Quantitative assessment of sense and antisense transcripts from genes involved in antigenic variation (*vsp* genes) and encystation (*cwp 1* gene) of *Giardia lamblia* clone GS/M-83-H7

N. VON ALLMEN[1], M. BIENZ[2], A. HEMPHILL[1] *and* N. MÜLLER[1]*

[1] *Institute of Parasitology, University of Berne, Länggass-Strasse 122, CH-3012 Berne, Switzerland*
[2] *Hematology, Inselspital (University Hospital), University of Berne, Switzerland*

(*Received 5 August 2004; revised 14 September 2004; accepted 14 September 2004*)

SUMMARY

Antigenic variation of the intestinal protozoan parasite *Giardia lamblia* is caused by an exchange of the parasite's variant surface protein (VSP) coat. Many investigations on antigenic variation were performed with *G. lamblia* clone GS/M-83-H7 which produces surface antigen VSP H7. To generate novel information on giardial *vsp* gene transcription, *vsp* RNA levels were assessed by quantitative reverse transcription-(RT)-PCR in both axenic VSP H7-type trophozoites and subvariants obtained after negative selection of GS/M-83-H7 trophozoites by treatment with a cytotoxic, VSP H7-specific monoclonal antibody. Our investigation was not restricted to the assessment of the sense *vsp* transcript levels but also included an approach aimed at the detection of complementary antisense *vsp* transcripts within the two trophozoite populations. We found that sense *vsp H7* RNA predominated in VSP H7-type trophozoites while sense RNA from only one (*vsp IVg*) of 8 subvariant *vsp* genes totally analysed predominated in subvariant-type trophozoites. Interestingly, the two trophozoite populations exhibited a similar relative distribution regarding the *vsp H7* and *vsp IVg* antisense RNA molecules. An analogous sense versus antisense RNA pattern was also observed when the transcripts of gene *cwp 1* (encoding cyst wall protein 1) were investigated. Here, both types of RNA molecules only appeared after *cwp 1* had been induced through *in vitro* encystation of the parasite. These findings for the first time demonstrated that giardial antisense RNA production did not occur in a constitutive manner but was directly linked to complementary sense RNA production after activation of the respective gene systems.

Key words: *Giardia lamblia*, antigenic variation, encystation, transcription, antisense RNA.

INTRODUCTION

Giardia lamblia (*Giardia duodenalis*, *Giardia intestinalis*) is a zoonotic protozoan parasite which resides in the small intestine of human and various mammalian hosts. In humans, *G. lamblia* is a common cause of endemic and epidemic diarrhoea throughout the whole world. Transmission of the parasite from one to another host individual occurs through peroral ingestion of cysts which, following excystation in the small intestine, release 2 trophozoites each. The life-cycle is completed when cysts are formed through encystation of proliferating trophozoites and subsequently excreted in the feces. Both, en- and excystation can also be triggered *in vitro* by applying *in vitro* growth conditions which – to a certain extent – simulate the intestinal (for encystation) and gastric (for excystation) milieu of the mammalian hosts.

* Corresponding author: Institute of Parasitology, Länggass-Strasse 122, CH-3012 Berne, Switzerland. Tel: +41 31 631 24 74. Fax: +41 31 631 26 22. E-mail: nmueller@ipa.unibe.ch

As extensively assessed in the *in vitro* cultivation system, stage conversion is associated with induction of various genes, some of which are structurally involved in cyst formation of the parasite (Hehl *et al.* 2000; Sun *et al.* 2002; Davis-Hayman *et al.* 2003; Lujan & Touz, 2003; Marti *et al.* 2003). Here, particularly the *cwp* genes encoding the cyst wall proteins are massively upregulated during encystation. Previous studies on cyst wall proteins (CWP) 1 (Hehl *et al.* 2000) and 2 (Davis-Hayman *et al.* 2003) indicated that control of the corresponding genes, *cwp 1* and *cwp 2*, occurs on the transcriptional level and involves *cis*-acting elements in the 5′ flanking sequence of the gene. Transcriptional activation of encystation-inducible genes involves a nuclear protein which is related to the Myb family transcription factors and initiates transcription by interacting with a specific binding site in the promoter region (Sun *et al.* 2002). As demonstrated for gene *cwp 1*, steady-state levels of cellular mRNA are modulated by a cis-element in 3′ untranslated region which seems to be crucial for the processing of the transcripts during the late stage of the encystation (Hehl *et al.* 2000).

Parasitology (2005), **130**, 389–396.
DOI: 10.1017/S0031182004006742 Printed in the United Kingdom

In the past decade, *G. lamblia* has been especially investigated in terms of the parasite's ability to continuously change its surface antigen coat (Müller & Gottstein, 1998; Nash, 2002). These studies have revealed that antigenic variation is associated with a unique family of surface antigens, named VSP (variant surface protein). By using trophozoites of *G. lamblia* clone GS/M-83-H7 (expressing VSP H7) and the neonatal mouse model for experimental infections, we recently quantitatively assessed the process of antigenic variation of the parasite on the transcriptional level (von Allmen *et al*. 2004). In this study, variant-specific regions identified on different GS/M-83-H7 *vsp* sequences served as targets for quantitative reverse transcription (RT)-PCR to monitor alterations in *vsp* mRNA levels during infection. Respective results demonstrated that antigen switching of both the duodenal trophozoite and the caecal cyst populations was associated with a strong reduction in *vsp H7* mRNA levels. The same study also explored giardial variant-type formation and *vsp* mRNA levels after infection of mice with antigenically diversified (VSP H7-negative) GS/M-83-H7 cysts. This infection mode led to an antigenic reset of the parasite in that a VSP H7-negative inoculum 'converted' into a population of intestinal trophozoites that essentially consisted of the original VSP H7-type.

