Parasitol Res (2003) 89: 113–119
DOI 10.1007/s00436-002-0643-2

ORIGINAL PAPER

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Gene expression changes in *Schistosoma mansoni* sporocysts induced by *Biomphalaria glabrata* embryonic cells

Received: 10 February 2002 / Accepted: 21 February 2002 / Published online: 14 September 2002

Abstract *Biomphalaria glabrata* embryonic (Bge) cells have been shown to provide favourable environmental conditions for the development of *Schistosoma mansoni* sporocysts. We investigated the effect of Bge excretory-secretory products on metabolic activity and gene transcription in *S. mansoni* mother sporocysts. Using the differential-display technique, we identified several sporocyst transcripts regulated by exposure to Bge soluble components. Research in databases indicated that six of the eight differential products analysed were homologous to sequences already present in databases. Two transcripts appeared of interest for schistosome development since they could be associated with cell division and protein synthesis in developing sporocysts. Their up-regulation following contact with cell products was confirmed by semi-quantitative RT-PCR. The first fragment coded for a part of the chaperonin containing T-complex protein gamma subunit-like protein of *S. mansoni* (SmTCP 1-C). The second one represented a new *S. mansoni* expressed sequence tag encoding a protein homologous to various glutaminyl-tRNA synthetases (GlnRS). The full-length sequence of SmGlnRS was cloned from adult schistosomes and its primary sequence was compared to other GlnRS. The overexpression of SmTCP-1 and SmGlnRS could be correlated with the metabolic changes observed in Bge-exposed sporocysts.

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Introduction

The transmission of trematode parasites is dependent on snail intermediate hosts that assure the intense asexual multiplication of the larval forms. The remarkable specificity of parasites for their hosts indicates the importance of an adaptive molecular dialogue between the two partners. Molecular studies of the interactions between the parasite *Schistosoma mansoni* and its intermediate host *Biomphalaria glabrata* have been facilitated by the development of a model of co-culture of sporocysts with mollusc cells in vitro. *B. glabrata* embryonic (Bge) cells (Hansen 1976) have been shown to provide a beneficial influence on schistosome development. Yoshino and Laursen (1995) demonstrated that the production of *S. mansoni* daughter sporocysts could be achieved from in vitro-derived mother sporocysts by cultivating miracidia with the Bge cell line. Bge cells share characteristics with haemocytes and their interaction with primary *S. mansoni* sporocysts in culture was characterized by their adherence and encapsulation of larvae, followed after several weeks by the growth and differentiation of the emergent daughter sporocysts. However, further results indicated that an intimate contact between cells and parasites was not required for larval reproduction in vitro. The positive effect of Bge-conditioned medium on the survival and development of sporocysts (Yoshino and Laursen 1995; Ivanchenko et al. 1999) indicated that excretory-secretory (ES) products from Bge cells could provide the nutritional and/or signalling factors necessary for the multiplication and propagation of intramolluscan stages. The role of soluble factors was also confirmed in experiments in which various trematode species were co-cultured with Bge cells. The ability of sporocysts to survive and develop in the presence of Bge cells was independent of cell adherence (Coustau et al. 1997; Ataev et al. 1998; Coustau and Yoshino 2000).

In this work, we investigated at the molecular level the role of Bge cells on schistosome development and analysed the effect of Bge ES products on metabolic activity

and gene transcription in *S. mansoni* mother sporocysts. The differential-display (DD)-RT-PCR technique was applied to the analysis of the parasite transcriptome, allowing the identification of several genes regulated in Bge-exposed sporocysts.

Materials and methods

Bge cell culture

Bge cells (ATCC CRL 1494) were grown at 27°C in Bge medium supplemented with 10% heat-inactivated fetal calf serum (Sigma) and antibiotics (Hansen 1976). Flasks which were 80% confluent were extensively washed in minimal salt saline (MSM) medium (Schallig et al. 1990). Successive washing media were analysed by SDS-PAGE in order to check for the presence of residual serum molecules. Cells were washed in MSM until the contaminating albumin traces were no longer detectable following silver nitrate staining of the electrophoresis gels. They were then incubated for 24 h at 27°C in MSM. Culture supernatants, referred to as ES products, were used immediately. Protein contents were determined using the BCA protein assay (Pierce, USA).

