or daughter sporocysts, including those encoding the anti-inflammatory protein Sm16 and paramyosin.

The intravascular stages of schistosome parasites have a complex and highly adapted relationship with the mammalian host [61,62]. To support this relationship, intravascular stages upregulate surface proteins or receptors, some of which have homology to mammalian receptors and/or factors involved in signaling cascades (Additional data file 6). These include the anti-Mullerian hormone type II receptor [63] and the thyroid receptor interacting protein [64].

Developmental regulation of cell number and type by programmed cell death appears to be an important function in all stages of schistosome development analyzed, as it is in the nematode C. elegans [65]. Homologues for genes involved in programmed cell death include TC11294 and the DAP-1 homolog TC18024 (type II analysis).

Since the initial stages of the genome analysis of schistosomes, it has been clear that transposon-like sequences are common. Efforts to exploit these elements for genetic manipulation are ongoing [66,67]. It is noteworthy that one transposon (TC17720) is more abundantly expressed in the sporocyst than in any other developmental stage. It is also expressed in adults, albeit at low levels, as corroborated in the study by Gobert et al. [25]. There is also another retroelement (TC7011) highly enriched in adult worms.

This expanded array data set, focusing on life cycle stage transitions, should aid in current attempts to develop transfection and gene knockout studies for schistosomes by identifying those genes that are stage-specific versus others shared among different developmental states. For example, the gene encoding cytochrome C is expressed at comparable levels in all stages studied. In contrast, genes such as those encoding the cercarial elastase in the sporocyst stage, or eggshell components in the adult female stage, represent potential models for identifying and characterizing key spatial and temporal promoter elements, and ultimately molecular mechanisms of gene regulation. Fitzpatrick et al. and Moertel et al. also have studied gender-specific gene transcription in S. mansoni using a 7,335 oligonucleotide array [53] and a 22,575 combined S. japonicum and S. mansoni array [68]. The results reported here for genes that can be attributed to either male or female worms largely validate these studies.

Analysis of the stage-specific transcriptional program of schistosomes also helps to validate and underscore differences in the levels of gene products noted in proteomic studies of schistosome life cycle stages. The levels of many gene products identified by proteomic analysis parallel transcription levels in this microarray analysis. In contrast to protozoan parasites like Leishmania [69], this may indicate that less post-transcriptional regulation operates in schistosomes. Having transcriptome data for three major stages, combined with proteomic data, should now facilitate a more focused analysis to determine to what extent RNA stability or other post-transcriptional mechanisms play a role in schistosome gene regulation. It was noted from proteome analysis that many genes are expressed across stages and these were, therefore, seen as less attractive vaccine targets [26,70]. The identification of several stage-specific patterns of expression in this present study should help to redirect efforts aimed at finding the most logical candidates for a subunit vaccine, and also identify new targets to explore for drug therapy.
Conclusion
We show that the daughter sporocyst stage in the intermediate host snail functions primarily to support the development of invasive larvae (cercariae) by up-regulating expression of genes involved in protein synthesis, cellular differentiation, and programmed cell death. Many of the major structural and functional components of cercariae, utilized to later invade the mammalian host, are expressed and packaged prior to larval release from the snail. The aquatic cercariae themselves are less transcriptionally active than other stages, with an emphasis on production of proteins involved in energy metabolism and motility. This is in keeping with the function of these larvae in swimming from snail to mammalian host. Finally, adult parasites, which have adapted to survival in the mammalian host bloodstream, have a complex transcriptional program that supports adaptation to a new host temperature and chemical environment, evasion of the host immune response, acquisition of nutrients, and production of eggs for transmission to a new host.

Materials and methods
All experiments were performed with *S. mansoni* of the NMRI Puerto Rican strain maintained routinely through *Biomphalaria glabrata* snails and Syrian golden hamsters as previously detailed [71].

Schistosome stage collection
Daughter sporocysts were collected by dissection of whole hepatopancreas from six-week old infected *B. glabrata* snails, during maturation of daughter sporocysts and prior to cercarial release. Uninfected snail hepatopancreas was also collected as a control. Cercariae were collected in distilled water from infected *B. glabrata* snails using the light induction method as previously described [71,72]. Following exposure to light for 2 hours, 50-60 snails shed about 200-300 cercariae/snail. Several collections were pooled and used for RNA extraction. Twenty-four hour schistosomula were mechanically transformed as previously described [73]. Adult worms were recovered from the mesenteric veins by portal perfusion as previously described [74].