Antigenic variation and the VSPs have been extensively investigated on the molecular, biochemical and immunological level (Müller *et al*. 1998; Nash, 2002). Conversely, the genetic mechanisms controlling antigen switching are, as yet, poorly understood and only little information on this process is available in current literature. Nash *et al*. (2001) demonstrated that the shift of VSP expression coincides with transient appearence of few trophozoites which simultaneously express at least two VSPs. Furthermore, preliminary data suggested that VSP expression may be regulated at the transcriptional level (Nash & Mowatt, 1992). However, another study tackling the genome organization of a novel family of cysteine-rich proteins (CRP65, CRP136) in *G. lamblia* may allow the consideration of a gene rearrangement effect as a possible mechanism responsible for antigen switching of the parasite (Cheng, Upcroft & Upcroft, 1996; Upcroft *et al*. 1997). Since Elmendorf, Singer & Nash (2001) recently found that *G. lamblia* contains sterile antisense *vsp* RNA, reverse RNA transcription, and more specifically, RNA interference (RNAi) (Tijsterman & Plasterk, 2004) is currently also discussed as a potential mechanism that might be involved in *vsp* gene regulation (Ullu, Tschudi & Chkrabortx, 2004). In this context, the review of Ullu *et al*. (2004) refered to as yet unpublished findings from H. Lujan and colleagues, which suggested that RNAi controls expression of the *vsp* genes and that an RNA-dependent RNA polymerase is involved in restricting expression of the *vsp* gene repertoire to a single gene at one time. Interestingly, database mining provided clear evidence that the genome of *G. lamblia* encodes key functions of the RNAi pathway (Ullu *et al*. 2004). The functionality of this pathway in *G. lamblia* has already been experimentally proven in that transfection of the parasite with a vector carrying an antisense sequence of a target gene was demonstrated to downregulate corresponding gene expression (Touz *et al*. 2002). RNAi is now basically considered to be an ideal molecular biological tool to achieve targeted silencing of any gene function in various protozoan parasites, including *G. lamblia* (Ullu *et al*. 2004).

Elmendorf *et al*. (2001) found that *G. lamblia* trophozoites generate a substantial amount of antisense RNA molecules which correspond to approximately 20% of the total RNA content. Antisense RNA production in *G. lamblia* is highly diversified and involves *vsp* genes as well as many other constitutive and regulated genes, which e.g. are (possibly) relevant to developmental stage conversion, cell division, and gene transcription. The antisense transcripts are polyadenylated and they essentially consist of sterile RNA molecules that do not participate in protein synthesis of the parasite. The study described by Elmendorf *et al*. (2001) clearly demonstrated that antisense RNA production represents a striking molecular biological feature of *G. lamblia*. However, this investigation was not focused on the possible gene-regulatory function of antisense transcripts and a preliminary Northern blot analysis included in the respective experimentation was not able to reveal a participation of these RNA molecules in developmental gene regulation.

The major aim of the present study was to evaluate the possibility of a bidirectional *vsp* gene transcription in association with *in vitro* antigenic variation of *G. lamblia* clone GS/M-83-H7. For this purpose, VSP H7-type and subvariant-type GS/M-83-H7 trophozoite cultures were generated and subsequently analysed by quantitative reverse RT-PCR for their relative production of sense and antisense *vsp H7* or subvariant *vsp* RNA, respectively. The quantitative RT-PCR approach was also applied for investigating the giardial sense and antisense transcription in the encystation-inducible gene system *cwp 1* encoding the major cyst component CWP 1 of the parasite.

MATERIALS AND METHODS

Parasite and in vitro *growth conditions*

The origin, axenization and cloning of *G. lamblia* clone GS/M-83-H7 has been described by Aggarwal *et al*. (1989). This clone expresses a major 72 kDa antigen (VSP H7) on its surface which is recognized by MAb G10/4. Trophozoites from *G. lamblia* clone GS/M-83-H7 were cultivated in modified TYI-S-33

https://doi.org/10.1017/S0031182004006742 Published online by Cambridge University Press

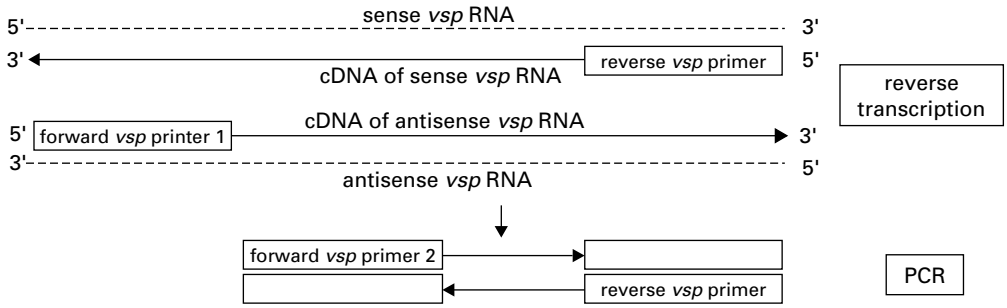

Fig. 1. Schematic illustration of the RT-PCR-based quantification of sense and antisense *vsp* RNA. First, sense and antisense RNA (within total RNA preparations) from VSP H7-type and subvariant-type *G. lamblia* clone GS/M-83-H7 trophozoites were reverse transcribed into cDNA using reverse *vsp* primer (synthesis of cDNA from sense *vsp* RNA) or forward *vsp* primer 1 (synthesis of cDNA from antisense RNA). The cDNAs were taken as template for quantitative PCRs which allowed specific amplification of different *vsp* cDNA molecules. Amplification of *vsp* cDNA was achieved by performing the PCR with forward *vsp* primers listed in Table 1 and the reverse *vsp* primer as indicated.

medium with antibiotics as previously described (Keister, 1983). *In vitro* growth conditions for induction of encystation of the parasite were described by Kane *et al*. (1991).