Parasite culture

A Puerto-Rican strain of *S. mansoni* was maintained by passage through albino *B. glabrata* snails and *Mesocricetus auratus* hamsters. Miracidia were transformed in vitro into sporocysts by incubation in MSM at 27°C for 8 h. They were incubated for 24 h in 96-well microtitre plates with Bge cell ES products or in MSM at a concentration of 1,000 parasites ml^{-1} in the presence of labelled precursors. A total of 200 parasites were labelled with 2 μCi [^{35}S]methionine (> 1,000 Ci $mmol^{-1}$) or 1 μCi [methyl-^{3}H]thymidine (55 Ci $mmol^{-1}$) (Amersham). Parasites were collected and ^{35}S radioactivity was determined in the TCA-precipitable material using a scintillation liquid (Beckman) and an LKB scintillation counter. [^{3}H]Thymidine incorporation was determined following filtration onto GF/C filters (Packard) using a cell harvester (Packard) and a Packard microplate scintillation counter.

RNA extraction

Sporocyst pellets were resuspended in TRIzol LS reagent (Life Technologies) and total RNA was prepared according to the manufacturer's instructions including a DNAse I (Boehringer Mannheim) step in order to eliminate contaminating genomic DNA. Aliquots of each RNA preparation were run on a formaldehyde agarose gel to check RNA integrity. The material from three independent RNA preparations was pooled.

DD-RT PCR technique

Messenger RNA differential display (Liang and Pardee 1992) was performed using the RNAimage kits 1 and 2 (GenHunter). First strand oligo d(T)-primed cDNA synthesis was performed according to the manufacturer's instructions. For PCR amplification, 2 μl cDNA template was used in a 20 μl reaction mixture consisting of 10 mM Tris-Cl, pH 8.4, 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatin, 2 μM dNTP, 0.2 μM random primer, 2 μCi [a-^{33}P] dATP (specific activity > 3,000 Ci/mmol, ICN Pharmaceuticals), 2 U AmpliTaq Gold DNA polymerase (Perkin Elmer) and 0.2 μM of the anchored oligo d(T) primer. Cycling conditions were an initial denaturation at 94°C for 10 min, then 40 amplifications performed at 94°C for 30 s; 40°C for 2 min; 72°C for 1 min; and a final elongation of 5 min at 72°C. PCR fragments were separated on a 6% denaturing polyacrylamide gel with a genomyxLR DNA sequencer (Beckman-Coulter). Gels were dried and autoradiographed.

Bands of interest, representing differentially expressed mRNAs, were excised from dried gels, eluted in 50 μl water for 1 h at 37°C, then overnight at 4°C. Bands were re-amplified by PCR using the same set of primers and the initial PCR conditions. PCR fragments were electrophoresed on agarose gels and DNA from excised bands was extracted using the JetSorb kit (Genomed), then cloned in the pCR 4-TOPO vector (InVitrogen). For each fragment, two or three positive clones were chosen and sequenced using the Big Dye terminator kit (Applied Biosystems). Sequences were compared to different databases using BLAST searches (Altschul et al. 1990; Gish and States 1993).

