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Screening the *Schistosoma mansoni* transcriptome for genes differentially expressed in the schistosomulum stage in search for vaccine candidates

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Abstract Schistosomiasis affects more than 200 million people worldwide; another 600 million are at risk of infection. The schistosomulum stage is believed to be the target of protective immunity in the attenuated cercaria vaccine model. In an attempt to identify genes up-regulated in the schistosomulum stage in relation to cercaria, we explored the *Schistosoma mansoni* transcriptome by looking at the relative frequency of reads in EST libraries from both stages. The 400 genes potentially up-regulated in schistosomula were analyzed as to their Gene Ontology categorization, and we have focused on those encoding-predicted proteins with no similarity to proteins of other organisms, assuming they could be parasite-specific proteins important for survival in the host. Up-regulation in schistosomulum relative to cercaria was validated with real-time reverse transcription polymerase chain reaction (RT-PCR) for five out of nine selected genes (56%). We tested their protective potential in mice through immunization with DNA vaccines followed by a parasite challenge. Worm burden reductions of 16–17% were observed for one of them, indicating its protective potential. Our results demonstrate the value and caveats of using stage-associated frequency of ESTs as an indication of differential expression coupled to DNA vaccine screening in the identification of novel proteins to be further investigated as potential vaccine candidates.

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Introduction

Schistosomiasis is an important parasitic disease, caused by trematode worms of the genus *Schistosoma*, affecting more than 200 million people worldwide, with a further 650 million individuals living at risk of infection (WHO 2002). *Schistosoma mansoni* is one of three schistosome species that cause the vast majority of human infections. This species is transmitted by *Biomphalaria* snails and found in Africa, the Arabian peninsula and South America (Gryseels et al. 2006). Treatment of the disease is based on the anti-helminthic drug, praziquantel, but chemotherapy cannot prevent re-infection, and there is evidence supporting the development of drug-resistant parasites (Fallon and Doenhoff 1994; Ismail et al. 1996), limiting the success of strategies based on chemotherapy alone and emphasizing the need for a complementary approach. Vaccination combined with drug treatment is considered to be the most cost-effective strategy against schistosomiasis (Bergquist 2002; Chitsulo et al. 2004).

Identification of effective vaccine antigens and drug targets has been hindered by the complexity of the parasite

life cycle and an incomplete comprehension of its immune evasion strategies (Williams et al. 2007; Wilson and Coulson 2006). Proof of principle for vaccine development is indicated by the existence of individuals resistant to infection by schistosomes (Butterworth et al. 1985) and by the high levels of protection induced by immunization with the radiation-attenuated cercariae in rodents and primates (Coulson 1997). The lung schistosomulum seems to be the main target of immune mechanisms, in response to the radiation-attenuated vaccine in the mouse model (Hewitson et al. 2005; Wilson and Coulson 1989). It is important to keep in mind that proteins which are exclusive to the adult worm may not be necessary to induce immunity in the mouse model, since radiation-attenuated cercariae do not develop into the adult stage. In addition, it also seems that antigens specific to the cercaria may not be important, since attenuated 7-day schistosomula administered intradermally, are capable of inducing high levels of protection (Coulson and Mountford 1989; Harrop et al. 2000). Therefore, genes that are differentially expressed in the transition from cercaria to schistosomulum intra-host lung stage may be relevant for the parasite's survival in the host and therefore may be potential vaccine candidates.

Microarray and serial analysis of gene expression (SAGE) methods have been used for differential gene expression studies, however most microarray studies have not looked at S. mansoni schistosomulum expression (DeMarco et al. 2006; Fitzpatrick and Hoffmann 2006; Fitzpatrick et al. 2004, 2005; Gobert et al. 2006; Moertel et al. 2006; Vermeire et al. 2004). Dillon et al. (2006) used a microarray based on a set of ~6,000 EST probes representing ~3,000 unique gene fragments from schistosomulum for analyzing differential expression profile among stages (Dillon et al. 2006). More recently a study using an oligonucleotide designed microarray (~7,132 probes representing ~4,371 contigs) was performed to profile gene expression throughout seven developmental stages of Schistosoma japonicum (Gobert et al. 2009). SAGE analyses have only been applied to identify genes differentially expressed in sub-adult liver stage, adult worms, miracidia, and sporocysts (Ojopi et al. 2007; Williams et al. 2007). In another approach, bioinformatics and microarrays have been applied in rapid genome mining coupled to DNA immunization protocols to evaluate antigenicity of selected genes, effectively identifying potential vaccine candidates in Trypanosoma cruzi and Leishmania (Almeida et al. 2002; Bhatia et al. 2004; Stober et al. 2006). In the search for novel vaccine candidates and drug targets, potential stage-specific or stage-regulated genes have been characterized based on the frequency of reads in the transcriptome for different species (Isokpehi and Hide 2003; Li et al. 2003, 2004; Lizotte-Waniewski et al. 2000; Mitreva et al. 2005; Williams et al. 2000). Schistosomes transcriptome have been studied previously, however with limited numbers of sequenced ESTs (8,000 to 16,000 ESTs)

(Franco et al. 1997; Fung et al. 2002; Peng et al. 2003; Santos et al. 1999). In October of 2003, two large-scale transcriptome databases of S. mansoni (163,000 ESTs) and S. japonicum (48,000 ESTs) were independently published by our group (Verjovski-Almeida et al. 2003) and by others (Hu et al. 2003), providing the first large repository of schistosome genes, bringing insights into several aspects of schistosome biology in parasite survival and host-parasite interactions. More recently, the whole genome sequence of S. mansoni and S. japonicum were reported by (Berriman et al. 2009) and (Liu et al. 2009), respectively. These data opened the avenue for molecular characterization of the parasite life cycle and the search for vaccine antigens and drug targets. The challenge is to identify a limited number of antigens that prove to be worth investigating more thoroughly from an enormous amount of data.