Immunofluorescence assays

Expression of the major surface antigen VSP H7 in *in vitro* cultivated GS/M-83-H7 trophozoites was tested by immunofluorescence using VSP H7-specific MAb G10/4 as described (Gottstein *et al*. 1990). CWP 1 synthesis in encysting trophozoites was detected by incubation of cells with 1 : 20 Texas Red-conjugated mouse MAb A300-TR (Waterborne, New Orleans, LA, USA), an anti-CWP 1 antibody. For staining of nuclei, trophozoites were incubated for 3 min in the presence of 1 : 300-diluted double strand (ds) DNA-specific fluorescent dye Hoechst 33258 (Sigma, Steinheim, Germany) stock solution (1 mg/ml in distilled H_2O) and subsequently washed twice in PBS and once in distilled H_2O.

Specimens were inspected on a Nikon Eclipse E800 digital confocal fluorescence microscope at a 600-fold magnification. Processing of images was performed using the Openlab 3.11 software (Improvision, Heidelberg, Germany). Percentages of VSP H7- or CWP 1-positive parasites were determined by inspection of approximately 400 parasites.

Antigen switching by GS/M-83-H7 trophozoites in vitro*, sample collection and total RNA extraction*

The procedure used for cytotoxic (VSP H7-specific) MAb G10/4-mediated antigen switching of *in vitro* cultivated *G. lamblia* clone GS/M-83-H7 trophozoites from a VSP H7-positive to a – negative (subvariant-type) parasite population was previously described (Bienz *et al*. 2001). After confirming by immunofluorescence that the resulting MAb G10/4-resistant parasite population was essentially VSP H7-negative (> 99%), about 10^6 of these negatively-selected variant trophozoites were resuspended in 100 μl of lysis buffer-β-ME mixture from the StrataPrep^{TM} Total RNA Microprep Kit (Stratagene, La Jolla, CA, USA). In parallel, an analogous cellular lysate was prepared from about 10^6 VSP H7-type trophozoites that had not been treated with MAb G10/4. Both cellular lysates were then further processed for total RNA extraction as described above. Finally, total RNA preparations were solubilized in 30 μl of elution buffer and stored at $-80\ ^{\circ}C$ until further used.

Analysis of vsp *mRNA by quantitative reverse transcriptase (RT)-PCR*

Complementary DNA (cDNA) was synthesized by reverse transcription from total RNA, prepared from VSP H7-type and subvariant-type trophozoites by using 17·5 μM of degenerate forward *vsp* primer 1 (5′-ACIAAYGGIGTITGYACIGC-3; positions I = deoxyinosine, Y = C,T; Invitrogen, Basle, Switzerland) (reverse transcription of antisense *vsp* RNA) or reverse *vsp* primer (5′-GAACCACCAGC AGAGGAA-3′) (reverse transcription of sense *vsp* RNA) (see Fig. 1) as well as M-MLV reverse transcriptase (Promega, Madison, WI, USA) and other components as instructed by the manufacturer of the reverse transcriptase. The forward *vsp* primer 1 covers a relatively conserved *vsp* region located between nucleotides (nt) 1147 and 1166 of gene *vsp H7*. The reverse *vsp* primer is complementary to nt 1636 to 1653 of the *vsp H7* encoding part of the highly conserved transmembrane domain of the surface protein. The reverse transcription reaction for generating *cwp 1* cDNA included forward *cwp 1* primer 1 (5′-CACCTGGACTGCAACCAGCTG-3′) (reverse transcription of antisense *cwp 1* RNA) and reverse *cwp 1* primer (5′-AGTACTCTCCGCAGT CCGGATC-3′) (reverse transcription of sense *cwp 1* RNA). The forward *cwp 1* primer 1 corresponds to nt positions 6 to 26 and the reverse *cwp 1* primer to nt positions 207 to 228 of a 3′ terminal GS/M-83-H7 *cwp1* gene segment (GeneBank^{TM} Accession

https://doi.org/10.1017/S0031182004006742 Published online by Cambridge University Press

Table 1. Forward *vsp* primers used for quantification of *Giardia lamblia* GS/M-83-H7 *vsp* transcripts by RT-PCR

vsp gene	GeneBank™ Accession no.	Nt position of primer on *vsp* sequence	Forward *vsp* primer sequence
H7	M80480	1282–1305#	5′CAAGATAAAGACAGCAATGGTTCA3′
IVc	AF354540	117–144*	5′CAAACAGCAGACAGTGGGACAGGATCC3′
IVe	AF354542	37–59*	5′CAACAGCCAGCTAGTGGTGTG3′
IVf	AF354543	146–165*	5′CAACAGCCAGCTAGTGGGGTC3′
IVd	AF354541	142–162*	5′CAAACATATGCTAATAACAAC3′
IVh	AF354545	142–162*	5′CAACAGCCAGCTAGTGGGGTC3′
IVb	AF354525	184–207*	5′CAAAGTGCAAATGGTCAAGGCGTG3′
IVg	AF354544	138–165*	5′TATGTCAAGCTCAGCAACGCTCAAACT3′
IVa	AF354539	46–66*	5′CAGAATCCTACTAATGGCAAT3′

Nucleotide (nt) positions from complete *vsp H7* sequence (Nash & Mowatt, 1992).
* Nt positions from a partial sequence proximal to the 3′ terminus of the *vsp* gene (von Allmen *et al*. 2004).

number: AY676465). Reverse transcription of sense RNA from gene *gdh* (encoding glutamate dehydrogenase) was performed as previously described (von Allmen *et al*. 2004).

