Research

Identification of *Schistosoma mansoni* gender-associated gene transcripts by cDNA microarray profiling

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Published: 25 July 2002

Genome ***Biology*** 2002, **3(8)**:research0041.1–0041.12

The electronic version of this article is the complete one and can be found online at http://genomebiology.com/2002/3/8/research/0041

(Print ISSN 1465-6906; Online ISSN 1465-6914)

Received: 23 April 2002
Revised: 24 May 2002
Accepted: 11 June 2002

Abstract

Background: Parasitic helminths of the genus *Schistosoma* mate, achieve sexual maturity and produce eggs in the bloodstream of their definitive hosts, and the most important pathological consequences of the infection are associated with this process. We have used cDNA microarray technology to initiate genome-wide gene-expression studies of sex and sexual development in mature *Schistosoma mansoni* parasites.

Results: An *S. mansoni*-specific cDNA microarray was fabricated using 576 expressed sequence tags selected from three cDNA libraries and originating from two different parasite developmental stages. Five independent cDNA microarray hybridizations were analyzed using stringent filtering criteria and careful quality control, leading to the identification of 12 new female-associated and 4 new male-associated gene transcripts in the mature adult schistosome. Statistical analysis of variation demonstrated high levels of agreement within a cDNA microarray (correlation coefficient 0.91; median coefficient of variation 11.1%) and between cDNA microarrays (correlation coefficient 0.90; median coefficient of variation 14.4%). RT-PCR analysis confirmed the cDNA microarray results, thereby supporting the reliability of the system.

Conclusions: Our study expands the list of *S. mansoni* gender-associated gene transcripts from all previous studies by a factor of two. Among the new associations identified, a tyrosinase ortholog was preferentially expressed in the adult female, and a dynein light-chain ortholog was highly induced in the adult male. cDNA microarrays offer the potential for exponential leaps in the understanding of parasite biology and this study shows how molecules involved in sexual biology can be rapidly identified.

Background

Schistosomes are members of a medically important group of parasitic helminths that contribute to severe morbidity and mortality in people in 74 tropical and subtropical developing nations [1]. A virtually unique trait of all species of *Schistosoma* (phylum Platyhelminthes) is their evolution from hermaphrodite ancestors into sexually dimorphic species. Although the selective pressures that initiated this heterogametic adaptive radiation are presently unknown, gender-specific gene-expression investigations have begun to shed some light on sexual biology within the schistosomes. Most gender-associated investigations have focused on female gene-expression patterns because this sex produces the eggs that cause the main pathological changes

within the infected host. Thus, in developing approaches to prevent egg-induced pathology (as well as blocking transmission), knowledge about the molecules associated with sexual maturation of the female parasite and development of the viable egg will be critical. To this end, past studies have provided insight into eggshell organization [2-4], reproductive duct morphology [5] and vitellarium biology [6,7] as well as gastrodermis composition [8], and cysteine-protease enzymatic activity [9]. However, only a few other detailed studies into female gene expression have added to our knowledge of this sex's genetic complexities [10,11].

Even less is known about the expression of male-associated transcripts, although these molecules clearly exist [12]. As female worms of some *Schistosoma* species (including *S. mansoni*) are incapable of reaching sexual maturity in the absence of sexually mature males [13], identifying the gene products expressed by adult male schistosomes may uncover molecules integral to female sexual maturation. Therefore, identifying gene transcripts associated with sexually mature male and female adult schistosomes represents an important first step in developing strategies for blocking the worm's reproductive life cycle, blocking their potential to induce host morbidity and ultimately preventing successful transmission.

The databases of available schistosome expressed sequence tag (EST) sequence information [14-16] offer an excellent source for genomic investigations and have led to the characterization of numerous individual gene products [17-21]. However, the information that can be obtained by this 'molecule by molecule' approach is limited in respect of subsequent interpretation of gene-expression studies in the modern genomic age. Gene discovery and expression studies can now be carried out on a much larger scale through the use of cDNA microarrays [22]. These tools (as well as genomic DNA arrays) have proved a major contributor in various disciplines ranging from cancer phenotyping [23] to host-pathogen interactions [24] and have slowly begun to have an impact on parasite genomic investigations [25,26]. Using DNA sequences deposited in the schistosome EST databases we have fabricated a schistosome-specific cDNA microarray useful for large-scale gender-specific gene discovery studies.

This first-generation schistosome cDNA microarray consisted of 576 putatively nonredundant elements from three different cDNA libraries (mixed adult (male/female); female-enriched; cercarial libraries) and two different stages of the parasite's life cycle (adult; cercarial stages). Of the 576 arrayed cDNA elements (representative examples of the *S. mansoni* EST database), 36% shared sequence similarity to known molecules in the GenBank databases, whereas the remaining 64% had no significant homology matches. The studies described here show that cDNA microarrays are a reproducible, rapid and highly efficient method for profiling schistosome gender-associated gene expression. Furthermore, the genes identified in this study will contribute to our understanding of schistosome sexual biology and will lead to the identification of associations, processes and pathways previously unappreciated during the development of schistosome parasites.

Results

Male and female gender-specific gene transcripts are reproducibly detected by *S. mansoni* cDNA microarrays

As our report represents the first use of cDNA microarrays to study gene expression in schistosomes, we investigated the reproducible nature of this functional genomics tool. Our first objective, however, was to ensure that similar levels of high-quality total RNA were isolated from each sexually mature parasite population. As differences in the starting quality and/or quantity of input RNA from each sample being compared can have dramatic effects on the measurement of gene expression, we took extreme care in isolating total parasite RNA from 7-week, sexually mature schistosomes. Denaturing gel electrophoresis of equivalent amounts of total male and female RNA showed that each sample pool was harvested intact, was of high quality, and displayed minimal degradation (Figure 1a), as judged by the characteristic staining pattern of schistosome 18S rRNA [27]. Additional evidence that equivalent quantities of male and female total RNA were used for each cDNA microarray hybridization experiment was obtained by examining the mean fluorescent intensities generated from hybridization to the schistosome genomic DNA spots contained on the cDNA microarray in three representative independent experiments (Figure 1b). No significant difference in mean fluorescent intensities was observed between male and female cDNA samples hybridizing to genomic DNA elements, suggesting that equivalent amounts of total RNA were used to prime cDNA synthesis. Therefore, the variation in gene expression between genders observed in this study is due to true biological differences and not to disparity between starting input RNA quantities.