We have previously used the frequency of normalized reads in EST libraries to identify genes that appeared to be differentially expressed along six S. mansoni stages (Verjovski-Almeida et al. 2003). The present study used a similar bioinformatics screening approach, now focused on the comparison between schistosomulum and cercaria, aiming to identify potential vaccine candidates in the S. mansoni transcriptome. This approach comprised five steps: (1) analysis of the published S. mansoni transcriptome database (Verjovski-Almeida et al. 2003) to identify genes potentially up-regulated in the schistosomulum stage in relation to cercaria; (2) Gene Ontology (GO) functional categorization by biological process; (3) selection of genes encoding-predicted proteins with no similarity to proteins of other organisms, suggesting they could be involved in parasite adaptation, immune evasion or survival in the mammalian host, (4) validation of up-regulation predictions through real-time reverse transcription polymerase chain reaction (RT-PCR) assays; (5) the use of DNA-vaccine technology to provide a first estimate of the protective potential of the selected vaccine candidates.

Materials and methods

Parasite material

The life cycle of S. mansoni (BH and PR isolates) was maintained in the laboratory by routine passage through mice and intermediate snail host Biomphalaria glabrata. Adult worms (both sexes) were perfused from BALB/c mice 7–10 weeks after infection with cercariae and washed thoroughly in phosphate-buffered saline (PBS). S. mansoni eggs were extracted from S. mansoni infected mouse livers and miracidia were hatched from S. mansoni eggs, both as previously described (Dalton et al. 1997). Cercariae were harvested from infected B. glabrata snails exposed to light.

Following in vitro transformation of cercariae, schistosomula were cultured for 7 days prior to recovery (Basch 1981). All materials were preserved in RNALater buffer (Ambion) for subsequent RNA extraction.

Selection and characterization of putative differentially expressed genes

Most of the cDNA libraries in the *S. mansoni* transcriptome project (Verjovski-Almeida et al. 2003) were obtained with the ORESTES method. In order to generate what is called a cDNA mini-library, the method uses a single arbitrarily selected non-degenerate primer for reverse transcription and subsequent low-stringency PCR; this is followed by cloning the amplicons and sequencing of a few hundred ESTs from each mini-library. Dozens of such mini-libraries were generated for six different life-cycle stages and were sequenced in the project (Verjovski-Almeida et al. 2003). While many different arbitrary primers were used, there was a set of six primers which were used for generating both cercariae and schistosomula mini-libraries (Verjovski-Almeida et al. 2003). The EST sequences obtained from these six cercariae and schistosomula mini-libraries were analyzed here. As previously demonstrated, when the same primer is used among different life-cycle stages, one can use the number of reads per stage obtained for a given gene as an indirect inference of the expression level of that gene in that stage (Verjovski-Almeida et al. 2003). Contigs with a minimal number of eight EST reads from the schistosomula stage and a higher frequency of reads from schistosomula than from cercariae were identified by automated processing and were subsequently manually screened for: (1) encoding an unknown protein; (2) having additional reads from an adult worm cDNA library that was generated by reverse transcription with poly-dT primer, thus marking the poly-(A) tail and revealing the direction of transcription of the original mRNA; (3) having an open reading frame (ORF) encoding more than 150 amino acids.

Gene Ontology terms were assigned based on BLASTX hits (cut-off $E \leq 10^{-9}$) against a database locally built from public sequences associated with Gene Ontology terms. The public GO annotated datasets used were from the following organisms: *H. sapiens, Drosophila melanogaster, Arabidopsis thaliana, Oryza sativa, Caenorhabditis elegans, Saccharomyces cerevisiae, Schizosaccharomyces pombe*, and *Vibrio cholera,* plus a curated sequence database (GO Annotation at EBI) available at the Gene Ontology Consortium website (http://www.ebi.ac.uk/GOA/). The clusters potentially differentially expressed were manually curated for their automated Gene Ontology categorization concerning biological processes. Additionally, hits with an *E* value $>10^{-9}$ were assigned as proteins with unknown functions.

Software packages at online bioinformatics websites were employed for prediction of domain, signal peptide, transmembrane segment, and GPI-anchor motifs, using SMART (http://smart.embl-heidelberg.de/), SignalP (http://www.cbs.dtu.dk/services/SignalP/), TMHMM (http://www.cbs.dtu.dk/services/TMHMM-2.0/) and big-PI Predictor (http://mendel.imp.ac.at/sat/gpi/gpi_server.html), respectively.

Real-time RT-PCR

Total RNA was extracted from cercariae, 7-day-old schistosomula and adult parasites using TRIzol reagent (Invitrogen) as per the manufacturer's recommendations. The RNA was quantified by spectrophotometry using a NanoDrop instrument (Wilmington, USA), and the integrity was analyzed by electrophoresis with an Agilent 2100 Bioanalyzer. RNA samples (3 μg) were treated with amplification grade DNAse RQ1 (Promega) for 1 h at 37°C to eliminate any genomic DNA contamination just prior to proceeding with cDNA synthesis. First-strand complementary DNA was synthesized from total RNA using SuperScript III Reverse Transcriptase (Invitrogen) with random hexamer primers. First-strand cDNA was synthesized in a total volume of 20 μL containing 3 μg total RNA either with reverse transcriptase or without the enzyme. The PCR reactions were carried out in 96 well microtiter plate wells in a 20 μL reaction volume with SYBR Green Master Mix (Applied Biosystems), with optimized concentrations of specific primers designed using Primer Express software (Applied Biosystems) the settings were adjusted to the highest possible stringency to generate 50–100 bp amplicons, as recommended. A GeneAmp®PCR System 9600 (Corbett Research) was programmed for: 95°C for 10 min and 40 cycles of 95°C for 10 s, 60°C for 1 min. Primers targeting *S. mansoni* actin (Accession: U19945), α-tubulin (Accession: M80214), and GAPDH (Accession: M92359) (Electronic supplementary Table 1—Online Resource 1) were used to evaluate the best endogenous house-keeping gene for qPCR, comparing cercariae, schistosomula and adult stages in triplicates of the same biological sample. The primers were tested as to efficiency in comparison to primers of the house-keeping gene, shown to be $\geq 95\%$. The generation of PCR products was measured in real time by the GeneAmp®5700 Sequence Detection System; specific PCR products were confirmed by dissociation curve analysis and agarose gel electrophoresis. Quantitation of relative differences in expression were finally calculated using the comparative $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001). Statistical comparisons were done with` a one-way analysis of vasriance (ANOVA) followed by a Tukey's pairwise comparison, as required. A *p* value <0.05 was considered statistically significant.