RNA extractions
Total RNA was extracted from all biological samples for CombiMatrix microarray analysis. Multiple uninfected and infected hepatopancreas, cercariae, and adult worms were homogenized in 1 ml Trizol (Invitrogen, Carlsbad, CA, USA) and RNA was extracted using the standard Invitrogen protocol. RNA was eluted in deionized water, quantified, and checked for RNA quality by UV spectrometry and agarose gel analysis. Total RNA (15 μg) for individual stages (coupled to Cy3 or Cy5) was hybridized to CombiMatrix custom array microarray chips, as described in detail below (CombiMatrix Corporation, Mukilteo, WA, USA).

*S. mansoni* DNA oligonucleotide probe design
All probes were designed using the CombiMatrix software CombiMatrix Automated Probe-design Suite (PDS), based on 11,997 TC groups from sequences at TIGR. TCs represent a virtual assemblage of ESTs. Of the 12,717 TIGR TC groups, 11,998 were used for the 12,000 spot array based on size of the TC source cDNA. Those with the smallest TC source cDNA sequences were excluded. The 12,000 sequences were loaded into the CombiMatrix Custom Array content probe array system for probe design, one of which was spotted in triplicate using different oligonucleotide designs constructed by hand. The probe design system took each TC sequence and designed 45-50-mer probes to be unique to each gene with predictable thermodynamic behaviors.

Microarray hybridization and data analysis
CombiMatrix Custom Array microarray chip hybridizations were stringently performed in duplicate or triplicate. Hybridizations (3× SSC, 0.025 M HEPES, 0.28 μg/μl polyA, 0.05% SDS) occurred at 63°C for 18 hours to maintain specific binding efficacy. DNA microarray chips were scanned using an Axon4000B scanner at 5 μ resolution and 100% laser power and the images were analyzed with GenPix Pro 4 (Molecular Devices, Sunnyvale, CA, USA). Microarray data were stored in the NOMAD microarray database [78] and normalized by a global normalization using unflagged features with a regression correlation coefficient ≥0.75 and median intensity value >0 as previously described [75]. Data were clustered using Cluster 3.0 [76] and visualized in Java TreeView version 1.0.11 [77]. Clustering was done as below: all data were log transformed and filtered for the presence of 100% of genes and a standard deviation value of log_{2} 1.5 or 3-fold gene expression, followed by correlation uncentered clustering of genes and arrays by complete linkage analysis. A second and less stringent analysis was repeated using the same methodology, except clustering was done with a standard deviation value of log_{2} 0.6, or 1.5-fold gene expression (Additional data file 3). Each CombiMatrix array has 12,500 spots; 148 are empty controls (no oligonucleotides) and 352 represent various small oligonucleotide controls. For a less sophisticated analysis of gene expression, on or off, each channel (635 and 532) was treated independently. The data were reanalyzed and normalized as described above. The average median value of the 148 empty spots for each chip was used as background and subtracted from the initial intensity unit value to produce a 'normalized value'. Spots from the 'normalized value' (IU) were retained as expressed genes. We refer to this type of analysis as type II analysis. To compare all three stages, the average median of all cercariae median values was used as a standard (Additional data file 4). Transcripts with an IU >2,000 are defined as enhanced.

RT-PCR
A total of 1 μg parasite RNA from each stage was used to prepare double stranded cDNA using SuperScript™ II reverse transcriptase (Invitrogen) in the presence of oligo dT.
Quantitative PCR (qPCR) experiments were repeated in duplicate using cytochrome c as an internal control gene. The relative quantification of our genes of interest was carried out by mixing cDNA from different parasite stages with SYBR Green PCR master mix in 96 well plates, which was then incubated in the Applied Biosystems (Foster City, CA, USA) 7500 Real-Time PCR System. The program included an initial melting phase for 2 minutes at 95°C, denaturation and hot start for 10 minutes at 95°C, followed by 40 amplification cycles (95°C, 15 s; 60°C, 1 minute). The sequences of the primers and names of the studied genes are given in Additional data file 2.

Additional data files
The following additional data are available with the online version of this paper. Additional data file 1 is an analysis of Cy3 and Cy5 dye-switch experiments comparing sporocyst to cercariae, and adults to cercariae. Additional data file 2 is a table of primer sequences used for real-time PCR. Additional data file 3 is a table listing the 431 genes highly enriched from the clustering analysis shown in Figure 2. Additional data file 4 lists all genetic data analyzed by type II analysis. Additional data file 5 is a table listing the 431 genes highly enriched from the clustering analysis shown in Figure 2. Additional data file 6 is a table listing S. mansoni receptor-like proteins and log2 gene expression ratios. Additional data file 7 lists all oligonucleotide sequences used for the schistosome array. Additional data file 8 is a key to compare the schistosome TC sequence to the updated TIGR schistosome gene index (Version 6).