Replication of gene-expression measurements is an important aspect of cDNA microarray hybridization experiments [28]. To address this issue, we carried out five independent cDNA microarray hybridizations using the prepared 7-week sexually mature adult male and female schistosome samples (Figure 2). One representative hybridization experiment clearly illustrates the layout of the schistosome cDNA microarray, with each DNA element being spotted twice within each subarray (Figure 2a). Examination of sub-arrays 3 and 4 from the five independent hybridization experiments showed that gene-expression measurements across multiple microarrays were also reproducible, even when the cDNA targets were labeled with different fluorochromes (Figure 2b). Although these data provided visual confirmation of gene-expression reproducibility, we also carried out two statistical analyses of variation in our studies,

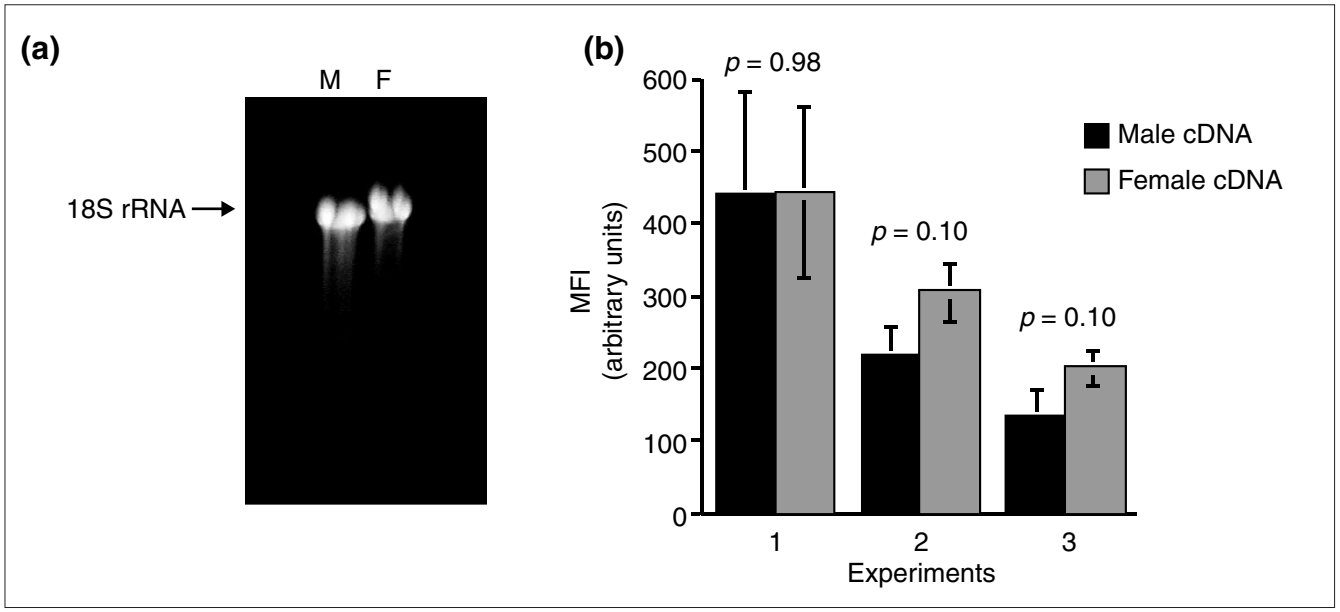

Figure 1
High-quality total RNA was isolated from adult male and female schistosomes and reverse transcribed into cDNA which hybridized with equal affinity to control *S. mansoni* genomic elements. **(a)** Total RNA was isolated from 7-week-old male (M) and female (F) parasites by iterative phase separation and affinity chromatography techniques. A 10 μg sample of each RNA preparation was electrophoresed under denaturing conditions on a 0.1% agarose gel [58]. Intense 18S rRNA bands are visible with minimal apparent sample degradation. **(b)** Analysis of mean fluorescent signal intensities (MFI) detected from all arrayed *S. mansoni* genomic elements after hybridization with cDNA generated from 10 μg male and female RNA samples. Three representative hybridization experiments (out of five) are shown, which show no statistically significant difference in the mean fluorescent intensity (MFI) between male and female cDNA samples, and support the contention that equivalent quantities of both sample materials were used in each experimental hybridization. Standard errors of the MFI are indicated.

intra-array and inter-array replication analyses (Figure 2c and d). Intra-array replication analysis compared the calibrated ratio (male/female gene-expression measurement) of each spotted DNA element to its printed duplicate within a single, representative cDNA microarray hybridization experiment (Figure 2c). As can be seen, the regression line generated from this comparison (natural log calibrated ratio spot 1 versus natural log calibrated ratio spot 2) was almost identical to the line of symmetry. Furthermore, almost every duplicated measurement (median coefficient of variation = 11.1%) was found within the 99% confidence interval (generated from the predicted values and measured away from the slope of the regression line). The coefficient of variation measure generated in this comparison is consistent with those values previously reported for replicate sample analyses [29,30]. On the basis of this comparison, the correlation coefficient of 0.91 represents a good measure of agreement between observations and, therefore, the intra-array calibrated ratios obtained from duplicated spots on our cDNA microarrays were highly reproducible.

Inter-array replication analysis compared the average calibrated ratio (average of duplicate measurements on each microarray) obtained from one representative cDNA microarray hybridization (hybridization 1) to those average calibrated ratios obtained from an independent hybridization (hybridization 2) (Figure 2d). Again, the regression line generated from this comparison (natural log average calibrated ratios from microarray experiment 1 versus natural log average calibrated ratios from microarray experiment 2) was almost identical to the line of symmetry. Furthermore, the correlation coefficient was approaching 1 (r = 0.90), the median coefficient of variation was 14.4%, and the vast majority of all measurements fell within the 99% confidence interval. Therefore, very little variability was observed in the measurement of gene expression between two independent cDNA microarray hybridizations (that is, the inter-array reproducibility was high).