DNA vaccine construction

Specific oligonucleotides shown in the Electronic supplementary Table 1 (Online Resource 1) were designed using the *S. mansoni* transcriptome assembly (Verjovski-Almeida et al. 2003) sequences (Dif 2—SmAE 604153, Dif 3—SmAE 605704, Dif 4—SmAE 606856, Dif 5—SmAE 611133, Dif 6—SmAE 602523, Dif 7—SmAE 609023, Dif 8—SmAE 609541). For optimal full-length expression of the cDNA sequences, a Kozak consensus sequence (CACCATG) was inserted in the sense primer flanking the start codon of the gene. The cDNAs were amplified by the PCR with specific primers from clones of the *Schistosoma* transcriptome project, or by RT-PCR from total mRNA from adult worms using the SuperScript™ Preamplification System for First-strand cDNA Synthesis" (Invitrogen). The PCR reaction was performed using Platinum *Pfx* enzyme (Invitrogen), and initiated with one cycle of 5 min at 94°C, followed by 30 cycles of 30 s at 94°C, 1 min at different annealing temperatures, and 3 min at 68°C. PCR products were purified from agarose gel electrophoresis and cloned into the eukaryotic expression vector pTARGET (pTG) (Promega).

Confirmation of expression of the gene products in mammalian cells

Expression of the genes cloned into pTG was confirmed via transient transfection of baby hamster kidney (BHK) cells by using Lipofectamine 2000 transfection reagent (Invitrogen). Transfections were performed according to the manufacturer's instructions. Briefly, 80–90% confluent BHK cells per 60-mm-diameter dish were cultured in RPMI 1640 (Life Technologies, Grand Island, N.Y.) containing 10% fetal bovine serum and glutamine at 37°C and 5% CO_2. The cells were brought to 90–95% confluency and then overlaid with a complex made with 8 μg of plasmid DNA (pTG-Dif and 20 μl of Lipofectamine transfection reagent). After 24 h of incubation, the cells were washed with PBS and harvested. The expression of pTG-Dif in transfected BHK cells was analyzed by RT-PCR using total RNA as template.

Immunization of mice

Five to six-week-old female C57BL/6 mice from Simonsen Laboratories, Gilroy, California, EUA, were supplied with food and water ad libitum. Animal experimental protocols were approved by Animals Use Ethics Committee of Instituto Butantan (São Paulo, Brazil). Groups of ten mice were lightly anesthetized and inoculated intramuscularly (im) with 50 μl of 10 μM cardiotoxin (Laxotan, Valence, France) into each tibialis anterior muscle, 5 days before starting the immunization with the DNA vaccines. The vectors (pTG) expressing the full-length sequence of Dif 2, Dif 3, Dif 4, Dif 5, Dif 6, and Dif 7 were used in this work. C57BL/6 mice were injected (im) with two doses of 50 μg of pTG-Dif in PBS (100 μl) on days 0 and 21. Additionally, C57BL/6 mice were injected (im—quadriceps muscle) with one dose of 30 ug coencapsulated pTG-Dif4 and trehalose dimicolate into biodegradable poly (DL-lactide-co-glycolide) microspheres (Nanocore, Techno Park Campinas, Brazil).

Challenge infections and parasite loads

Challenge infections were initiated 2 weeks following the final immunization. Mice were anesthetized with 65 mg sodium pentobarbital (Cristália, São Paulo, Brazil) per kg body weight and exposed percutaneously to 130 cercariae by the ring method on their shaved abdomens. Six weeks after percutaneous challenge infections, ten mice per group were sacrificed with 800 μL of a saline solution containing 5 mg/mL of ketamine and 2 mg/mL of xylazine. Perfusion fluid (Saline solution, 500 units/L of heparin) was pumped into aorta artery, and perfused worms were collected from the hepatic portal vein. Adult male and female worms were counted using a stereomicroscope, the protection was calculated by comparing the number of worms recovered from each vaccinated group with its respective control group. Statistical analysis was performed applying the normality test of Kolmogorov–Smirnov to check if data follows a normal distribution. ANOVA and Bonferroni post-test were used to compare experimental and control groups on challenge infections response analysis.

Results

Analysis of transcriptome data for differentially expressed genes

The frequency of reads in a *S. mansoni*-assembled EST (SmAE) database obtained from any two different life-cycle stages can reflect differential gene expression between these stages, when the same set of primers is used for generating the cDNA mini-libraries. This in silico approach was employed here, now focusing on the published cercariae and schistosomula ESTs (Verjovski-Almeida et al. 2003), to identify 5,052 SmAEs that were enriched with ESTs from the schistosomulum stage in relation to cercaria. In an attempt to search for more consolidated clusters, we empirically selected those that had at least eight reads from schistosomulum cDNA libraries, which is twice the average number of ESTs per cluster in the entire database (namely four ESTs per cluster) (Verjovski-Almeida et al. 2003); the

idea was that by restricting the analysis to the clusters with at least twice the average number of ESTs per cluster, one would increase the chance that the abundance of ESTs does reflect the relative abundance of that gene in the life-cycle stage of interest, and not be biased by an overall low EST coverage of that gene. Applying the cut-off filter of eight reads from schistosomulum per cluster, a set of 400 SmAEs (Fig. 1) was identified, which represents genes predicted to be up-regulated in schistosomula. A complete list of these SmAEs is shown in Electronic supplementary Table 2 (Online Resource 2).

Initially, these 400 clusters were manually curated for their automated Gene Ontology categorization concerning biological processes. The distribution of SmAEs among the categories is shown in the pie chart (Fig. 1). The most prominent aspects of schistosomulum biology highlighted by GO analysis were the expression of genes associated with nucleic acid metabolism (20.6%), cell organization (13.5%), protein biosynthesis (7.2%), protein catabolism (6%), chaperones (stress response; 5.0%), and proteins of unknown function (no match in GenBank; 20.0%). Among the genes associated with nucleic acid metabolism, there were many transcription factors and nuclear enzymes. Concerning the genes associated with cell organization, there were structural proteins of the cytoskeleton such as proteins similar to human cadherin, alpha spectrin, and dynein heavy chain. Regarding protein biosynthesis, the

ribosomal proteins were the majority. Another marked group was the stress response proteins, named as chaperones and exemplified by the HSP70 and HSP20 transcripts. The next most prominent GO categories were genes related with transport, signal transduction, and carbohydrate metabolism, although in smaller percentages.