Quantitative RT-PCR was carried out on a LightCycler™ Instrument (Roche Diagnostics, Rotkreuz, Switzerland) by using SYBR™ Green I as a dsDNA-specific fluorescent dye and continuous fluorescence monitoring as described (Wittwer *et al*. 1997). Amplification reaction mixes for the *vsp* PCRs included forward primers specific for individual *vsp* genes (indicated in Fig. 1 as forward *vsp* primer 2 and listed in Table 1), and reverse *vsp* primer. For the *cwp 1* PCRs, forward *cwp 1* primer 2 (5′-GTCCC AGTTGGCCTTATGACTCT-3′, corresponding to nt positions 36 to 58 of 3′ terminal GS/M-83-H7 *cwp 1* gene segment) and reverse *cwp 1* primer (see above) were applied. Quantitative PCR was done with 4 μl of 1 : 100-diluted cDNA using the Quanti Tect™ SYBR Green PCR Kit (Qiagen) in a 10 μl standard reaction containing a 0·5 μM concentation of forward and reverse primers (Invitrogen). All PCRs containing cDNA were performed in triplicates. Furthermore, a control PCR included RNA equivalents from samples that had not been reverse transcribed into cDNA (not shown) to confirm that no DNA was amplified from any residual genomic DNA that might have resisted DNase I digestion (see above). PCR was started by initiating the 'Hot-Start' Taq DNA-polymerase reaction at 95 °C (15 min). Subsequent DNA amplification was done in 50 cycles including denaturation (94 °C, 15 s), annealing (48 °C, 30 s), and extension (72 °C, 30 s); temperature transition rates in all cycle steps were 20 °C/s. Fluorescence was measured at 82 °C during the temperature shift after each annealing phase in the 'single' mode with the channel setting F1. Fluorescence signals from the amplification products were quantitatively assessed by applying the standard software (version 3.5.3) of the LightCycler™ Instrument. Quantification of PCR products was performed during the log phase of the reaction and was achieved by using the secondary derivative maximum mode for plotting of the fluorescence signals versus the cycle numbers. As external standards, serial 10-fold dilutions (4 μl aliquots) of previously generated amplification products from the different target sequences were included in the quantitative PCR analyses. The standard curves from the different assays (*vsp*- and *cwp 1*-PCRs) were run in duplicates and contained 4 log units within a linear range that essentially covered the maximal and minimal concentrations of the *vsp*- and *cwp1*-cDNA sequences within the different samples. Linearity among the standard reactions was reflected by the correlation coefficient which was calculated by the computer program to be extremely high (between 0·99 and 1·0) for all PCR assays applied.

Lack of PCR-inhibitory effects and overall comparability of the different standard and sample reactions were evidenced by the quasi-identity of the slopes from the amplification plots (monitoring amplification rates).

Control experiments for identification of PCR products included a DNA melting point analysis (Ririe, Rasmussen & Wittwer, 1997) (not shown). The DNA melting profile assay was run after the final PCR cycle by gradually increasing the temperature to 95 °C at a transition rate of 0·1 °C/s with continuous acquisition (determination of the melting profile by measuring loss of fluorescence). Data from the DNA melting profile assay were processed by using the standard software (version 3.5.3). In all PCR tests performed, identical melting temperatures of amplicons from samples and respective standards indicated identical and specific amplification reactions without unwanted primer-dimer formation (not shown). This overall identity and specificity of reactions was confirmed by subsequent agarose gel electrophoresis (2% gels) (Sambrook & Russel, 2001) which monitored the PCR products as single DNA bands of expected sizes (not shown). In the cases of

https://doi.org/10.1017/S0031182004006742 Published online by Cambridge University Press

the PCRs amplifying segments of *vsp H7*, *vsp IVg*, and *cwp 1*, nucleotide sequence authenticity of the amplification products was confirmed by automated sequencing through a commercial sequencing service (Microsynth, Balgach, Switzerland).

In order to compensate for variations in input, RNA amounts and efficiencies of reverse transcription, sense RNA of the 'housekeeping' gene *gdh* was quantitated as recently described (von Allmen *et al*. 2004). Respective mean values from triplicate determinations were taken for the calculation of the relative sense and antisense *vsp* RNA levels (*vsp* RNA level/*gdh* RNA level) or sense and antisense *cwp 1* RNA levels (*cwp 1* RNA level/*gdh* RNA level), respectively.

Sequence alignments

Alignment of the *vsp* nucleotide sequences derived from a previous study (Bienz *et al*. 2001) and accessible in GenBank™ (Accession numbers, see Table 1), was done using the MultAlin™ and the ESPript1.9™ computer software, available at the ExPASy™ Molecular Biology Server.