Gene-expression profiling reveals several novel gender-specific associations in sexually mature adult *S. mansoni*

After carrying out five independent cDNA microarray hybridizations and subjecting the accumulated data to stringent quality-control measures (summarized in Materials and methods), we assigned novel, sex-associated transcription to 12 female-associated and 4 male-associated transcripts in the 7-week adult schistosome (Figure 3a). In addition to these specific gender associations, all the positive control DNA elements (chorion, p48, mucin-like protein) printed on the fabricated schistosome cDNA microarray showed expression profiles similar to those of previous observations

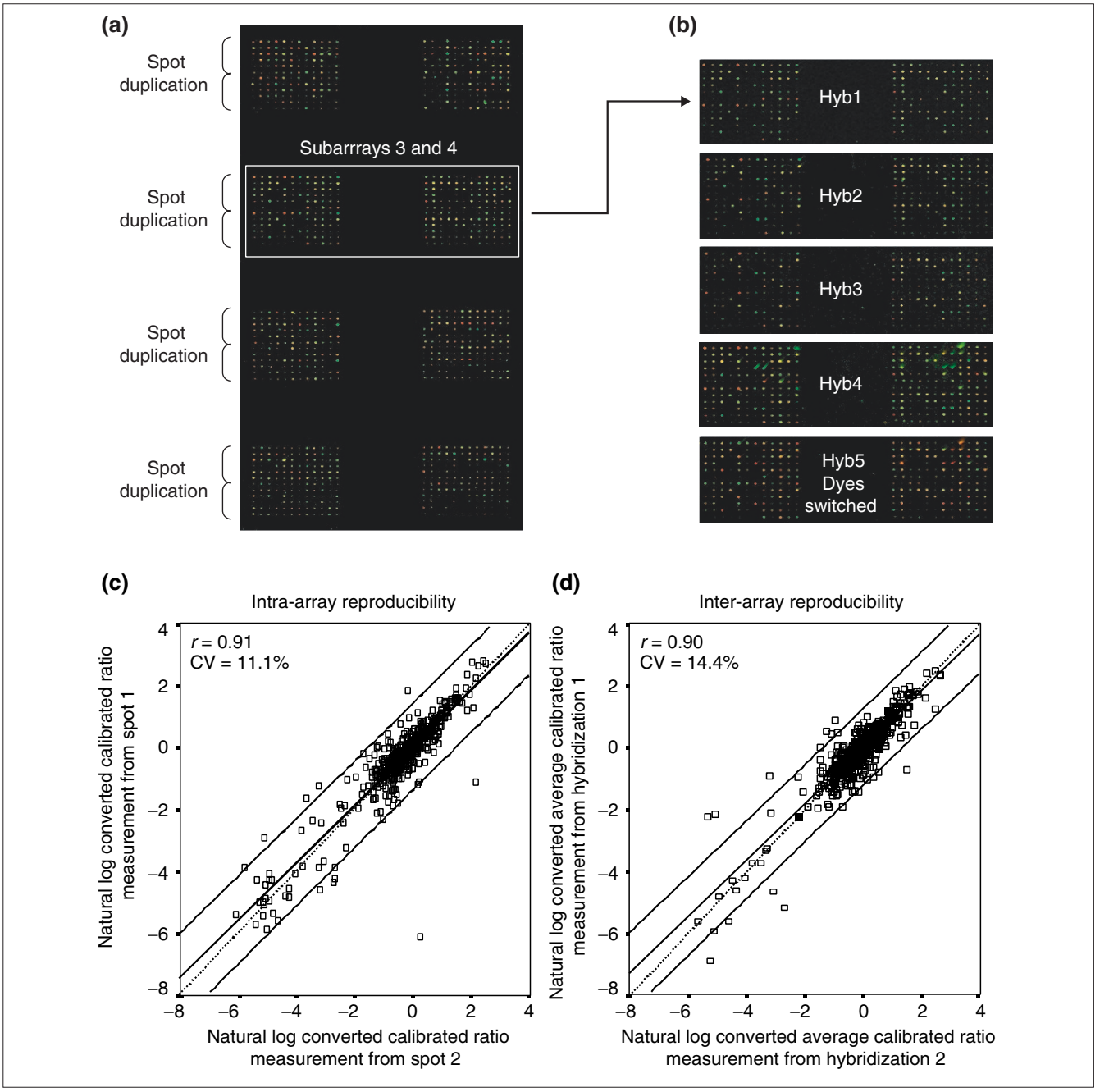

Figure 2

Measurement of adult *S. mansoni* male and female gene expression using cDNA microarrays was highly reproducible. **(a)** *S. mansoni* cDNA microarrays were constructed on glass slides in a format consisting of eight individual subarrays (arranged in four rows by two columns). Each subarray consisted of 144 DNA elements (arranged in 12 rows by 12 columns) with each DNA element being spotted twice (6 rows by 12 columns) within each subarray (indicated by brackets). Images were obtained by hybridization of Cy5-conjugated male cDNA and Cy3-conjugated female cDNA. **(b)** Analysis of subarrays 3 and 4 from five independent hybridization experiments (Hyb 1-Hyb 5) demonstrated consistent patterns of gene expression in adult male and female parasites. In experiments 1-4, male parasite cDNA was labeled with Cy5 (red) and female parasite cDNA was labeled with Cy3 (green), whereas in experiment 5, the fluorescent dyes were switched. **(c)** Statistical analysis of intra-array variation (comparing the natural log converted calibrated ratio from one DNA element to the natural log converted calibrated ratio of its corresponding duplicate DNA element for one representative experiment) demonstrated high reproducibility and tight associations between duplicates. The median coefficient of variation (CV = 11.1%) and the correlation coefficient (r = 0.91) indicated a high degree of agreement. **(d)** Statistical analysis of inter-array variation (comparing the mean natural log converted calibrated ratio of each DNA element in one hybridization experiment to the mean natural log converted calibrated ratio of the same DNA element in a second hybridization experiment) indicated a high degree of association between measurements and high reproducibility. The median coefficient of variation (CV = 14.4%) and the correlation coefficient (r = 0.90) supported this contention. Dashed lines in (c) and (d) represent the line of symmetry. The remaining solid lines in both (c) and (d) represent the regression line and the predicted 99% confidence intervals of the supplied data.