Within the list of 400 genes with increased frequency of EST reads from schistosomula, we could identify several putative *Schistosoma* vaccine antigens, such as Sm97 (paramyosin), Sm28, Sm23, Sm14, TSP-1, 14-3-3 protein, triose phosphate isomerase, GAPDH, and fructose 1,6 bisphosphate aldolase; these molecules have been shown to elicit protective immune responses against schistosome infection in mice (Argiro et al. 2000; Boulanger et al. 1995; Harn et al. 1992; Marques et al. 2008; Pearce et al. 1988; Reynolds et al. 1992; Schechtman et al. 2001; Tendler et al. 1996; Tran et al. 2006).

Worthy of notice was the category of genes encoding proteins with no similarity to proteins of other organisms and therefore with unknown functions (20.0%). We reasoned that these genes may encode proteins exclusive to the parasite, which could be involved in its adaptation and survival, ranking them as possible vaccine candidates. A total of 93 sequences were found in this category. Since most of the EST reads in the database were obtained by the low-stringency RT-PCR method, no information about the direction of transcription of the corresponding mRNA was

Fig. 1 Analysis of transcriptome data for differentially expressed genes. Schematic representation of the general procedure to identify genes differentially expressed in the transition from cercariae to schistosomula stage using the *S. mansoni* transcriptome database in order to search for potential vaccine candidates
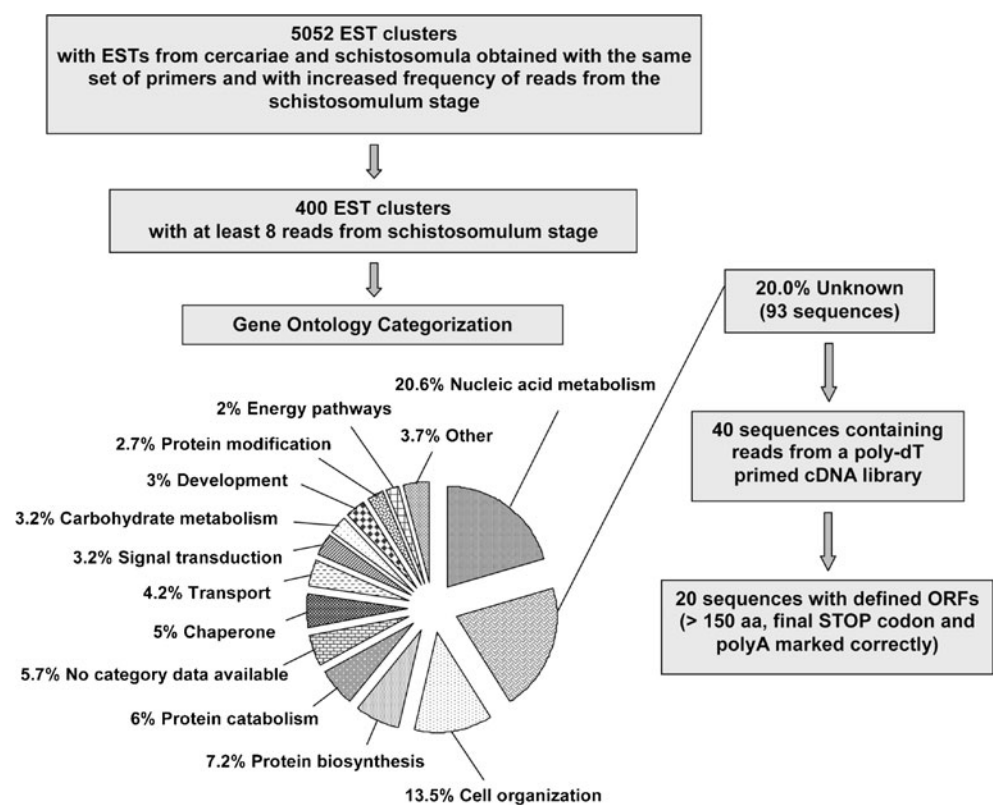
available. In order to determine the orientation of these messages, we additionally selected the clusters containing reads from a poly-dT primed *S. mansoni* cDNA library (showing the correct position of the poly-A tail); a total of 40 SmAEs fulfilled this criterion. Finally, we chose SmAEs containing ORFs with at least 150 amino acids and showing a STOP codon at the 3′ end. From the previously selected 40 sequences, 20 of them showed these features (Fig. 1).

Further characterization of the selected genes by an updated search against GenBank

A total of 20 SmAEs with unknown function were identified after the selection steps described above. From this list, nine sequences were randomly chosen to be investigated as vaccine candidates and were named Dif 1 to Dif 9.

Since the initial gene selection process was based upon the annotation from the large-scale sequencing project (Verjovski-Almeida et al. 2003), the translated proteins for Dif 1 to Dif 9 were re-annotated using an updated version of GenBank. In a search for more information on the selected genes, the translated amino acid sequences were analyzed for the presence of predicted protein domains, signal peptides, transmembrane domains, and GPI-anchor sites (Table 1). The Dif 1 gene was shown to encode a protein that contains a putative transmembrane domain. No significant domains were identified on Dif 2-predicted protein. The Dif 3 gene encodes a protein that does not present any significantly similar domain, but shows 48% similarity with the tick *Ixodes scapularis* protective antigen, 4D8, an endopeptidase (Almazan et al. 2005). Interestingly, although initially we had not found orthologues to the Dif 4 sequence in the public databases, the availability of substantially more sequence information for the cloned full-length ORF for this gene, resulted in the identification of a Pfam SPFH/Band 7 domain (E value=1.7×10^{-47}). The Dif 5 gene encodes a protein that displays a LY-6/CD59 domain (*E* value=1.80×10^{-02}), belonging to a family of glycosyl-phosphatidylinositol-anchored surface proteins, which may be associated with mechanisms of complement system evasion. The Dif 6 gene encodes a protein that contains a cystatin domain (*E* value=2.59×10^{-03}) and a signal peptide. Dif 7 and Dif 8 genes encode proteins that showed only a putative transmembrane domain. Finally, the Dif 9 gene encodes a protein that presents a signal peptide, a sperm coating protein domain (*E* value=2.4×10^{-19}) and shows similarity to venom allergens; additionally the translated gene presents low similarity (26%) to NaASP-2 vaccine candidate from *Necator americanus*.