Semi-quantitative RT-PCR

Reverse transcription (RT) was performed using the Thermoscript RT-PCR system with the oligo d(T) primer (Life Technologies) on the RNA samples previously analysed in DD-RT PCR. PCR conditions were optimized in order to obtain a product in the linear phase of the reaction (Murphy et al. 1993). Cycling conditions consisted of an initial denaturation at 94°C for 10 min, followed by 19–31 amplifications. Different temperatures were used for annealing (54°C for a-tubulin, 50°C for SmCOX II, 50°C for β-tubulin, 48°C for SmGlnRS and 49°C for SmTCP-1C). Elongation took place for 1 min. Specific forward (F) and reverse (R) primers were used for α-tubulin (F: TGG AAC TTA TCG TCA ACT TTT CCA TCC, R: GAA GTG GAT ACG AGG ATA AGG TAC CAG), for SmCOX II (F: GGG TTT TTA TTC CGT TAT GAT G, R: GCA TAA CTC TGA ACA ATA ACC), for β-tubulin (F:CGA CAA TGT CTG GAG TCA C, R: CGT TTG AAG AGC TCT TGG ATG), for SmGlnRS (F: CCA TCA GGC CGT AGA AGA AAT A, R: CCG CGT AAC GAA GAC CAA C) and for SmTCP-1C, (F: GCA GAA TCA ATA TAT ATT TTA GG, R: CGA CTT ATT ACA GAG CTT CG). PCR fragments were electrophoresed on agarose gels and the intensity of the bands was measured using the Image Master 1D software (Pharmacia, Biotech).

Cloning of the complete *S. mansoni* glutaminyl-tRNA synthetase

The 5′ sequence of the SmGlnRS transcript was amplified by using the SMART RACE cDNA amplification kit from a cDNA prepared from adult worm or cercarial poly A+ RNA according to the manufacturer's instructions (Clontech). PCR reactions were performed using the Universal Primer Mix (UPM) and a primer specific for SmGlnRS (5′ ATA ACA AGG CCC GCG TAA CGA AGA CCA ACT G 3′). PCR products were cloned and sequenced as described above.

Northern blot analysis

RNA was electrophoresed on a formaldehyde agarose gel, blotted on a nylon charged membrane (Amersham) and prehybridized at 42°C for 3 h. The probe was prepared from SmGlnRS cDNA, labelled with ^{32}P using the Megaprime DNA labelling system kit (Amersham) and used for hybridization at 42°C for 18 h in a solution of 50% formamide, 5×SSC, 5× Denhardt's reagent, 0.5% sodium dodecyl sulfate (SDS) and denatured herring sperm DNA at 0.1 mg ml^{-1}. The membrane was washed twice in 2× SSC/0.1%SDS for 10 min at room temperature and once at 0.1×SSC/0.1%SDS for 10 min at 42°C and was then autoradiographed.

Results

Effect of Bge cell ES products on sporocyst metabolism

Experiments presented in Fig. 1 show that ES products from Bge cells stimulated both [^{35}S]methionine and

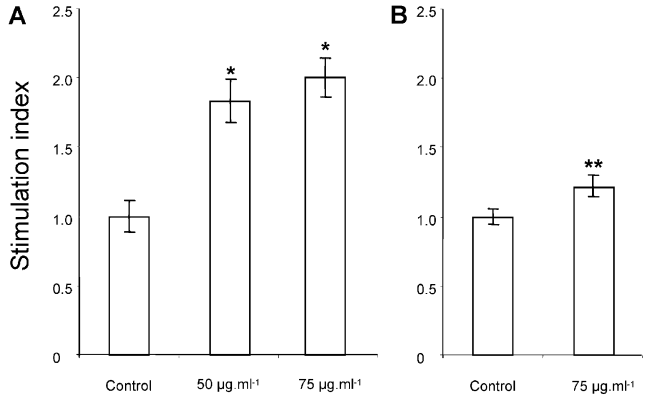

Fig. 1A, B Effect of *Biomphalaria glabrata* embryonic (Bge) cell excretory-secretory (ES) products on the metabolic activity of *Schistosoma mansoni* sporocysts. **A** [^{35}S]Methionine and **B** [^{3}H]thymidine incorporation in the absence (control) or presence of different concentrations of ES products corresponding to 50 µgml^{-1} or 75 µgml^{-1} protein content. Results are expressed as means ± SEM of the stimulation index (expressed as the ratio of cpm in sample/cpm in control) calculated from triplicate values obtained in three (**A**) and two (**B**) independent experiments. Statistical analyses of data were performed using the Kruskal-Wallis one-way analysis of variance (**A**) or Mann-Whitney (**B**) tests. * = $P < 0.001$ and ** = $P < 0.01$

[^{3}H]thymidine incorporation into *S. mansoni* sporocysts. A twofold stimulation was observed for protein synthesis in exposed parasites as compared to controls. However, even in the presence of high concentrations of Bge products, DNA synthesis was only moderately enhanced (15%) although this was statistically significant.