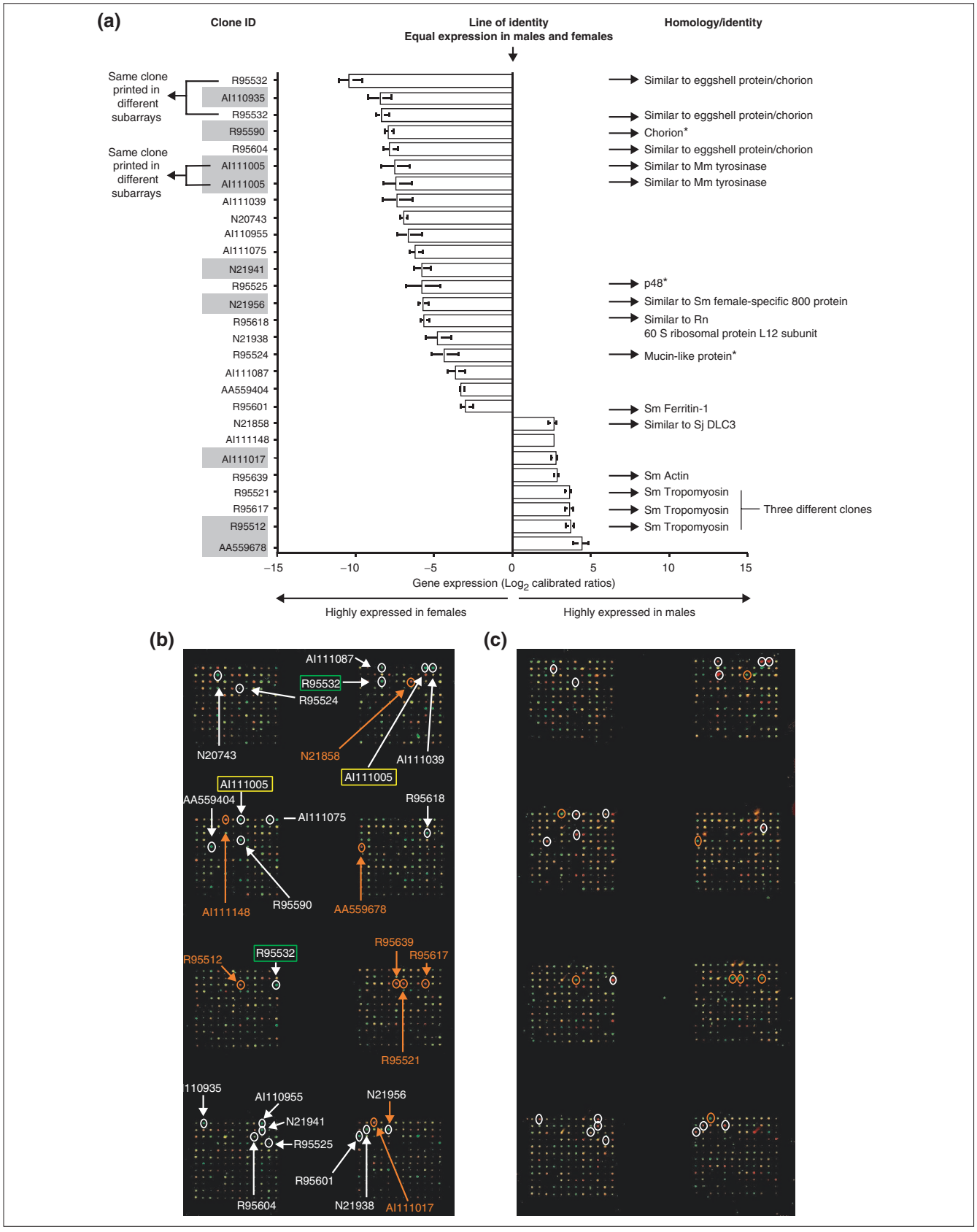

Figure 3 *(see legend on the next page)*

[3,5,31]. This can also be extended to cytochrome oxidase subunit I, where no difference in gene expression was observed between sexes (data not shown) as previously shown [32]. Every subarray contained at least one of the differentially expressed gene products, showing that no localized hybridization bias occurred during our experiments (Figure 3b). In addition, as all 23 differentially expressed gene products (16 novel, sex-associated transcripts in this study and control elements) had similar expression profiles when the dyes used to label the starting cDNA pools were switched (Figure 3c), fluorescent dye differences did not contribute to the observations. Note that multiple elements corresponding to tropomyosin and eggshell-protein genes were arrayed and detected in this study. This resulted from the fact that ESTs with these homologies formed multiple contigs under the assembly parameters used for EST clustering.

Independent confirmation of gender associations predicted by the schistosome cDNA microarrays by reverse transcription PCR

To verify the cDNA microarray predictions for a subset of gene products (gray-shaded clone IDs in Figure 3a), we examined gene expression in the sexually mature male and female schistosome RNA samples by RT-PCR analysis (Figure 4). All PCR reactions (except for alpha-tubulin and clone AA559631) were carried out for 35 cycles (well within the plateau of cycling parameters) to determine whether any product could be obtained in the sex where the cDNA microarray hybridizations detected minimal or no transcript. In every case, differentially transcribed gene products examined by RT-PCR analysis displayed the gender-specific associations predicted by the cDNA microarray hybridizations. Even subjecting the parasite cDNA to 35 rounds of amplification failed to yield significant product in the male sample for clones AI111005, AI110935, N21956, and N21941 or in the female sample for clones AI111017, AA59678 and R95512. This suggested that these clones (and probably all the others identified by the cDNA microarray hybridizations) were highly expressed in a sex-dependent manner. Control RT-PCR reactions also showed predicted patterns of gene expression (chorion and alpha-tubulin). Clone AA559631, which was shown to be expressed at similar levels in 7-week male and female schistosomes by cDNA microarray hybridization experiments, was also found to be expressed at similar levels by RT-PCR analysis.

Discussion

The modern tools of this post-genomic age are slowly developing alongside the well-established parasite sequencing efforts and will undoubtedly change the path and scope of all future, genome-wide, functional investigations. We describe the first use of one specific post-genomic tool (cDNA microarrays) in the study of schistosome biology and illustrate several key points. First, our fabricated schistosome cDNA microarrays provided an efficient means to examine gender-associated gene expression *en masse*. Second, the results obtained with these cDNA microarrays were highly reproducible and capable of independent confirmation, and finally, microarrays provided information previously unappreciated in schistosome sexual biology.