RESULTS

RT-PCR-based quantification of sense and antisense vsp *RNA associated with* in vitro *antigen switching of* G. lamblia *clone GS/M-83-H7*

To select for new variants within *G. lamblia* clone GS/M-83-H7 originally consisting of approximately 95% VSP H7-type (MAb G10/4-positive) trophozoites (not shown), cultivated parasites were treated twice with VSP H7-specific, cytotoxic MAb G10/4. The efficacy of this selection was assessed by immunofluorescence which demonstrated that the treated culture contained more than 99% VSP H7-negative (MAb G10/4-negative) trophozoites (not shown).

For RT-PCR-based quantitative assessment of sense and antisense *vsp* RNA levels in VSP H7-type and subvariant-type GS/M-83-H7 trophozoites, total RNA was prepared and then used as a templete for synthesis of cDNA representing either sense or antisense *vsp* RNA (Fig. 1). The primers for reverse transcription were designed on the basis of previously generated GS/M-83-H7 *vsp* gene sequence information and allowed synthesis of cDNAs from sense and antisense RNA templates covering the 3′ terminal region of the individual *vsp* genes (Fig. 1). For specific reverse transcription of sense RNA, a primer (reverse *vsp* primer) was used that was complementary to nts 1636 to 1653 close to the 3′ terminus of the *vsp H7* encoding sequence. This sequence encodes part of the transmembrane domain of the surface protein and had previously been revealed to be highly conserved within the *vsp* genes of clone GS/M-83-H7 and other *G. lamblia* isolates

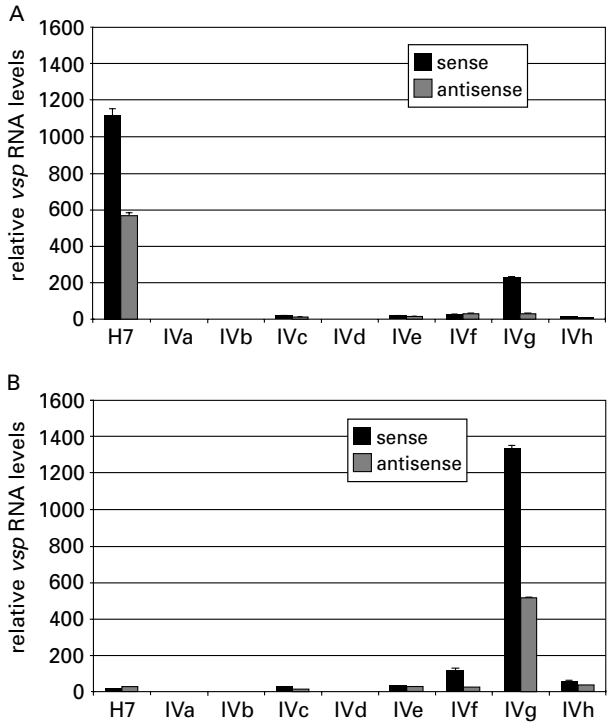

Fig. 2. Quantitative RT-PCR-based assessment of relative sense (black bars) and antisense (grey bars) *vsp* RNA levels (*vsp* RNA level/*gdh* RNA level) in VSP H7-type (A) and subvariant-type (B) trophozoite cultures from *Giardia lamblia* clone GS/M-83-H7. The relative levels of mRNA from *vsp* genes H7 and IVa-IVh (see Table 1) represent mean values (plus standard deviations) from triplicate determinations.

(Bienz *et al*. 2001). For specific reverse transcription of antisense *vsp* RNA, we applied a previously described degenerate primer (forward *vsp* primer 1) corresponding to nt positions 1147 to 1166 of *vsp H7* and covering a relatively conserved region of GS/M-83-H7 *vsp* genes (von Allmen *et al*. 2004).

To establish a PCR system for quantitative and differential amplification of cDNA synthesized from sense and antisense *vsp* RNA, a nucleotide sequence alignment was performed with 3′ regions of gene *vsp H7* and subvariant *vsp* genes that had previously been identified in VSP H7-negative trophozoite cultures of *G. lamblia* clone GS/M-83-H7. This comparative sequence analysis led to the identification of a relatively variable *vsp* region which allowed to design forward primers (forward *vsp* primer 2) that were specific for individual *vsp* genes (Table 1). Each of these forward *vsp* primers in combination with a reverse primer (reverse *vsp* primer) targeted to a conserved *vsp* stretch close to the 3′ terminus (Fig. 1) were used for PCR-based specific quantification of *vsp* cDNA molecules generated from VSP H7-type and subvariant-type trophozoite cultures outlined above.

As can be seen in Fig. 2, sense *vsp H7* RNA predominated in VSP H7-type trophozoites. Subvariant-type trophozoites, however, did not

https://doi.org/10.1017/S0031182004006742 Published online by Cambridge University Press

produce significant amounts of sense *vsp H7* RNA but exhibited predominance of subvariant sense *vsp IVg* RNA. The low, but clearly detectable, amount of sense *vsp IVg* RNA in the VSP H7-posititive culture was indicative for the existence of a minor subvariant-type population representing approximately 5% of the total trophozoite population (see above). Taken together, these finding were in concordance with results from the immunofluoresence analysis (not shown) indicating that *in vitro* antigen switching 'converted' an essentially VSP H7-positive into a VSP H7-negative (subvariant-type) trophozoite culture. Furthermore, data evidenced at least on the transcriptional level that the *in vitro*-switched trophozoite culture represented a relatively homogeneous subvariant-type population which preferentially expressed *vsp IVg*.