DD profile analysis

The DD-RT-PCR method was applied to the identification of genes that are differentially expressed in *S. mansoni* sporocysts exposed to ES products from Bge cells during a 24 h period. For each RNA sample, RT reactions were performed in duplicate. PCR reactions were carried out using three one-base anchored oligo(dT) primers and 16 random primers (from the RNAimage kit) giving for each RT product a total number of 48 PCR combinations. Duplicates were analysed side by side on the gel in order to consider only the bands that were identical in both lanes. Nine bands that showed differences in intensity between unexposed and exposed parasites were considered of interest. Five of these (EA31, EA32, Eg2, EC2, EC9) were found to be overexpressed in stimulated parasites whereas four of them (MC11, MC9, Mg5, MA10) were underexpressed. The nine bands were excised from the gels and reamplified with success, except for the MA10 band.

Sequence analysis of differential products

The eight differential products were subcloned and their sequence was determined. Sequences varied in length from 300 bp to 650 bp and all contained a stop codon and a mRNA 3′-end. Their comparison with nucleotide and protein databases showed that at least six of them were significantly homologous to sequences in databases, one of them (EC9) corresponding to the ribosomal RNA sequence. Three sequences (EA31, EA32 and Eg2) from exposed-parasite displays matched known expressed sequence tags (EST) from *S. mansoni*. EA31 (571 bp) showed a very high identity (99%) to the EST (GenBank: AI975572) corresponding to β-tubulin. EA32 (398 bp) was identical to the EST (GenBank: AI395663) with an open reading frame of 81 residues encoding an unknown protein homologous to (41% identity) a *Thermus aquaticus* hypothetical protein (GenBank: P74897). Eg2 (329 bp) was identical to the EST (GenBank: AI974971) that encoded a 91 amino acid sequence of the *S. mansoni* chaperonin containing T-complex protein gamma subunit-like protein (SmTCP-1C) (57% identity with the human TCP-1C protein (GenBank: P49368, Walkley et al. 1996). The two last sequences were found to correspond to new *S. mansoni* sequences.

MC11 was down-regulated whereas EC2 was up-regulated following exposure to Bge products. The MC11 fragment (521 bp), with a 146 amino acid open reading frame, was highly homologous with proteins from various species, including *Mus musculus* (67%, Carninci and Hayashizaki 1999) and human (65%) (GenBank: BAB23024 and NP_057146, respectively) and whose function remains unknown. EC2 contained a fragment of 364 bp encoding a 86 amino acid sequence homologous to the glutaminyl-tRNA synthetase (GlnRS) of different species, including *Drosophila melanogaster* (GenBank: AF145668), human (GenBank: P47897, Lamour et al. 1994), *Caenorhabditis elegans* (GenBank: O62431) and *Escherichia coli* (GenBank: J01617, Hoben et al. 1982). Identity levels varied from 54% for *C. elegans* to 45% for *E. coli*.

Confirmation of differential displays

Semi-quantitative RT-PCR was performed in order to confirm that selected bands effectively corresponded to differentially expressed genes. In these assays, α-tubulin (Duvaux-Miret et al. 1991) was used as an internal standard (Mei and Loverde 1997) but our results showing that β-tubulin could be differentially expressed in sporocysts led us to consider an additional standard, the cytochrome *c* oxidase subunit II (SmCOXII), a mitochondrial enzyme shown to be transcriptionally stable in schistosomula (Oliveira et al. 1998). Results in Fig. 2 demonstrate clearly the overexpression (threefold) in stimulated parasites of glutaminyl tRNA synthetase (GlnRS) transcripts and to a lesser extent (1.3-fold) of chaperonin-γ (SmTCP-1C). However, they did not confirm changes in the transcription of β-tubulin.