Evaluation of house-keeping genes to be used as internal controls in real-time RT-PCR

The expression levels for three house-keeping genes, Actin (GeneBank: U19945), α-tubulin (GeneBank: M80214) and GAPDH (GeneBank: M92359), were assessed in the cercariae, schistosomula and adult worms. The α-Tubulin

Table 1 Selected genes with increased frequency of reads in the transition to schistosomulum stage to be investigated as vaccine candidates

Gene[a]	SmAE[b]	Gene DB Contig[c]	Number of reads[d]		Additional information on the gene	
			Cer	Sch	Domain[e]	Primary structure analysis[f]
Dif 1	C604371.1	Smp_174490	0	8	–	Transmembrane helix
Dif 2	C604153.1	Smp_030830.2	0	15	–	–
Dif 3	C605704.1	Smp_076040.3	0	16	–	–
Dif 4	C606856.1	Sm01732	0	8	SPFH	–
Dif 5	C611133.1	Smp_105220	0	57	LY-6 Antigen	Signal peptide/GPI anchor
Dif 6	C602523.1	Smp_034420.2	2	77	Cystatin	Signal peptide
Dif 7	C609023.1	Smp_002740.2	5	74	–	Transmembrane helix
Dif 8	C609541.1	Sm01331	3	125	–	Transmembrane helix
Dif 9	C604241.1	Smp_070240	0	17	SCP	Signal peptide

SCP Sperm coating protein

[a] Gene names adopted in this work

[b] Accession number from the *S. mansoni* ORESTES database (http://bioinfo.iq.usp.br/schisto6/)

[c] Schisto GeneDB version 4 systematic ID (http://www.genedb.org/genedb/smansoni/)

[d] Number of reads obtained from cercariae (Cer) and schistosomula (Sch) from normalized cDNA libraries using the same set of primers

[e] SMART and Pfam predict the presence of motifs or domains in a protein sequence

[f] SignalP, TMHMM and DGPI for analyzing the presence of signal peptide, transmembrane helix, GPI-anchor and cleavage sites, respectively

and GAPDH genes showed statistically significant variation in the levels of transcription among the stages analyzed, while the Actin gene seems to be a slightly better internal control because its Ct values were less variable (Fig. 2). Thus, we have chosen the Actin gene as normalizer in real-time RT-PCR experiments to validate the putative differentially expressed genes.

Validation of differential expression by real-time RT-PCR

Up-regulation in the schistosomulum stage of the nine selected Dif genes was measured by real-time RT-PCR assays, using cDNA produced from total RNA of cercariae, in vitro 7-day-old schistosomula and adult worms of *S. mansoni*. From the total of nine genes examined, five of them (Dif 3, Dif 4, Dif 5, Dif 8, and Dif 9) confirmed the differential expression in schistosomulum stage when compared with cercaria (Fig. 3). Thus, the approach of searching for differentially expressed genes by analyzing the number of reads in a library was successful for 56% of the genes. The expression patterns could be grouped into a few profiles:

• Dif 5 gene was characterized by 4-fold increased expression in schistosomula in relation to cercariae, and further increase in adults in relation to schistosomula, with statistical differences among all stages;
• Dif 3 and Dif 8 genes had an increased expression in schistosomula in relation to cercariae, 4.7-fold and 2.4-fold, respectively, and maintained their expression levels in the adult worms;
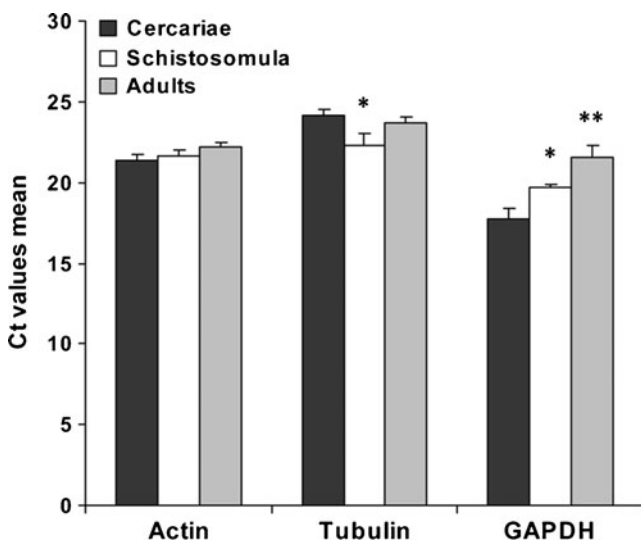
Fig. 2 Quantification of actin, α-tubulin, and GAPDH genes from *Schistosoma mansoni* cercariae, schistosomula, and adult worms. *Numbers* represent the mean of cycle threshold values observed among the RNA sample from different stages. The data are the means ±SD of triplicates of the same biological sample. Statistical analyses performed according to ANOVA and Tukey's, *$p<0.05$

• Dif 4 and Dif 9 genes showed 6-fold and 14-fold increased expression in schistosomula compared with cercariae, respectively, and reduction in the adult stage.
• Dif 1, Dif 2, Dif 6, and Dif 7 genes presented similar profiles of expression, with no statistical differences of expression between cercariae and schistosomula, while significantly higher levels of expression could be detected in adult worms (Fig. 3).

Expression of Dif genes in vitro in transfected mammalian cells

In an attempt to evaluate the protective potential of the selected Dif genes against experimental schistosome infection in the murine model, we chose a DNA-vaccine strategy, which is a simple and direct approach to screen for vaccine candidates. We successfully cloned the cDNA fragments for Dif 2, 3, 5, 6, 7, 8, 9 (GenBank accession no. Dif_2 FJ490611, Dif_3 FJ490612, Dif_5 FJ490613, Dif_6 FJ490614, Dif_7 FJ490615, Dif_8 FJ490616, Dif_9 FJ490617), and Dif 4 (GenBank accession no. EU531730) genes. DNA vaccine vectors containing these genes were constructed, and they were named pTG-Dif 2–9. These were evaluated for in vitro expression by transfection into BHK cells. The expression of Dif 2, 3, 4, 5, 6, 7, 8, and 9 was analyzed by testing for the presence of the respective mRNAs by RT-PCR. In the RT-PCR assays, PCR products of the expected sizes (611, 660, 1077, 375, 447 and 570 bp) were obtained, using cDNA derived from cells transfected with 8 μg of pTG-Dif 2, 3, 4, 5, 6, and 7 (Fig. 4). No product was observed for pTG-Dif 8 and 9 (data not shown), suggesting no transcriptional expression; therefore, we excluded these constructs from further investigation. To eliminate the possibility that the PCR product was produced because of plasmid DNA contamination, the extracted RNA (with no reverse transcription) was used as a template for PCR. No product was amplified without reverse transcription of the RNA (Fig. 4). Similarly, no product was obtained using cDNA derived from cells transfected with a control vector without insert. These results demonstrate that the pTG-Dif 2, 3, 4, 5, 6, and 7 constructs were expressed in mammalian cells.