RT-PCR-based assessment of the antisense *vsp* RNA contents in both VSP H7-type and subvariant-type trophozoites provided analogous results although cellular concentrations of these molecules were lower than those of corresponding sense RNAs. Antisense *vsp H7* RNA predominated in VSP H7-positive trophozoites and antisense *vsp IVg* RNA in -negative trophozoites. Sequence analysis revealed that sense and antisense RNAs from these two genes were fully complementary, and the corresponding PCR products exhibited the expected sizes of 377 base pairs (bp) (sense and antisense *vsp H7* RNA) and 361 bp (sense and antisense *vsp IVg* RNA), respectively.

RT-PCR-based quantification of sense and antisense cwp 1 *RNA associated with encystation of* G. lamblia *clone GS/M-83-H7*

To test whether the simultaneous production of sense and complementary antisense RNA is unique to the *vsp* genes or rather a more general phenomenon of giardial gene transcription, we tested an encystation-inducible gene system, *cwp 1*, regarding its sense versus antisense transcription profile. Gene *cwp 1* encodes cyst wall protein (CWP) 1 which represents a major component of the giardial cyst (Lujan *et al*. 1995; Mowatt *et al*. 1995). As assessed by immunofluorescence assay using an antibody against CWP 1 for immunostaining of VSP H7-positive GS/M-83-H7 trophozoites, *in vitro* stage conversion resulted in approximately 12% CWP 1-positive parasites after 24 h growth in encystation medium (not shown). For RT-PCR-based quantitative determination of sense and antisense *cwp 1* RNA levels in trophozoites, corresponding cDNA was generated from parasites sampled at 0, 6, 12 and 24 h of the encystation period. The RT-PCR approach monitoring relative sense and antisense *cwp 1* levels in encysting trophozoites was designed in analogy to the procedure applied for the assessment of sense and antisense *vsp* RNA (see above). For specific reverse transcription of sense RNA, reverse *cwp 1* primer was used that was complementary to nts 207 to 228 of a 3′ terminal GS/M-83-H7 *cwp 1* gene segment. For specific reverse transcription of antisense *vsp* RNA, we applied forward *cwp 1* primer 2 corresponding to nt positions 6 to 26 of the same gene segment.

RT-PCR-based quantification of *cwp 1* RNA revealed that *in vitro* encystation of clone GS/M-83-H7 induced simultaneous synthesis of sense and, to a lower extent, antisense *cwp 1* RNA. While sense *cwp 1* RNA levels increased for at least 12 h during encystation, antisense *cwp 1* RNA already reached its maximal level at 6 h. At 24 h, a strong reduction in the cellular contents of both sense and antisense *cwp 1* RNA was detected. Sequence analysis demonstrated full complementarity between the sense and antisense *cwp 1* RNAs, and sizes of the *cwp 1* amplicons were determined to be 192 bp (not shown).

DISCUSSION

The discovery of giardial antisense *vsp* transcription has implications for the current thinking about the as yet poorly understood genetic mechanism controlling surface antigen alterations in *G. lamblia* (Ullu *et al*. 2004). Considering still unpublished data presented by H. Lujan and colleagues at the 2002 Molecular Parasitology Meeting in Woods Hole (MA, USA), particularly RNA interference (RNAi) (Tijsterman & Plasterk, 2004) has to be discussed as a potential mechanism regulating giardial *vsp* gene activity. Such a mechanism could favour transcription of a primary *vsp* gene in that all other genes from the *vsp* repertoire are silenced by overproduction of complementary antisense *vsp* RNA molecules, and small (20–26 nucleotide pairs) interfering RNA (siRNA) (Tijsterman & Plasterk, 2004), respectively. According to this scenario, antigen switching could occur through a spontaneous, or controlled, mechanism which up-regulates antisense RNA from the primary *vsp* gene and down-regulates antisense RNA from a secondary *vsp* gene. In the present study, we demonstrated for the first time that *in vitro* antigen switching of *G. lamblia* is associated with alterations in both sense and antisense *vsp* RNA production. In our analysis, we found that sense and complementary antisense *vsp* RNA production in both VSP H7-type and subvariant-type trophozoites occurred in a simultaneous, and not a reciprocal, manner. This finding may be taken as an argument against the above-mentioned speculation suggesting that RNAi could act as a mechanism involved in regulation of *vsp* gene expression during antigen switching of the parasite. The solution of this problem relies on the determination of the relative *vsp H7* versus *vsp IVg* RNA composition (see above) on the level of the extremely fragmented siRNA sequences. Respective experimentation will be rather difficult because the

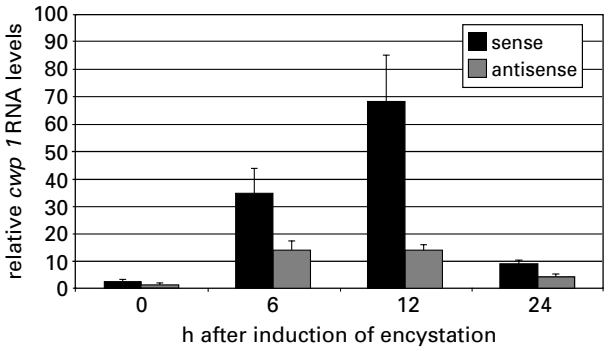

Fig. 3. Quantitative RT-PCR-based assessment of relative sense (black bars) and antisense (grey bars) *cwp 1* RNA levels (*cwp 1* RNA level/*gdh* RNA level) in *Giardia lamblia* clone GS/M-83-H7 trophozoites sampled at 0, 6, 12, and 24 h after induction of encystation. The relative levels of mRNA from *cwp 1* gene represent mean values (plus standard deviations) from triplicate determinations.

standard RNA isolation protocols were not designed to efficiently recover small RNA pieces. Furthermore, methods for detection of small RNA molecules are supposed to be relatively insensitive.