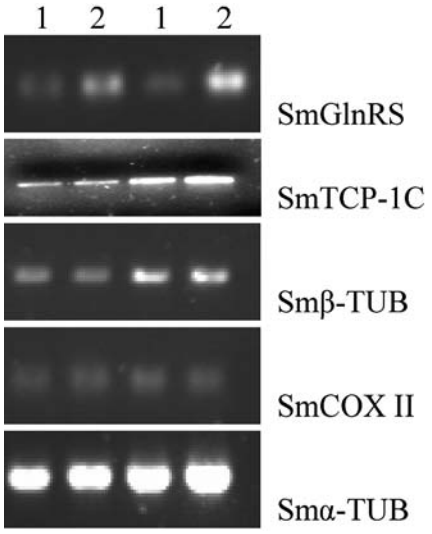

Fig. 2 Semi-quantitative RT-PCR results using specific primers for *S. mansoni* glutaminyl-tRNA synthetase (*SmGlnRS*, 880 bp, 28 and 31 cycles), chaperonin-γ (*SmTCP-1C*, 302 bp, 28 and 31 cycles), β-tubulin (*Smβ-TUB*, 447 bp, 22 and 25 cycles), cytochrome oxydase subunit 2 (*SmCOX II*, 478 bp, 25 and 28 cycles) and α-tubulin (*Smα-TUB*, 872 bp, 28 and 31 cycles). *1* Control sporocysts, *2* sporocysts exposed to Bge ES products. Results indicated three- and 1.3-fold excess of PCR products for SmGlnRS and SmTCP-1C, respectively (mean of three experiments)

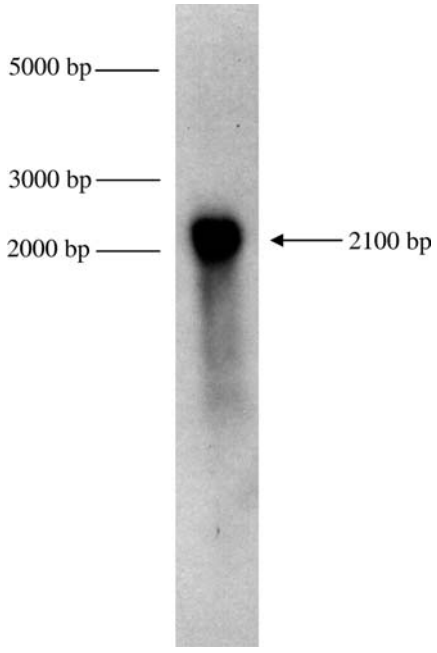

Fig. 3 Size determination of SmGlnRS transcripts by Northern blot analysis of *S. mansoni* adult worm total RNA

Molecular cloning of the *S. mansoni* glutaminyl tRNA synthetase

PCR experiments were performed in order to characterize the 5′ end of the SmGlnRS cDNA sequence by amplification of a SMART library of adult worm RNA using an EC2-derived sequence as a gene-specific primer. These experiments allowed us to obtain a total cDNA sequence of 1,812 bp (GenBank: AF358445) containing a unique and continuous open reading frame of 531 residues with an ATG start codon (nt 114–116) positioned in the *PuNNAUGG* favourable context (Hughes and Andrews 1997). Northern blot data demonstrated that the EC2 sequence hybridized with a 2,100 bp *S. mansoni* mRNA species in adult worms (Fig. 3), thus indicating that a part of the 5′-untranslated SmGlnRS sequence was probably missing. However, amplification of a SMART library of cercarial RNA led to obtaining a cDNA identical to that obtained from adult worm RNA. The complete 60.8 kDa protein showed significant homology with different, already known GlnRS, varying from 42% to 63% identity with GlnRS of *E. coli* and *D. melanogaster*, respectively. The predicted SmGlnRS sequence, presented in Fig. 4, contained the two homology boxes constituting the dinucleotide binding domain of class I aminoacyl-tRNA synthetases (AARS) and separated by the connective peptide (Delarue and Moras 1993). The first box of SmGlnRS (residues 46–130) was highly similar to insect (75%), human (76%) and bacterial (53%) sequences and included the HIGH consensus motif (residues 62–65) known to be involved in ATP binding (Freist et al. 1997). The second half of the dinucleotide binding domain contained at the position 249–252 a SKRK motif similar to that found in the other GlnRS and corresponding to the consensus sequence of class I AARS also involved in ATP interaction (Freist et al. 1997). This second box overlapped with a gap of 31 residues that was only found in the parasite sequence. A major difference also exists between prokaryotic and eukaryotic GlnRS concerning the presence of an N-terminal extension of about 220 residues in all eukaryotic sequences. Surprisingly, this extension was not detected with SmGlnRS.