Schistosomes are complex parasitic metazoans that have coevolved with humans since before historical records were kept. They have excelled at parasitism by adopting developmental strategies virtually unique amongst the Platyhelminthes. The separation of sexes is one of these traits and so is perfectly suited for the initiation of schistosome cDNA microarray investigations. In this study, we developed a schistosome cDNA microarray from a small subset of EST elements deposited in one of the current schistosome EST databases [14]. The resultant 576-element schistosome cDNA microarray contained only about 4% of the predicted open reading frames (ORFs) encoded by the schistosome genome [33], but was sufficient to identify 23 differentially expressed genes between the sexes. Some of these genes were positive control elements intentionally printed on the cDNA microarray (chorion, p48, mucin-like protein), but 16 genes demonstrated novel sex-specific associations in the adult schistosome (12 female- and 4 male-associated

Figure 3 *(see figure on the previous page)*
Identification of 28 differentially expressed gender-associated gene products in the adult schistosome by cDNA microarray analysis. Adult male and female cDNA samples were used to probe five schistosome cDNA microarrays. Those 23 arrayed cDNA clones that passed the iterative filtering process (described in Materials and methods) were described as being differentially expressed in a gender-dependent manner. Among the 23 differentially expressed cDNAs, 12 novel female associations and 4 new male associations were identified. **(a)** The 23 cDNAs demonstrating gender-associated gene-expression profiles were plotted away from the line of identity (equal expression between sexes) on a $\log_2$-based scale. Clone identifiers (GenBank accession numbers) and common gene names (as annotated by database homologies/identities) define each differentially transcribed cDNA. cDNA clones without common gene names have no known database homologies. Those clones chosen for independent verification by RT-PCR analysis are indicated by gray shading (AI110935, R95590, N21941, N21956, AI111017, AA559678, and R95512). Common gene names indicated with an asterisk (chorion, p48 and mucin-like protein) define the positive control female-associated clones printed on the cDNA microarrays. Mm, *Mus musculus*; Rn, *Rattus norvegicus*; Sm, *Schistosoma mansoni*; Sj, *Schistosoma japonicum*. These 23 differentially expressed cDNAs hybridized to DNA elements located in every subarray and maintained this characteristic when the fluorochromes were interchanged. **(b)** Male cDNA labeled with Cy5 compared with Cy3-labeled female cDNA. R95332 (green box) and AI111005 (yellow box) are cDNA clones that were deposited in two different subarrays as an additional quality-control measure.**(c)** Male cDNA labeled with Cy3 compared with Cy5-labeled female cDNA. cDNA elements indicated by orange circles and arrows are associated with differential expression in the adult male schistosome. cDNA elements indicated by white circles and arrows are associated with differential expression in the adult female.

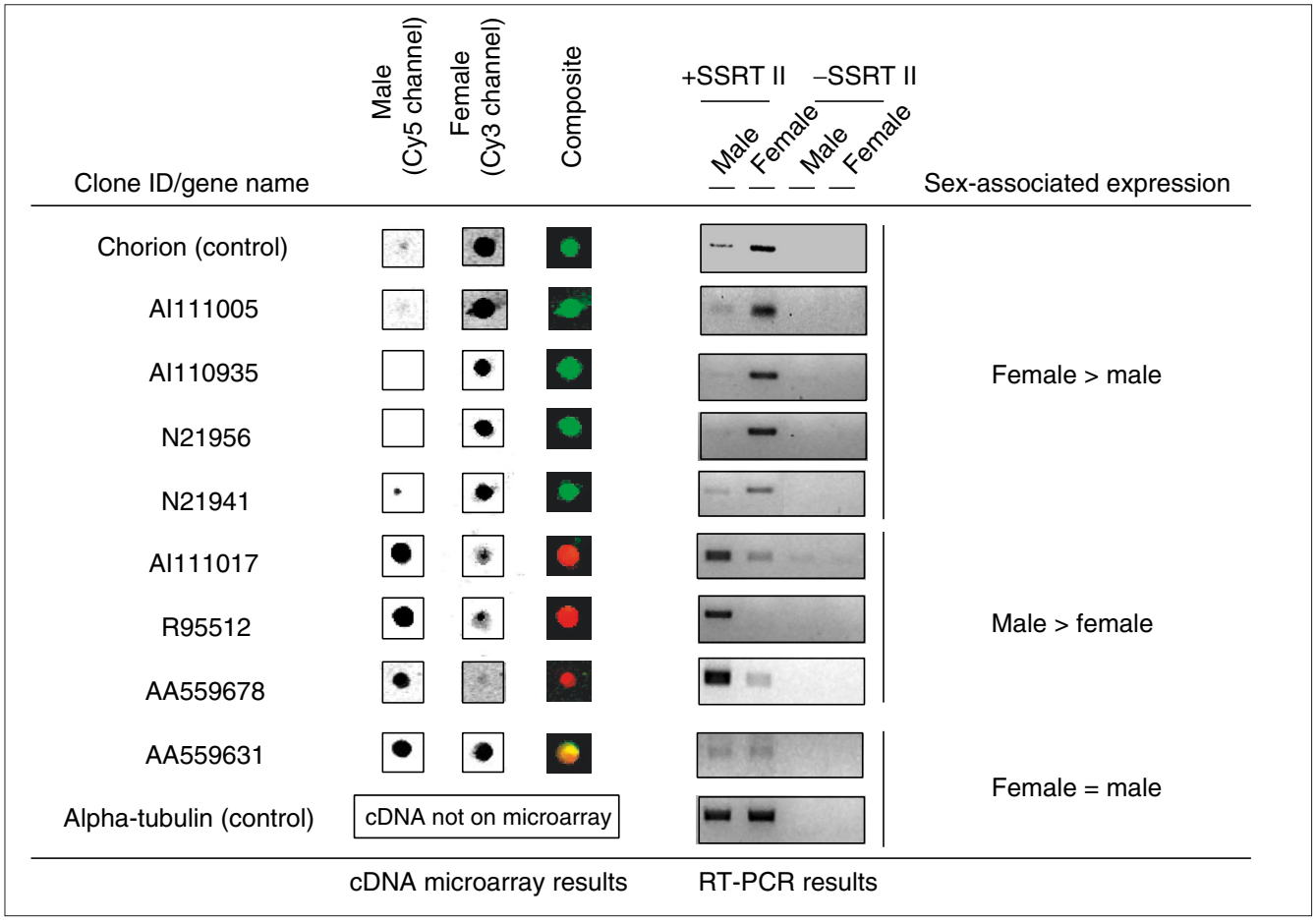

Figure 4
RT-PCR analysis confirmed gene expression results obtained by cDNA microarray hybridizations. A 1 μg sample of total RNA was used to prime cDNA synthesis for both adult male and female samples (in the presence or absence of reverse transcriptase; +SSRT or -SSRT). PCR primers (Table 1) were designed for cDNA clone IDs AI111005, AI110935, N21956, N21941, AI111017, R95512, AA59678, AA559631 as well as chorion and alpha-tubulin. PCR amplification was performed for 35 cycles (except for alpha-tubulin and clone AA559631: 23 cycles). Spot morphology and color intensity for each RT-PCR-verified clone, obtained from one representative cDNA microarray hybridization, are included for comparison (except for alpha-tubulin which is not present on the cDNA microarray). The sex-associated expression of each transcript, as classified by both techniques, is also indicated.

transcripts) (Figure 3a). In comparison to all previous gender-associated genomic investigations undertaken in *Schistosoma*, this one study, which exploited a very limited subset of the available EST clone archive, was able to double the number of genes identified as possessing sex-specific transcription.