Acknowledgements
We thank Dr Ping Loke, Dr Adam Carroll, Dr Kael Fisher, Dr Anjan Debnath and members of the McKerrow lab for technical support and helpful discussions. This research was supported in part by a UNCF/Merck Postdoctoral Fellowship (ERJ), the Sandler Family Supporting Foundation, NIAID ROI AI53247 and a US-Egypt Joint Science and Technology grant for discussions. This research was supported in part by a UNCF/Merck Distinguished Scientist Award. The work was supported by NIAID ROI AI53247 and a US-Egypt Joint Science and Technology grant for discussions. This research was supported in part by a UNCF/Merck Distinguished Scientist Award.

References
1. Chitsulo L, Engels D, Montresor A, Savioli L: The global status of schistosomiasis and its control. Acta Trop 2000, 77:41-51.
2. Savioli L, Albonico M, Engels D, Montresor A: Progress in the prevention and control of schistosomiasis and soil-transmitted helminthiasis. Parasitol Int 2004, 53:103-113.
3. Haas W, Grabe K, Geis C, Pach T, Stoll K, Fuchs M, Haberl B, Loy C: Piano and drug resistance of schistosomes. Parasitol 2002, 124:153-167.
4. Grabe K, Haas W: Navigation within host tissues: cercariae orientate towards dark after penetration. Parasitol Res 2004, 93:111-113.
5. Shift CJ, Cermelk SH, Ley HE, Kriel RL: The influence of human skin lipids on the cercarial penetration responses of Schistosoma haematobium and Schistosoma mansoni. J Parasitol 1972, 58:476-480.
6. Saladin KS: Schistosoma mansoni: cercarial responses to irradiance changes. J Parasitol 1982, 68:120-124.
7. Chai M, McManus D, McInnes R, Moerel L, Tran M, Loukas A, Jones MK, Gobert GN: Transcriptome profiling of lung schistosomes, in vitro cultured schistosomula and adult Schistosoma japonicum. Cell Mol Life Sci 2006, 63:919-929.
8. Starrock RF: Schistosomiasis epidemiology and control: how do we get here and where should we go? Mem Inst Oswaldo Cruz 2001, 96(Suppl):17-27.
9. Wiess PM: The epidemiology of morbidity of schistosomiasis. Parasitol Today 1996, 12:215-220.
10. Ismail M, Farghaly A, Farghally A, Tao LF, Day TA, Bennett JL: Resistance to praziquantel: direct evidence from Schistosoma mansoni isolated from Egyptian villagers. Am J Trop Med Hyg 1999, 60:932-935.
11. Tran MH, Pearson MS, Smyth DJ, Jones MK, Duke M, Don TA, McManus DP, Correa-Oliveira R, Loukas A: Tetratransfins on the surface of Schistosoma mansoni are protective antigens against schistosomiasis. Nat Med 2006, 12:835-840.
12. Pearce EJ: Progress towards a vaccine for schistosomiasis. Acta Trop 2003, 86:309-313.
13. Simpson AJ, Sher A, McCutchan TF: The genome of Schistosoma mansoni: isolation of DNA, its size, bases and repetitive sequences. Mol Biochem Parasitol 1989, 34:303-304.
14. Short RB, Menzel MY, Pathak S: Somatic chromosomes of Schis-
15. hosa mansoni. J Parasitol 1979, 65:471-473.
16. Grossman AI, McKenzie R, Cain GD: Sex heterochromatin in Schistosoma mansoni. J Parasitol 1980, 66:368-370.
17. Mike KA, Bizzarro JW, Blake RD, Haien Tsai M, Fang Tao L: Experimental DNA melting behavior of the three major Schisto-
18. soma species. Mol Biochem Parasitol 2000, 107:303-307.
19. Johnson DH, Tsao J, Luo M, Carson M: GCCeDb: a flexible database and web interface integrating experimental results and analysis for structural genomics focusing on Ce
20. onchoditis elegans. Nucleic Acids Res 2006, 34:D471-474.
21. Verjovski-Almeida S, DeMarco R, Martins EA, Guimaraes PE, Ojopi EP, Paucola AC, Piazza JP, Nishiyama MY Jr, Kitajima JP, Adamson RE, et al: Transcriptome analysis of the acelomate human parasitic Schistosoma mansoni. Nat Genet 2003, 35:148-157.
22. Hoffmann KF, Johnston DA, Dunne DW: Identification of Schisto-
23. soma mansoni gender-associated gene transcripts by cDNA microarray profiling. Genome Biol 2002, 3:RESEARCH0041.
24. Fitzpatrick JM, Johansson MV, Johnston DW, Dunne DW, Hoffmann KF: Gender-associated gene expression in two related strains of Schistosoma japonicum. Mol Biochem Parasitol 2004, 136:191-209.
25. DeMarco R, Oliveira KC, Venancio TM, Verjovski-Almeida S: Gender biased differential alternative splicing patterns of the transcriptional cofactor CA150 gene in Schistosoma mansoni. Mol Biochem Parasitol 2006, 150:123-131.
26. Fitzpatrick JM, Hoffmann KF: Dioecious Schistosoma mansoni express different gender repertoire regulated by pairing. Int J Parasitol 2006, 36:1081-1089.
27. Vermeire JJ, Taft AS and Hoffmann KF, Fitzpatrick M, Yoshino TP: Schisto-
28. soma mansoni: DNA microarray gene expression profiling during the miracidium-to-mother sporocyst transformation. Mol Biochem Parasitol 2006.
29. Gobert GN, McInnes R, Moertel L, Nelson C, Jones MK, Hu W, McManus DP: Transcriptomic tool for the human schistosome blood flukes using microarray gene expression profiling. Exp Parasitol 2006, 114:160-172.
30. Liu F, Lu J, Hu W, Wang SY, Cui SJ, Chi M, Mao Q, Wang XR, Song HD, Xu XN, et al: New perspectives on host-parasite interplay by comparative transcriptomic and proteomic analyses of Schistosoma japonicum. PLoS Pathog 2006, 2:e29.
31. (TIGR), the Institute for Genomic Research 2003 [http://www.tigr.org].
32. Lueders GM, Medzhirdashsky F, Lim KC, Hansell E, McKerrow JH: Proteomic analysis of Schistosoma mansoni cercarial secretions. Mol Cell Proteomics 2005, 4:1862-1875.
33. Valle C, Fustucci A, Calogero A, Macri P, Mecozzi B, Liberii P, Cioli D: Stage-specific expression of a Schistosoma mansoni polyamine similar to the vertebrate regulatory protein spm. J Biol Chem 1999, 274:33869-33874.
34. Boghst B, Dalton JP, Brady CP, Brindley PJ: Gut-associated immuno-localization of the Schistosoma mansoni cysteine proteases, SmCL1 and SmCL2. J Parasitol 2001, 87:237-241.
35. Huynh S, Gilliano DB, Okose Y, Lustigman S: Cathespin L is essential for embryogenesis and development of Ce
36. onchoditis elegans. J Biol Chem 2002, 277:3477-3486.
Carceral elastase is encoded by a functionally conserved gene family across multiple species of schistosomes. 