Challenge with cercariae

Mice were immunized with two doses of the vectors pTG-Dif 2, 3, 4, 5, 6, or 7 to evaluate their protective potential as compared with mice immunized with the control vector. Mice were challenged with 130 cercariae and the adult worms were recovered 7 weeks later. The results of the challenge experiments are presented as scattergrams,
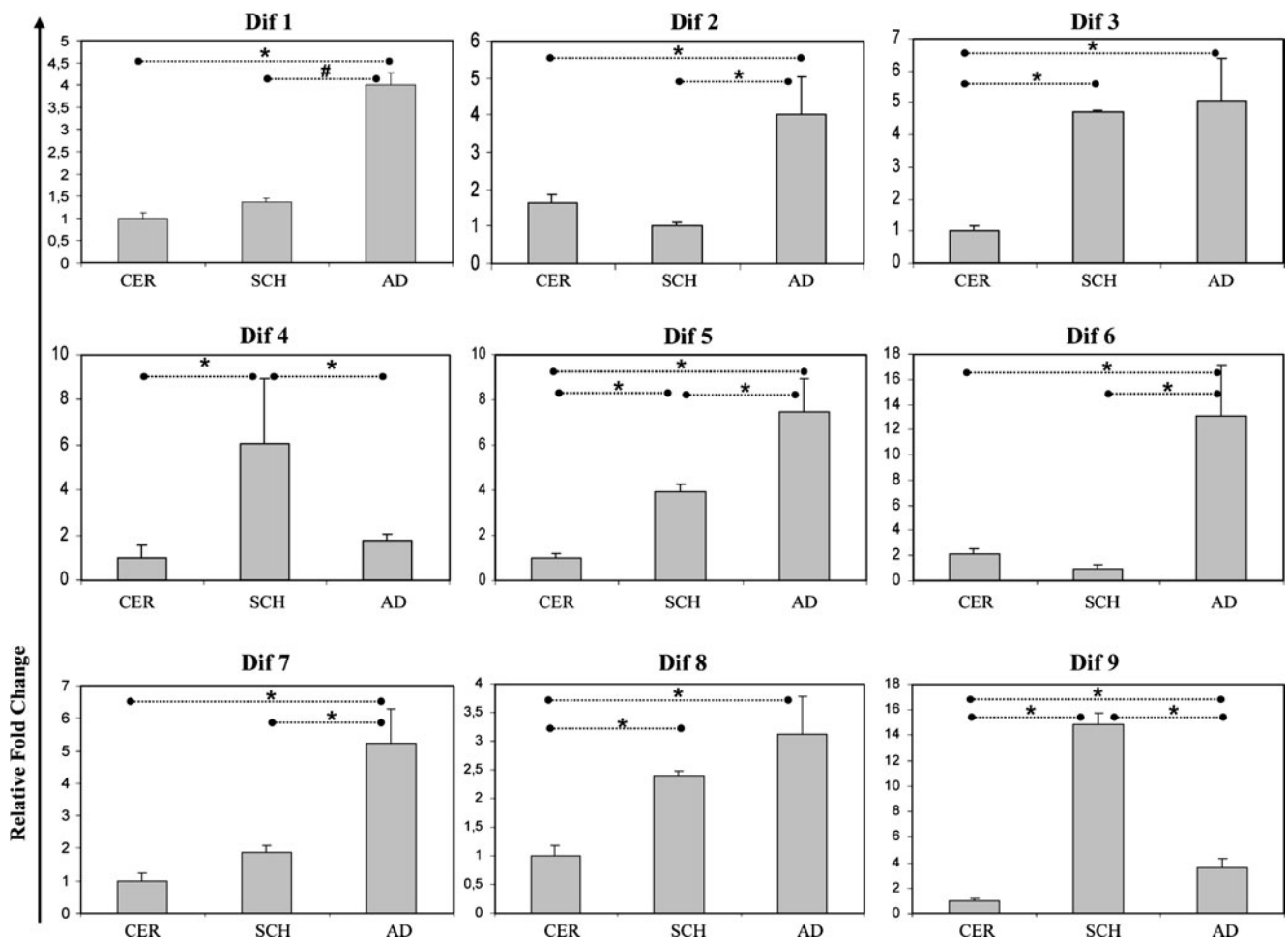
Fig. 3 Analysis of gene expression of Dif 1 to Dif 9 genes in the cercaria, schistossomulum, and adult stages. Total RNA from the different life-stages were transcribed to cDNA and analyzed by real-time RT-PCR to quantitate the differences in expression levels of the genes between stages. An internal house-keeping control gene (Actin) was used as normalizer. Calculated according to the relative $2^{-\Delta\Delta Ct}$ method and shown as relative fold change in relation to stage with less expression (calibrator—usually cercaria). The data are the means± SEM of triplicates of the same biological sample. *Asterisks* statistical analyses performed according to ANOVA and Tukey's. *Number signs* according to Student's *t* test

together with the "mean worm burden" and "percentage of protection" (Fig. 5).