Of the 18 cDNA clones (Figure 3a) that showed female-associated gene expression in our study, most of the annotated DNAs (those that had a positive match in the GenBank databases) that passed the applied filters were (unsurprisingly) either selected control elements or components involved in schistosome egg production (Figure 3a). Mature females devote a tremendous amount of metabolic effort to producing eggs and thus a large proportion of mRNA generated by this sex is for this purpose. It has been estimated that the eggshell-protein genes of *S. mansoni*, encoding p14 and p48, contribute 5-10% and 0.3-0.5%, respectively, of the total mRNA pool in a mature female [3]. These genes only represent two of the many that contribute to the production, assembly and laying of the approximately 100-300 eggs per day often observed in *S. mansoni* infections. Thus, it is not surprising that molecules associated with this fundamental biological process would be a large component of the mature female RNA population and subsequently be identified in our cDNA microarray study (especially when compared to mature male parasite RNA pools). Ferritin-1 (clone R95601), a clone related to *S. mansoni* female-specific 800 protein (clone N21956), and a tyrosinase ortholog (clone AI111005) are three such molecules. Ferritins are involved in hemoglobin metabolism where they serve as anchoring proteins for iron [34]. Schussler and colleagues demonstrated the expression of ferritin-1 predominantly in the female schistosome (possibly in yolk platelets in the vitellarium) [35] and

our results support this finding. The exact function of ferritin-1 in vitellarium biology remains unclear. Clone N21956 has a moderate degree (BLAST *E* value 6 x 10^{-4}) of sequence similarity to a female-specific cDNA previously characterized [7]. Reis *et al*. reported that this cDNA (female-specific 800 protein) contained two large ORFs and the corresponding protein product(s) localized to vitelline cells [7]. Clone N21956 may represent a novel *S. mansoni* female-specific 800 protein family member (divergent ORF), but the function of any of these cDNA products in vitelline cell biology is still unknown. The finding here that a tyrosinase ortholog (clone AI111005) is differentially transcribed in the adult female is intriguing. Vitelline cells avidly take up tyrosine, where it is stored in vitelline droplets and endoplasmic reticulum [36]. In addition, the tyrosine residues on eggshell precursor proteins have been hypothesized to be substrates for enzymes and enzyme activities during the tanning process of eggshell formation (reviewed in [37]). One of these enzymatic activities has been characterized by Eshete and LoVerde in extracts of *S. mansoni* and is related to those described for tyrosinases [38]. We now show here that adult female parasites transcribe the mRNA for a tyrosinase ortholog at a much higher rate than adult males, and so this enzyme may be responsible for the phenol oxidase/tyrosinase activities described previously in *S. mansoni* [38-40]. It is also possible that this enzyme ortholog (or family member) participates in additional schistosome-related metabolic reactions (alkaloid biosynthesis I, riboflavin metabolism or melanin synthesis) described for other organisms [41]. This molecule is being characterized further.

In addition to these cDNAs, several other clones had novel female-specific associations. One clone had sequence similarity to a 60S ribosomal protein L12 subunit (R95618) and clearly has a role in protein production, but why it is preferentially transcribed in adult female schistosomes is, as yet, unclear. The remaining nine clones possessed no significant database match. The functional roles of these nine novel female-associated cDNA clones in schistosome biology are currently under investigation. They could be newly identified components of the female's egg-producing machinery or be involved in other sex-specific biological processes. These molecules represent a new collection of female-associated transcripts that may be useful in understanding the processes that lead to host immunopathology.

Three annotated proteins whose genes show male-associated expression are tropomyosin (clones R95521, R95617, and R95512), actin (clone R95639) and a dynein light-chain ortholog (Sj DLC3 - clone N21858) (Figure 3a). Although actin has previously been shown to be differentially expressed in adult males [12], our study provides information on the sex-associated expression of two other tegument-associated molecules, tropomyosin and the dynein light chain [42,43]. Tropomyosin and actin are also located in other schistosome tissues such as parenchyma, muscle and spines [44].

The coordinate expression of tropomyosin and actin in the adult male schistosome is probably due to the fact that these two molecules interact intimately. Actin polymers (microfilaments) provide mechanical stability for the cytoskeleton of eukaryotes and serve as tracks for motor proteins such as tropomyosin [45]. As male schistosomes are significantly larger than females, contain a greater volume of tegument and muscle, and are more physically active, our expression results may reflect this bias. It is also possible, however, that the preferential transcription of tropomyosin, actin and dynein light chains in the male has an important developmental role for copulating worm pairs. Evidence for this comes from studies where unpaired female parasites are developmentally and sexually stunted in comparison to paired female parasites [46-48]. In the paired state, male schistosomes are thought to provide mechanical (among other types of) support to the maturing female [49]. This mechanical support enables the female to acquire proper nutritional supplements from the hepatic portal system, which allows the development of full sexual maturity. As a function of tropomyosin (binding to actin) is in contractile muscle regulation and a hypothesized function of dynein light chains is in tegument biology [43], our results suggest that these molecules, preferentially transcribed in the male, may participate in this mechanical and support maintenance. With the male firmly securing the female in its gynecophoral canal, she can devote more of her metabolic and transcriptional machinery to egg production (as our results indicate). Several other schistosome tegument-associated transcripts (EST clones corresponding to Sm DLC, Sm 20.8, Sm 22.6, Sm 21.7 and Sm 22) were also differentially expressed in the adult male in our study, further supporting this hypothesis. These molecules narrowly missed inclusion in our final dataset because they failed in one of the applied filtering criteria. Taken together, these data show that tegument-associated gene products are transcribed in a gender-related manner, which may reflect differences in size and/or physical activity, or a differential functional requirement for each of these transcripts. The identity and functional role of the three unknown male-associated transcripts identified in this study await further investigation.