**R65**

32. Salter JP, Choe Y, Albrecht H, Franklin C, Lim KC, Craik CS, McKerrow JH: Cercarial elastase is encoded by a functionally conserved gene family across multiple species of schistosomes. *J Biol Chem* 2002, **277**(24):23-24.

33. Sajid M, McKerrow JH: Cysteine proteases of parasitic organisms. *Mol Biochem Parasitol* 2002, **120**(1):1-21.

34. Caffrey CR, McKerrow JH, Salter JP, Sajid M: Blood ‘n’ guts: an update on schistosome digestive peptidases. Trends Parasitol 2004, **20**(8):241-248.

35. Sajid M, McKerrow JH, Hansell E, Mathieu MA, LCD, Hsieh I, Greenbaum D, Bogoy M, Salter JP, Lim KC, et al.: Functional expression and characterization of Schistosoma mansoni cathepsin B, and its trans-activation by an endogenous asparagine endopeptidase. *Mol Biochem Parasitol* 2003, **131**(6):65-75.

36. Dvorak J, Delcroix M, Rossi A, Vopalevsky V, Pospisek M, Sedinova M, Mikes L, Sajid M, Sal, McKerrow JH, et al.: Multiple cathepsin B isoforms in schistosomula of *Schistobilharzia regenti*: identification as a vaccine. *Parasitol Immunol* 2005, **23**(3):859-910.

37. Fantappie MR, Freebern WJ, Osman A, LaDuca J, Niles EG, LoVerde PT: Evaluation of Schistosoma mansoni retinoid X receptor (SmRXR1 and SmRXR2) activity and tissue distribution. *Mol Biochem Parasitol* 2001, **118**(1):1-9.