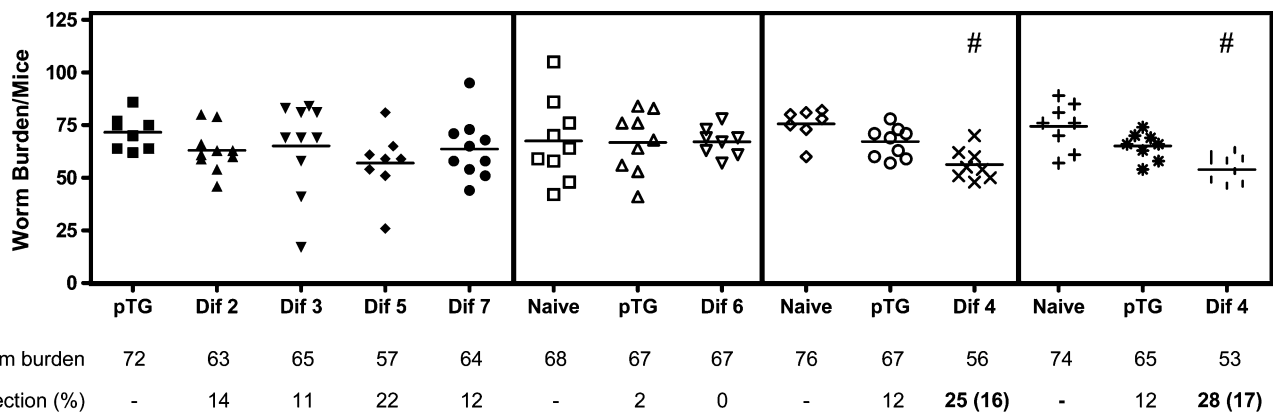In the first set of experiments, in which mice were immunized with pTG-Dif 2, 3, 5, or 7, only pTG-Dif 5 induced a reduction of worm burden of 22%, however displaying a borderline statistical significance (*p* value= 0.06). In another experiment, pTG-Dif 6 showed no protective potential. On the other hand, the immunization of mice with pTG-Dif 4 induced a reduction in worm burden of 25% in relation to the non-immunized group
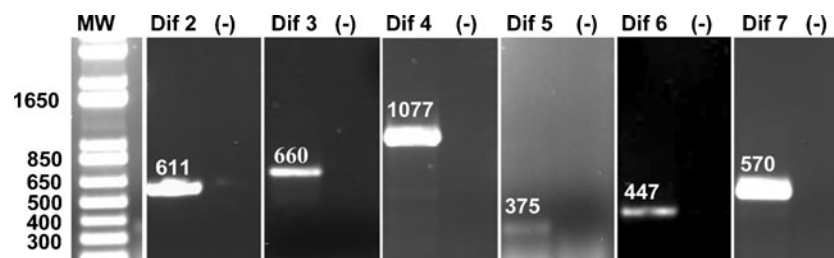
Fig. 4 Expression of Dif 2, 3, 4, 5, 6, and 7 genes at the transcriptional level. PCR products from cDNA of transfected mammalian cells. BHK cells were transfected with pTG-Dif 2, 3, 4, 5, 6, or 7. Total RNA was extracted 24 h later and RT-PCR performed with specific primers. PCR products are shown after separation on a 2% agarose gel

	pTG	Dif 2	Dif 3	Dif 5	Dif 7	Naïve	pTG	Dif 6	Naïve	pTG	Dif 4	Naïve	pTG	Dif 4
Worm burden	72	63	65	57	64	68	67	67	76	67	56	74	65	53
Protection (%)	-	14	11	22	12	-	2	0	-	12	**25 (16)**	-	12	**28 (17)**

Fig. 5 Scattergram of worm burden in mice immunized with DNA vaccines expressing Dif genes. Groups of ten mice were either non-immunized (Naïve), or immunized intramuscularly with control plasmid, pTG, or pTG-Dif 2–7 and challenged with 130 live *S.* *mansoni* cercariae. Statistical analyses of the protection rate were performed with ANOVA and Bonferroni multiple comparison test; significantly different ($p < 0.05$) compared with Naïve or (pTG) (*number signs*)

(p value < 0.001) and 16% in relation to the empty vector, pTG (p value < 0.005). We then repeated the experiment using pTG-Dif 4 encapsulated in microspheres, which induced a reduction in worm burden of 28% in relation to the non-immunized group (p value < 0.001) and 17% in relation to the empty vector, pTG (p value < 0.005). Overall, the results obtained with the DNA vaccines indicated a protective potential for Dif 4.

Discussion

Here we report the strategy of searching for potential vaccine candidates using the stage-associated frequency of ESTs in a *S. mansoni* transcriptome database coupled to a test of DNA vaccine immunization and parasite infection challenge. The advantage is that the relative abundance of ESTs from different stages is usually available as a by-product of transcriptome sequencing, before SAGE and Microarray data can be generated, and DNA vaccines obviate the necessity of protein expression and purification.

We compared our data with a microarray analysis that identified genes preferentially expressed in the same stage (Dillon et al. 2006). Twenty-one SmAEs (16 genes) from a total of 65 genes that were described as differentially expressed by microarray analysis were identified by our strategy (Table 2), which corresponds to 32% of the contigs described by Dillon et al. (2006). Both studies have used in vitro cultivated schistosomula, however the relatively low (32%) overlap in the contig list indicates that they are both partial sets of schistosomula genes, and probably reflect the fact that both studies have drawn the schistosomula genes from different pools of ESTs, namely a set of ~6,000 ESTs in the case of Dillon et al. (2006), and ~1,800 ESTs that were contained in the 400 SmAEs under study here.

In both strategies, genes encoding proteins postulated to be involved in the contraction and extension of the schistosomulum were found to be up-regulated, such as fimbrin, and different isoforms of cadherin and actin related protein-3 (ARP-3), which may be increased because the schistosomulum is growing and needs to reorganize its internal structures. The cathepsin genes identified indicate the change of schistosomulum for adaptation to its life in the blood circulation (Table 2), where it will start digestion of hemoglobin. Lactate-dehydrogenase gene expression was also identified as up-regulated, corroborating the described switch in larval metabolism from aerobic to anaerobic pathways after transformation (Lawson and Wilson 1980). Another group of genes with increased expression were the chaperones, e.g. HSP20 and HSP70. Expression of stress proteins is induced by exposure of an organism to stress factors such as heat, nutrient deprivation, and metabolic disruption. Such stress would occur when the parasite develops along its life cycle, e.g., when cercaria penetrates the skin and falls into circulation in the host blood stream.

It is interesting to note that, from the list of gene fragments up-regulated in schistosomula within the microarray data reported by Dillon et al. (2006), we can identify 60% of these within our 5,052 contigs. When we selected the 400 SmAEs with at least eight reads from schistosomulum, 32% of these SmAE gene fragments still overlap with the up-regulated microarray dataset (Dillon et al. 2006). This indicates that we have reduced by over 10-fold our working group, but still have a reasonable proportion of genes validated by another technique.