A few other critical points were uncovered during this investigation. First, the fabricated cDNA microarrays performed with a high degree of reproducibility (and low variation) when analyzed statistically (Figure 2). Additional proof of this was obtained when the expression ratios of the same cDNA clone (AI111005 and R95532), printed in different subarrays (Figure 3b), were compared and found to be highly similar (Figure 3a). Also, cDNA clones of different sequence and size but with the same database homology (for example, R95532, R95590, and R95604 (eggshell protein/chorion) and R95521, R95617, and R95512 (tropomyosin)), printed in different subarrays, also yielded very similar expression ratios (Figue 3a). Thus, the sex-specific transcriptional biases in this report were of high confidence, as verified by RT-PCR

(Figure 4). Second, the importance of fabricating cDNA microarrays from EST sequences derived from different developmental stages and libraries was evident. Orphan ESTs AA559404 and AA559678 were both derived from cercarial cDNA libraries, yet showed adult female- and male-associated gene expression, respectively (Figure 3a). Whether both male and female cercariae express these two molecules, and then alter their expression as the parasites mature, or whether a gender-specific association is already present at this immature developmental stage remains to be determined. This point could be addressed by examination of gene transcription in sexed cercariae [50]. Nevertheless, the gender-specific association of these two transcripts in adults would not have been identified if cercarial elements had not been included in our schistosome cDNA microarrays. Third, it was observed that two male-associated transcripts hybridized to cDNA clones that originated from an adult female-enriched cDNA library (clones AI111148 and AI111017, Figure 3a). This showed that associations not often looked for (differentially expressed male transcripts hybridizing to female-enriched cDNA clones) could be identified using cDNA microarrays. Together, these points show that cDNA microarrays constructed in a highly reproducible manner from diverse ESTs and different cDNA libraries are extremely useful for gene identification/expression purposes. Attention to these details will maximize the information obtained from future experimental hybridizations.

Conclusions

This report describes the first use of cDNA microarrays in functional analyses of schistosome biology. We have shown that these tools were of high quality, capable of reproducibly detecting gene-expression differences between genders, and useful for identifying new biological associations. The 16 novel, gender-associated transcripts identified here serve as starting points for functional investigations. Continued refinement and expansion of schistosome cDNA microarrays (fabrication of a 4,000-element array is underway) and development of new post-genomic techniques will provide the means to initiate numerous other investigations aimed at unraveling the biological complexities of this parasitic helminth and the pathology it induces.

Materials and methods

Parasites

A Puerto Rican strain of *S. mansoni* was used in this study. Adult male and female schistosomes were perfused from percutaneously infected TO outbred mice (Harlan) challenged 7 weeks earlier with 125 cercaria [51]. The parasite was passaged through *Biomphalaria glabrata* intermediate snail hosts.

Schistosome cDNA microarray fabrication

Parasite ESTs deposited on cDNA microarrays were selected from a putatively nonredundant sequence set contained in the March 2000 cluster-analysis database [14]. This particular arrayed clone set was selected for two main reasons: we could successfully grow this subset of bacterial clones at the time of array fabrication; and the set contained similar representative percentages of known and unknown annotated sequences deposited in the schistosome EST database. Clusters were assembled using an exhaustive search-and-compare algorithm in Sequencher v3.1.1 (Gene Codes Corp., Ann Arbor MI), set to assemble contigs from sequences displaying $\geq$ 90% homology over $\geq$ 60 bases, with a maximum of two allowable consecutive mismatched bases. EST products were generated via standard PCR techniques from cDNA clones archived by the Schistosome Genome Network and maintained at The Natural History Museum (London). The source libraries were constructed in LambdaZap vectors (Stratagene, La Jolla, CA) and inserts were amplified from phage suspension or from lysed bacterial colonies containing excised pBluescript plasmid using M13 forward and reverse primer pairs. EST PCR products were 1,000 bp on average as assessed by DNA gel electrophoresis (over 98% of the PCR reactions generated a single product). PCR products were purified, using Multiscreen-FB 96-well filtering units (Millipore, Bedford, MA), away from primers, salt and other potential contaminants. Purified EST products were diluted (1:4) in 4x spotting buffer (600 mM sodium phosphate, 0.04% sodium dodecyl sulfate) and printed in ordered arrays (8 subarrays composed of 12 columns x 12 rows) on gamma-aminopropyl silane-coated glass slides (Corning) using a Microgrid II robotic arrayer (BioRobotics Ltd, Cambridge, UK). Slides were processed after printing by baking for 2 h at 80°C and UV cross-linking at 450 mJ to permanently fix DNA on the glass slides, followed by boiling for 2 min to denature fixed DNA. The 1,152 elements (576 elements printed in duplicate) arrayed on each glass slide were composed of the PCR-amplified parasite ESTs, positive controls (*S. mansoni* genomic DNA and known female-specific cDNAs: chorion [2], mucin-like protein [5] and p48 [3]), and negative controls (yeast tRNA, pBluescript DNA, lambda DNA, and spotting buffer only).

Total RNA isolation, cDNA synthesis and hybridization

After perfusion of experimentally infected mice (7 weeks post-infection), male and female schistosomes were separated manually. Approximately equal numbers of adult male or female worms were pooled and used as the starting material for total RNA isolation. A procedure to isolate total RNA from the parasite was adapted from previous studies [52] and involved both phase extraction (TRIZOL reagent, Invitrogen, Life Technologies, Paisley, UK) and column chromatography (Qiagen RNeasy maxi affinity columns, Qiagen, Crawley, UK). Denaturing gel electrophoresis assessed RNA quality from each sample. Amino-allyl dUTP (Sigma-Aldrich) labeled cDNA targets (nomenclature recommendation described in [53]) were generated from a 10 μg male RNA sample and a 10 μg female RNA sample using Superscript

Reverse Transcriptase II (Invitrogen). Cy dye (Amersham Pharmacia Biotech, Little Chalfont, UK) conjugation to each cDNA target was carried out according to standard protocols [54]. Fluorescent cDNA targets were hybridized to the cDNA microarray probes in a modified hybridization buffer (40% deionized formamide, 5x Denhardt's reagent, 5x SSC, 1 mM sodium pyrophosphate, 50 mM Tris pH 7.4, 0.1% SDS, 0.25 μg/μl mouse Cot1 DNA (Life Technologies), 0.44 μg/μl poly(dA) (Amersham Pharmacia Biotech), and 0.22 μg/μl yeast tRNA (Sigma-Aldrich) at 48°C using hybridization chambers (TeleChem, Sunnyvale, CA) for a minimum of 16 h. Post-hybridization processing involved three successive 5-min washes in 0.5x SSC/0.1% SDS, 0.5x SSC/0.01% SDS and 0.06x SSC. Slides were spun dried (500*g* for 5 min) to remove all washing buffer, stored at room temperature in the dark, and scanned at 10 μm resolution using a Packard ScanArray Express microarray scanner (Packard BioScience, Pangbourne, UK).