Discussion

Differential display techniques have been widely used to investigate gene expression in cells or tissues and more recently applied to the analysis of gene transcript levels in total organs and complex organisms. For example, Liebau et al. (2000) have recently used DD-RT PCR to study the effects of oxidative insult on transcription in the parasitic nematode *Onchocerca volvulus*. A DD-RT PCR related technique was also used to detect changes in gene expression in *B. glabrata* upon infection with *S. mansoni* (Lockyer et al. 2000). Using this technique, differences were also observed between haemocytes from schistosome-resistant or susceptible *B. glabrata* phenotypes (Schneider and Zelck 2001).

In this work, we describe the use of this technique for the analysis of gene expression in the sporocysts of *S. mansoni* and the effect of soluble components from host cells on gene transcription. Synxenic cultivation of sporocysts with Bge cells has been shown to allow the

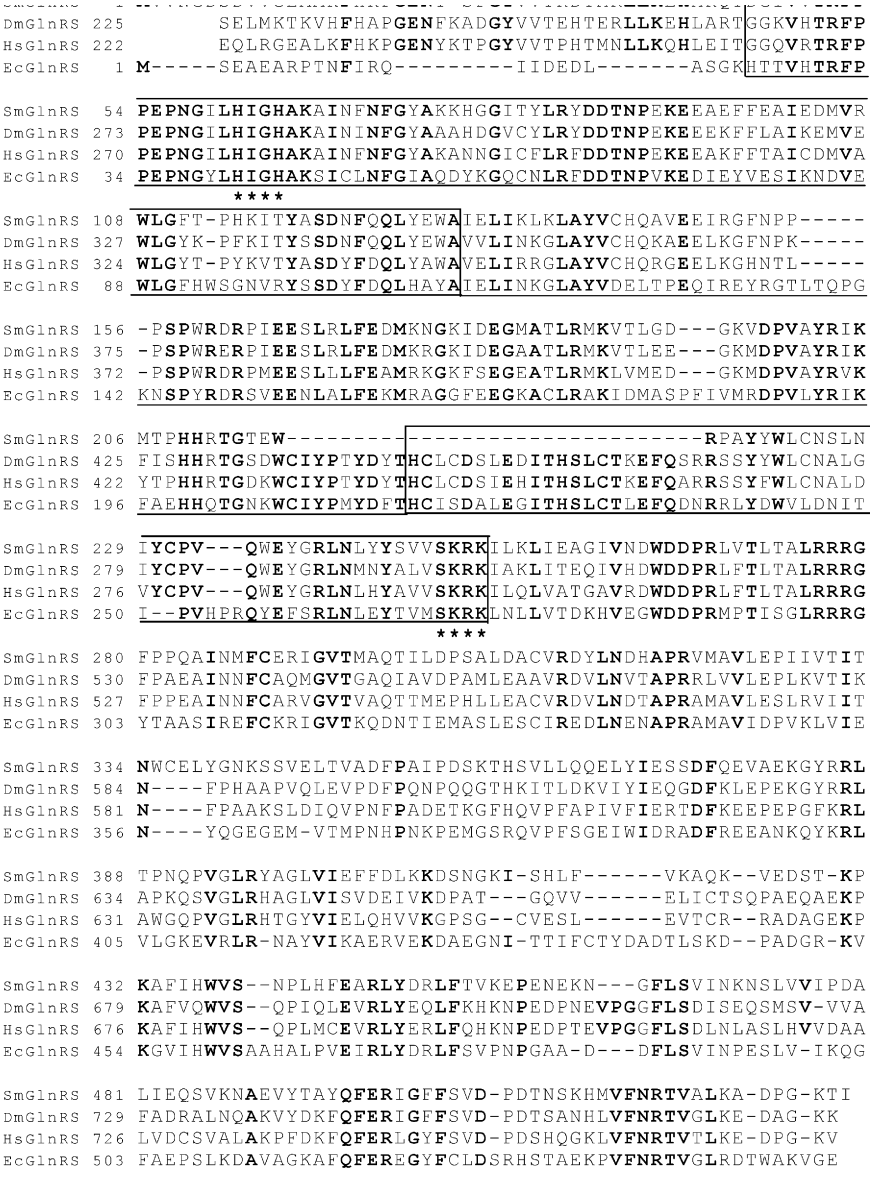

Fig. 4 Amino-acid sequence alignment by the Clustal method (Megalign, DNASTAR program) of glutaminyl-tRNA synthetases. Identical residues in the four sequences are in *bold letters*. Percentages of identity with the *S. mansoni* enzyme (SmGlnRS) are 63% for *Drosophila melanogaster* (DmGlnRS), 62% for *Homo sapiens* (HsGlnRS) and 42% for *Escherichia coli* (EcGlnRS). Gaps are indicated by *dashes*. HIGH and SKRK motifs are indicated by *asterisks*. The dinucleotide binding domains are *boxed* and the connective peptide is underlined

differentiation of in vitro-derived mother sporocysts into daughter sporocysts (Yoshino and Laursen 1995). In this model, the role of ES products from Bge cells was confirmed by the effect of Bge-conditioned medium on parasite growth as well as by the successful differentiation of sporocysts in membrane-separated cultures (Ivanchenko et al. 1999).

Preliminary results demonstrated that Bge ES products could influence the metabolic activity of *S. mansoni* sporocysts. The levels of nucleotide incorporation in total parasites did not clearly indicate an enhancement of DNA synthesis, but the labelling procedure is not sensitive enough to exclude the possibility that cell division only occurred in a limited number of cells, such as germinal cells. The results of amino acid incorporation, however, demonstrated a significant increase in protein synthesis in parasites exposed to cellular soluble factors that could be related to transcription modifications in the parasites. RT-PCR results showed that several transcripts were differentially present in exposed parasites and thus confirmed this hypothesis.