As outlined in the Materials and Methods section, the individual *vsp* primers used for the quantitative RT-PCRs target to a sequence stretch that is located in the 3′ terminal region of the corresponding *vsp* genes. In this region, the *vsp H7* sequence is indistinguishable from the sequence of another *vsp* gene (*vsp H7-1*) previously identified in *G. lamblia* clone GS/M-83-H7 (Nash, Conrad & Mowatt, 1995). Accordingly, our present RT-PCR approach was not suitable to discriminate between transcripts from these two closely related genes. However, since *vsp H7-1* had previously been shown to be silent in clone GS/M-83-H7 (Nash *et al*. 1995), we concluded that our approach must have exclusively monitored transcripts that originated from *vsp H7*.

By analysing *cwp 1* transcription of *G. lamblia* clone GS/M-83-H7, we detected maximal levels of sense and antisense RNA between 6 h (antisense RNA) and 12 h (sense RNA) after encystation had been induced (see Fig. 3). Interestingly, the *cwp 1* gene activation resulted in sense/antisense co-expression patterns that resembled those observed for transcription of the *vsp* genes. Based on this observation, a participation of RNAi in control of the *cwp 1* gene activity can still not be excluded but seems to be rather unlikely (see also above). This assumption is compatible with data from previous studies which demonstrated that the up-shift of the *cwp 1* mRNA levels during early encystation is the consequence of the induction of the transcriptional activity of a classical promoter (Hehl *et al*. 2000; Sun *et al*. 2002).

In agreement with previous data (Hehl *et al*. 2000), we found that late-stage encystation was associated with a dramatic decrease of the intracellular *cwp 1* mRNA concentration. In addition, we observed that the antisense *cwp 1* RNA levels started to decrease earlier (before 12 h) during encystation than complementary sense RNA levels (between 12 and 24 h). According to data from Hehl *et al*. (2000), the reduction in the sense *cwp 1* RNA concentration may be due to a post-transcriptional RNA degradation process which is modulated by a *cis*-acting element in the 3′ untranslated region of the RNA molecules. Since antisense *cwp 1* RNA is supposed to lack an equivalent regulatory element, it may even undergo faster degradation than sense *cwp 1* RNA. Such a differential RNA degradation process may have caused the observed bias in the relative sense and antisense *cwp 1* RNA levels at advanced encystation stages of the parasite.

As assessed by determination of the nucleotide sequences of RT-PCR amplification products, sense and antisense RNAs from *vsp H7*, *vsp IVg*, and *cwp 1* exhibited complete sequence complementarity. This suggested that the individual sense/antisense RNA pairs had been transcribed in *cis* from opposing DNA strands at the same genetic locus. A significant amount of antisense RNA molecules from many different genes including *vsp* genes had already been detected in *G. lamblia* clones GS/M-83-H7 and WB/1267, respectively (Elmendorf *et al*. 2001). In this previous investigation, the overall content of sense RNA was estimated to be approximately four times higher than the content of antisense RNA. Based on this finding, the authors concluded that this comparably low synthesis of antisense RNA was eventually driven by frequently occurring AT-rich sequences acting as cryptic promoters on the antisense DNA strand of the respective gene loci. This conclusion is compatible with our present data demonstrating that giardial antisense RNA contents were substantial, but unambigously lower than those of simultaneously produced sense RNA molecules.

It is certainly feasible that antisense RNA production in *G. lamblia* is only a tribute to the simplicity of this organism which perhaps cannot control 'unspecific' antisense gene transcription eventually occurring as a side-effect of DNA unwinding during regular gene transcription. Nevertheless, it has to be kept in mind that the parasite has evidently maintained abundant antisense RNA production during evolution although a high energy metabolism is needed for running this process. Accordingly, further studies will have to address the question whether antisense RNA production in *G. lamblia* is biologically significant in that it is relevant for the cell biology or even pathogenicity of the parasite.

We acknowledge A. Hehl, M. Marti (Institute of Parasitology, Zürich, Switzerland), N. Keller (Institute of Parasitology, Berne, Switzerland) and M. E. Weiland (Karolinska Institute, Stockholm, Sweden) for technical support, and T. E. Nash (NIH, Bethesda, Maryland,

USA) for his gift of MAb G10/4 and *G. lamblia* clone GS/M-83-H7. This work was financed through a grant obtained from the Swiss National Science Foundation (No. 31-066795.01).

REFERENCES

AGGARWAL, A., MERRITT, J. W. & NASH, T. E. (1989). Cysteine-rich variant surface proteins of *Giardia lamblia*. *Molecular and Biochemical Parasitology* **32**, 39–48.

BIENZ, M., SILES-LUCAS, M., WITTWER, P. & MÜLLER, N. (2001). *Vsp* gene expression by *Giardia lamblia* clone GS/M-83-H7 during antigenic variation *in vivo* and *in vitro*. *Infection and Immunity* **69**, 5278–5285.

CHENG, N., UPCROFT, J. A. & UPCROFT, P. (1996). A new cysteine-rich protein-encoding gene family in *Giardia duodenalis*. *Gene* **169**, 33–38.

DAVIS-HAYMAN, S. R., HAYMAN, J. R. & NASH, T. E. (2003). Encystation-specific regulation of the cyst wall protein 2 gene in *Giardia lamblia* by multiple *cis*-acting elements. *International Journal for Parasitology* **33**, 1005–1012.