RT-PCR analysis

For RT-PCR analysis, 1 μg male or female RNA was used for cDNA synthesis to confirm cDNA microarray results. RT-PCR was carried out as described [55] to confirm the cDNA microarray gender-specific gene-expression results for clones AI110935, N21956, N21941, AI11017, R95512, AA559678, AI111005 and AA559631. Additional cDNAs examined by this method included two well characterized controls - alpha-tubulin (housekeeping transcript [56]) and chorion (female-associated transcript [31]). The primers for all transcripts are included in Table 1. Thirty-five cycles of PCR were used to amplify all transcripts except for alpha-tubulin and clone AA559631 (to ensure that amplification was within the linear range of PCR in both male and female cDNA, 23 cycles were used for these clones). All amplicons were electrophoresed on a 1% agarose gel and stained with ethidium bromide. Images were captured by a digital camera and analyzed by gel-documentation software (Kodak 1D 2.0 electrophoresis documentation and analysis system 120, Eastman Kodak, New Haven, CT). Semi-quantitative analysis of the differentially expressed transcripts was not carried out owing to the cycling conditions used in this study (35 cycles). RT-PCR was used, therefore, to verify the cDNA microarray results and not used to obtain exact or relative amounts of gene transcript present in each sex.

Statistical analysis

Microarray Suite (Scanalytics, Fairfax, VA) software was used to process signal-intensity information from all 16-bit TIFF files generated in the cDNA microarray experiments. Hybridization signals representing gene expression (Cy5/Cy3 ratios) from cDNA microarray experiments were processed by several filtering criteria after global mode normalization (each experiment normalized to itself) and local background subtraction. Filters one and two evaluated target/probe spot morphology and were used to eliminate any questionable data (weak signals, poorly defined spots, and background noise). The first criterion required each spot to be greater in size than the lowest tenth percentile of all spots within an experiment (removal of small or irregularly shaped spots). The second criterion required one fluor (either Cy5 or Cy3) from each spot to be greater in intensity than one standard deviation above the mean intensity from all negative control array elements (removal of spots that were not significantly above background intensities).

Intra-array and inter-array reproducibility were next examined as filtering criteria. As each EST was printed twice on every array (intra-array reproducibility), only those ESTs that showed highly reproducible gene-expression ratios (within the 99% confidence interval of each experimental hybridization as predicted by the derived regression equation) were included. Each of the duplicates subsequently had to show expression levels significantly greater than the median gene-expression ratio for each cDNA microarray

Table 1

PCR primer pairs used for RT-PCR confirmatory assays on *S. mansoni* gender-associated transcripts identified by cDNA microarray analysis

Accession number/ gene name	PCR primer pairs	PCR product size
AI111005	5′ - **CTT CCG GAT GTA GAG GAT TTG** - 3′	301 bp
	5′ - **CGG GAT ATG CGT TTG GAC TAG** - 3′	
AI110935	5′ - **CGT TAA CAG TGC ACA GAT GCC** - 3′	182 bp
	5′ - **GGA ATC CGA CTA CAT CAT TTC** - 3′	
N21956	5′ - **CAG AAG AAC GAA ACT AAT CAC** - 3′	245 bp
	5′ - **TTC TGA TGA TAC GAT ACC GTG** - 3′	
N21941	5′ - **ATC ATC ATT ATG GTA CAT CAT** - 3′	236 bp
	5′ - **TGA ATT TCA ACG TAT ATT CTC** - 3′	
AI111017	5′ - **AAG TGT CCC TGA CAC AAC AAC** - 3′	137 bp
	5′ - **GCC TGT TTC ATT TTC ACC TTC** - 3′	
R95512	5′ - **TCG TTT ATT AGG AGG AAG ATC** - 3′	271 bp
	5′ - **GCT TTC TGC AGC TTC GAG ACG** - 3′	
AA559631	5′ - **TGT TAC ATA TAG TGG TGA GGG** - 3′	210 bp
	5′ - **CGA CAC TTA CTG CCT GGA TGG** - 3′	
AA559678	5′ - **GGT ATA TTT CGA GAA TGT GAG** - 3′	338 bp
	5′ - **CGC TTA GGT TAA TCA TGA ATG** - 3′	
Alpha tubulin	5′ - **GGC GGT GGT ACT GGT TCT GGG** - 3′	291 bp
	5′ - **CAT TTA GCG CAC CAT CGA AGC** - 3′	
Chorion	5′ - **GAA ACA GTC ACT CAC ACT CG** - 3′	461 bp
	5′ - **ATG GCT GGG TTT GTA AGT GC** - 3′	

RT-PCR reaction conditions are described in detail in the Materials and methods section. Thirty-five cycles were used for all primer pairs except for alpha-tubulin and clone AA559631 (23 cycles).

hybridization (outside the 99% confidence interval derived from all gene-expression ratios).

Finally, gene-expression measurements for each cDNA (including duplicates) had to have passed the previous filters in four out of five independent hybridizations to be included in the final dataset. Statistical analysis of gene-expression ratios between two of these five independent hybridizations showed low variation (that is, inter-array reproducibility). The 28 calibrated ratio values surviving these stringent filters were $\log_2$ transformed and stored in a table (rows, individual cDNA clones; columns, gene-expression ratios for each independent hybridization). All clones showing differential gene expression were resequenced to confirm their annotated identity, as human error can sometimes lead to well-to-well cross-contamination of bacterially derived clone sets [57].

Additional data files

Additional files containing the primary data for the individual microarray experiments are available with the online version of this article.

Acknowledgements

We thank Francis Jones, Maureen Laidlaw, Karen Plant, Mike Anderson, Viv Tuffney, and Susan Arnold for excellent technical assistance in maintaining the schistosome life cycle and separation of adult parasites. We also thank Rhian Hayward, Tom Wynn, and Thomas McCarty for critically reviewing this manuscript. Finally, we thank David Latto for many helpful discussions and in the fabrication of the cDNA microarrays. K.F.H. is supported by a 2-year, long-term research fellowship awarded by the European Molecular Biology Organization. This work was also supported by means of Wellcome Trust and Medical Research Council programme grants to the Cambridge laboratory and a UNDP/WORLD BANK/WHO Special Programme for Research and Training in Tropical Diseases (T.D.R.) grant (ID no 980502) awarded to D.A.J. at The Natural History Museum.

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