In our experiments, the electrophoretic patterns obtained were relatively simple probably due to the high A + T content of the schistosome genome (Musto et al. 1994). As was demonstrated for *Plasmodium yoelii* (Lau et al. 2000), the replacement of conventional primers by A + T rich primers should probably be helpful in optimizing amplification and obtaining more complex patterns.

Among the eight differential bands identified, two were of particular interest for studies of schistosome development. The up-regulation of their respective transcripts following contact with cell products was confirmed. The first fragment coded for a part of the CCT-complex protein γ subunit of *S. mansoni* (SmTCP-1C). The CCT is a heterooligomeric molecular chaper-

one assisting mainly in the folding of actin and tubulin (Kubota et al. 1995; Lewis et al. 1996; Willison and Horwitch 1996). Yokota et al. (1999) reported that CCT expression was closely correlated with cell growth in mouse and human cultured cells, but they also showed that the growth-dependent up-regulation of CCT did not always occur in cells abundant in tubulin or actin. These results suggested that the function of CCT in growing cells was not restricted to the folding of tubulin and actin and that CCT can interact with many other targets (Thulasiraman et al. 1999). In the human K14 cell line, CCT synthesis is down-regulated by agents affecting cell growth and differentiation (Hynes et al. 1996). Recently, Campos and Hamdan (2000) cloned a CCT component from *S. mansoni* corresponding to the α-subunit (SmTCP-1A) which was shown to be down-regulated under heat-shock and oxidative-stress conditions. The authors proposed that this down-regulation could reflect a switch in CCT subunits. It would be interesting to analyse a possible switch between SmTCP-1A and SmTCP-1C in stress conditions as well as in sporocysts exposed to Bge cells. Indeed, chaperone activity may be critical for the development and survival of the different life stages of *S. mansoni*, a parasite which is exposed to drastic environmental changes during its development.