ELMENDORF, H. G., SINGER, S. M. & NASH, T. E. (2001). The abundance of sterile transcripts in *Giardia lamblia*. *Nucleic Acids Research* **29**, 4674–4683.

GOTTSTEIN, B., HARRIMAN, G. R., CONRAD, J. T. & NASH, T. E. (1990). Antigenic variation in *Giardia lamblia*: Cellular and humoral immune response in a mouse model. *Parasite Immunology* **12**, 659–673.

HEHL, A. B., MARTI, M. & KÖHLER, P. (2000). Stage-specific expression and targeting of cyst wall protein-green fluorescent protein chimeras in *Giardia*. *Molecular Biology of the Cell* **11**, 1789–1800.

KANE, A. V., WARD, H. D., KEUSCH, G. T. & PEREIRA, M. E. (1991). *In vitro* encystation of *Giardia lamblia*: large-scale production of *in vitro* cysts and strain and clone differences in encystation efficiency. *International Journal for Parasitology* **77**, 974–981.

KEISTER, D. B. (1983). Axenic culture of *Giardia lamblia* in TYI-S-33 medium supplemented with bile. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **77**, 487–488.

LUJAN, H. D., MOWATT, M. R., CONRAD, J. T., BOWERS, B. & NASH, T. E. (1995). Identification of a novel *Giardia lamblia* cyst wall protein with leucine-rich repeats. Implications for secretory granule formation and protein assembly into the cyst wall. *Journal of Biological Chemistry* **270**, 29307–29313.

LUJAN, H. D. & TOUZ, M. C. (2003). Protein trafficking in *Giardia lamblia*. *Cellular Microbiology* **5**, 427–434.

MARTI, M., REGOS, A., LI, Y., SCHRANER, E. M., WILD, P., MÜLLER, N., KNOPF, L. G. & HEHL, A. B. (2003). An ancestral secretory apparatus in the protozoan parasite *Giardia intestinalis*. *Journal of Biological Chemistry* **278**, 24837–24848.

MOWATT, M. R., LUJAN, H. D., COTTEN, D. B., BOWERS, B., YEE, J., NASH, T. E. & STIBBS, H. H. (1995). Developmentally regulated expression of a *Giardia lamblia* cyst wall protein gene. *Molecular Microbiology* **15**, 955–963.

MÜLLER, N. & GOTTSTEIN, B. (1998). Antigenic variation and the murine immune response to *Giardia lamblia*. *International Journal for Parasitology* **28**, 1829–1839.

NASH, T. E. (2002). Surface antigenic variation in *Giardia lamblia*. *Molecular Microbiology* **45**, 585–590.

NASH, T. E., CONRAD, J. T. & MOWATT, M. R. (1995). *Giardia lamblia*: identification and characterization of a variant-specific surface protein gene family. *Journal of Eukaryotic Microbiology* **42**, 604–619.

NASH, T. E., LUJAN, H. T., MOWATT, M. R. & CONRAD, J. T. (2001). Variant-specific surface protein switching in *Giardia lamblia*. *Infection and Immunity* **69**, 1922–1923.

NASH, T. E. & MOWATT, M. R. (1992). Characterization of a *Giardia lamblia* variant-specific surface protein (VSP) gene from isolate GS/M and estimation of the VSP gene repertoire size. *Molecular and Biochemical Parasitology* **51**, 219–228.

RIRIE, K. M., RASMUSSEN, R. P. & WITTWER, C. T. (1997). Product differentiation by analysis of DNA melting curve during the polymerase chain reaction. *Analytical Biochemistry* **245**, 154–160.

SAMBROOK, J. & RUSSEL, D. W. (2001). *Molecular Cloning: A Laboratory Manual*, 3rd Edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

SUN, C. H., PALM, D., MCARTHUR, A. G., SVÄRD, S. G. & GILLIN, F. D. (2002). A novel Myb-related protein involved in transcriptional activation of encystation genes in *Giardia lamblia*. *Molecular Microbiology* **46**, 971–984.

TIJSTERMAN, M. & PLASTERK, R. A. H. (2004). Dicer at RISC: The mechanism of RNAi. *Cell* **117**, 1–4.

TOUZ, M. C., GOTIG, N., NASH, T. E. & LUJAN, H. D. (2002). Identification and characterization of a novel secretory granule calcium-binding protein from the early branching eukaryote *Giardia lamblia*. *Journal of Biological Chemistry* **52**, 50557–50563.

ULLU, E., TSCHUDI, C. & CHKRABORTX, T. (2004). RNA interference in protozoan parasites. *Cellular Microbiology* **6**, 509–519.

UPCROFT, P., CHENG, N. & UPCROFT, J. A. (1997). Telomeric organization of a variable and inducible toxin gene family in the ancient eukaryote *Giardia duodenalis*. *Genome Research* **7**, 37–46.

VON ALLMEN, N., BIENZ, M., HEMPHILL, A. & MÜLLER, N. (2004). Experimental infections of neonatal mice with cysts of *Giardia lamblia* clone GS/M-83-H7 are associated with an antigenic reset of the parasite. *Infection and Immunity* **72**, 4763–4771.

WITTWER, C. T., RIRIE, K. M., ANDREW, R. V., DAVID, D. A., GUNDRY, R. A. & BALIS, U. J. (1997). The LightCycler™: a microvolume multisample fluorimeter with rapid temperature control. *Biotechniques* **22**, 176–178.