The only other published microarray expression data that has compared schistosomula with all other stages was performed by (Gobert et al. 2009) with *S. japonicum*. However, the parasites were recovered after in vivo

Table 2 Genes expressed in the schistosomulum stage as identified by two different strategies

Gene DB Contig[a]	Microarray strategy (Dillon et al. 2006) Putative product[b]	Stage-associated frequency of ESTs (this work) SmAE[c]
Sm12907	Cathepsin B1 isotype 2	c607650.1
		c607658.1
		c600492.1
Sm01349	Cathepsin B1 isotype 1	c602627.1
Sm01393	Cathepsin L	c603452.1
Sm00165/Sm00493	Lactate dehydrogenase	c603066.1
		c605209.1
		c609596.1
Sm00989	Asparaginyl endopeptidase	c609789.1
Sm03904	Ectonucleotide pyrophosphatase	c602603.1
Sm12775	Wasp venom allergen	c604241.1
		c608323.1
Sm12764	Antigen 5	c612423.1
Sm03779	Lysosomal-associated membrane glycoprotein (LAMP)	c610914.1
Sm13240	Fimbrin	c603972.1
Sm01621/Sm04825	Hypothetical	c611956.1
Sm12913	Hypothetical	c600235.1
Sm03795	Hypothetical	c603536.1
Sm10117	Hypothetical	c604867.1
Sm12881	Hypothetical protein st13	c610076.1
Sm06895	HSP20-like	c602780.1

[a] Schisto GeneDB version 4 systematic ID (http://www.genedb.org/genedb/smansoni/)

[b] Putative identity obtained by BLAST analysis against the GenBank database

[c] Accession number from the *S. mansoni* ORESTES database (http://bioinfo.iq.usp.br/schisto6/)

transformation, and therefore, data cannot be directly compared with our results, due to intrinsic differences in the expression profile from in vitro and in vivo obtained schistosomula, as reported by (Chai et al. 2006). Unfortunately, we could not compare our data with those obtained from SAGE analysis (Williams et al. 2007), due to differences in the stages investigated. However, the identification by these authors of a large proportion of unknown or hypothetical sequences (~59%) among the differentially expressed genes in the mammalian stage, points to the importance of better investigating this category.

Real-time RT-PCR analysis has confirmed up-regulation in schistosomula compared with cercariae for approximately 56% of the selected Dif genes. This percentage is comparable to data from Li et al. (2004), who confirmed differential expression by real-time RT-PCR for 47% of the transcripts identified by an electronic selection based on the *Brugia malayi* EST database. Although this data is obtained directly from a transcriptome sequencing project, without involving additional investments, its predictive efficiency is lower than other more sensitive techniques, such as microarrays and SAGE. Therefore, it may be advantageous to increase the initial number of selected genes to be validated by RT-PCR.

It should be noted that Dif 9 (SmVAL-7) was confirmed to be up-regulated in schistosomulum by real-time RT-PCR; this is not in agreement with the findings of Chalmers et al. (2008), who described its expression peaking at the cercaria stage (Chalmers et al. 2008). On the other hand, microarray analyses by Dillon et al. (2006) have shown up-regulation of this gene (Wasp venom allergen) in schistosomula. Differences in experimental procedures or normalizers could account for this divergence and it will be important to investigate the protein expression profiles.

DNA vaccines are a cost-effective means of screening a reasonable number of candidate antigens from eukaryotic pathogens, in which correct protein expression and folding are more difficult to be achieved (Clark and Cassidy-Hanley 2005). Furthermore, presentation of antigens as DNA vaccines elicits strong cellular immune responses with Th1 cytokines profile, which is preferentially required in the murine model for schistosomiasis (Wynn et al. 1994). A DNA vaccine strategy was used to evaluate the selected genes as vaccine candidates. Seven genes were cloned in eukaryotic expression vectors, six of which showed transcriptional activity in vitro and were used to immunize mice for challenge assays.

Immunization with pTG-Dif 5 caused a 22% reduction in worm burden in the challenge assays (with a borderline statistical significance). Recently, Wilson and Coulson (2009) call attention for the identification in the schistosome genome of six homologues of human CD59/LY6 (which is a potent inhibitor of the Membrane Attack Complex); furthermore four of them were identified at the tegument surface by proteomics. Actually, one of these molecules corresponds to Dif 5 (A. Wilson and W. Borges, personal communication). Based on this context we believe that Dif 5 molecule and its paralogs deserve further studies; obtaining the respective recombinant protein and the specific antibody, will allow establishment of the protein expression profile along the parasite life cycle, as well as its tissue localization and different presentation systems should be explored to test its protective potential against schistosomes.

Immunization with pTG-Dif 4 induced a 16–17% reduction in worm burden upon *Schistosoma* infection, suggesting a protective potential for Dif 4 antigen. This gene encodes a protein that has a prohibitin domain and similarity with stomatin like-protein 2 (SLP-2) from *Danio rerio*; recently the human SLP-2 has been suggested to be involved in TCR signalling, being proposed as a potential immunotherapeutic target (Kirchhof et al. 2008). It is important to note, that in the search for vaccines against *Schistosoma*, many studies using DNA vaccines initiate with low protective values, which are then increased by the use of adjuvants or prime-boost strategies (Dai et al. 2009; Siddiqui et al. 2005; Wei et al. 2008, 2009). The protective effect of Dif 4 was recently confirmed with a recombinant protein formulation, which was able to reduce the worm burden in 30–32% after challenge in the murine model (Farias et al. 2010).

Assuming that genes up-regulated in the transition to intra-host stages may be relevant for the parasite's survival in the host and therefore may be potential vaccine candidates; we have screened a *Schistosoma* EST database to select genes up-regulated in the transition from cercariae to schistosomula. DNA vaccine technology identified an antigen with protective potential to be further evaluated. The advantage of the strategy is that the data is a by-product of a transcriptome project, and therefore involves low costs and is rapidly applicable; however, they present low to medium RT-PCR validation and the DNA vaccine method may not be the ideal presentation system, usually displaying modest protection rates. Our results illustrate the advantages and caveats of using bioinformatic analysis on raw EST data coupled to experimental immunization assays using the DNA vaccine strategy in the identification of novel proteins, which may have a potential as vaccine candidates against schistosomiasis.

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