38. Osman A, Niles EG, LoVerde PT: Identification and characterization of a SmAD2 homologue from *Schistosoma mansoni*, a transforming growth factor-beta signal transducer. *J Biol Chem* 2001, **276**(10072-10082).

39. Gamble-Leal LM, Guarner J, Todd CW, Da’Dara AA, Freeman GL Jr, Boyer AE, Harn DA, Secor WE: Comparison of *Schistosoma mansoni* irradiated cercariae and Sm23 DNA vaccines. *Parasite Immunol* 2005, **27**(3):341-349.

40. Simpson AJ: Cysticercosis of *Schistosoma mansoni*: complex biologic molecules and potent antigens. *Mol Biochem Parasitol* 1990, **35**(1):95-102.

41. Wright MD, Henkle KJ, Mitchell GF: An immunogenic Mr 23,000 integral membrane protein of *Schistosoma mansoni* worms that closely resembles a human tumor-associated antigen. *Parasitol Immunol* 1991, **13**(4):299-300.

42. Reynolds SR, Shoemaker CB, Harn DA: T and B cell epitope mapping of Sm23, an integral membrane protein of *Schistosoma mansoni*. *J Immunol* 1992, **149**(9):3995-4001.

43. Simpson AJ: *Schistosoma mansoni*: immunoreactivity of human sera with the surface antigen Sm23. *Exp Parasitol* 1993, **77**(2):282-294.

44. Inal J, Bickel Q: Sequence and immunogenicity of the 23-KDa transmembrane antigen of *Schistosoma haematobium*. *Mol Biochem Parasitol* 1995, **74**(2):217-221.

45. Koster B, Strand M: *Schistosoma mansoni*: Sm23 is a transmembrane protein that also contains a glycosylphosphatidylinositol anchor. *Arch Biochim Biophys* 1994, **310**(6):108-117.

46. Petzke MM, Suri PK, Bungiro R, Goldberg M, Taylor SD, Ronjan S, Taylor H, Boyer AE, Harn DA, Secor WE: Characterization of *Schistosoma mansoni* and *S. japonicum* glutathione S-transferases. *Mol Biochem Parasitol* 1995, **77**(2):195-203.

47. Ivens AC, Fatani B, Verbeke N, Lennard LC, Almeida MS, Thaumaturgo N, Simoes M, Villela-Ferreira R, Mehlhorn H, Thanh HL, Chevalier J, Ripoche P, Abrami L, Gobin R, Berthonaud V, Thanh VL, Chevalier J, Ripoche P, Verbazvat JM: Localization of the FA-CHIP water channel in frog urinary bladder. *Eur J Cell Biol* 1997, **73**(2):215-221.

48. Elendendorf JS: Signals that regulate GLUT4 translocation. *J Cell Biol* 2002, **159**(2):177-186.

49. Durlinger AL, Visser JA, Themmen AP: Regulation of ovarian function: the role of anti-Mullerian hormone. *Reproduction* 2002, **124**(6):601-609.

50. Josso N, di Clemente N, Goudard L: Anti-Mullerian hormone antagonism: a review of factors influencing the host-parasite system and its possible role in biological control. *Adv Parasitol* 1972, **10**(1):91-192.

51. McKerrow JH, Pino-Heiss S, Lindquist R, Wern G: Purification and characterization of an elastinolytic proteinase secreted by *Schistosoma mansoni*. *J Biol Chem* 1985, **260**(37):3703-3707.

52. Colley DG, Wilke SK: *Schistosoma mansoni*: simplified method.
for the production of schistosomules. Exp Parasitol 1974, 35:44-51.

74. Smithers SR, Rooodyn DB, Wilson RJ: Biochemical and morphological characteristics of subcellular fractions of male Schistosoma Mansoni. Exp Parasitol 1965, 16:195-206.

75. Llinas M, Bozdech Z, Wong ED, Adai AT, DeRisi JL: Comparative whole genome transcriptome analysis of three Plasmodium falciparum strains. Nucleic Acids Res 2006, 34:1166-1173.

76. Eisen MB, Spellman PT, Brown PO, Botstein D: Cluster analysis and display of genome-wide expression patterns. Proc Natl Acad Sci USA 1998, 95:14863-14868.

77. Saldanha AJ: Java Treeview - extensible visualization of microarray data. Bioinformatics 2004, 20:3246-3248.

78. S. mansoni Gene Index 2006 [http://compbio.dfci.harvard.edu/tgi].