The second gene up-regulated in Bge-exposed sporocysts encoded a protein homologous to the glutaminyl-tRNA synthetase, SmGlnRS, an enzyme that catalyses the ligation of glutamine to its cognate tRNA. Further cloning and sequencing of SmGlnRS indicated that the parasite protein was significantly homologous to the GlnRS of various species but differed from eukaryotic homologues essentially by the absence of the N-terminal extension. In human GlnRS, this extension is primarily involved in the formation of multi-enzymatic complexes associating different AARS activities (Lamour et al. 1994). In yeast, deletion experiments have shown that this extension was not essential for the catalytic activity of the enzyme (Ludmerer and Schimmel 1987). Therefore, we assume that SmGlnRS is an active enzyme that probably does not function in the context of multi-enzymatic complexes. SmGlnRS exhibits most of the characteristics of class I AARS (including GlnRS, GluRS, ArgRS, TrpRS, Tyr RS, MetRS, CysRS, IleRS, LeuRS and ValRS) (Delarue and Moras 1993) and particularly the two signature sequences (HIGH and SKRK) present, respectively, in the two homology boxes of the dinucleotide binding domain. However, SmGlnRS presents another particularity, i.e. the existence of a gap covering the last ten residues of the connective peptide and the first 21 residues of the second homology box. Different studies performed in *E. coli* have shown that extensive deletions in the connective peptide did not alter the catalytic activity of GlnRS (Schwob and Soll 1993) but to our knowledge the effects of limited deletion in the second homology box with conservation of the SKRK consensus are unknown. Molecular studies of the recombinant SmGlnRS need to be pursued in order to understand the implications of the particular features of the parasite enzyme for its specificity and catalytic activity.

In this work, the overexpression of SmGlnRS was associated with an increase of protein synthesis. In *Artemia*, Brandsma et al. (1997) have shown that the termination of quiescence was accompanied by an increase in the aminoacylation of transfer RNAs associated with higher levels of several AARS activities and concomitant with an activation of protein synthesis. AARS are considered to be housekeeping enzymes but in *E. coli* a metabolic control of the expression of the GlnRS gene occurs at the transcriptional level (Cheung et al. 1985). Similar results were also obtained by Hall and Yanofsky (1982) with *E. coli* and by Seshaiah and Andrew (1999) with the *D. melanogaster* tryptophanyl-tRNA synthetases. All these data show that the level of AARS, including GlnRS, increases during growth and is therefore in agreement with the higher levels of SmGlnRS transcripts observed in developing sporocysts.

In conclusion, our results have demonstrated significant effects of host cell components on the metabolic activity and the transcription of several genes in *S. mansoni* sporocysts. These differential transcripts probably represent only a limited sample of the panel of parasite genes regulated by mollusc signals. This work needs to be completed by the use of the other primers available in the GenHunter system and the design of primers better adapted to the DNA composition of schistosomes. It could also be interesting to investigate the kinetics of the expression of sporocyst genes following longer exposition to Bge cell products and during the differentiation of primary sporocysts into daughter sporocysts.

Acknowledgements The authors thank Raymond Pierce (Unite INSERM 547) for helpful discussions and Professor Michel Salzet (UPRESA 8017) for valuable help and encouragement. This work was supported by the Ministere Education Nationale Recherche Technologie (MENRT), the Institut National de la Sante et de la Recherche Medicale (INSERM), the Centre National de la Recherche Scientifique (CNRS) and the Institut Pasteur de Lille.

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