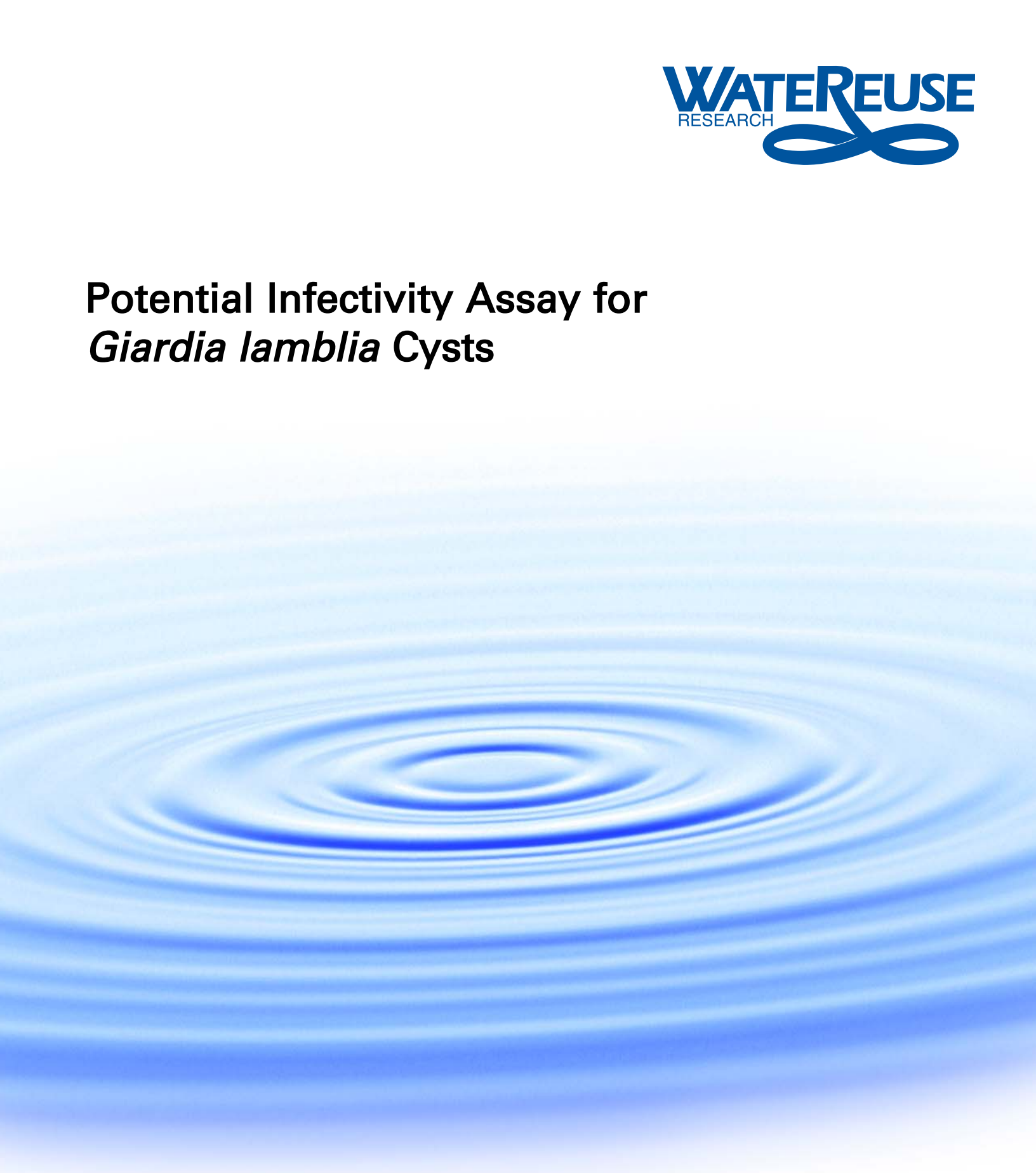
Potential Infectivity Assay for *Giardia lamblia* Cysts



WateReuse Research Foundation

Potential Infectivity Assay for *Giardia lamblia* Cysts

About the WateReuse Research Foundation

The mission of the WateReuse Research Foundation is to conduct and promote applied research on the reclamation, recycling, reuse, and desalination of water. The Foundation's research advances the science of water reuse and supports communities across the United States and abroad in their efforts to create new sources of high quality water through reclamation, recycling, reuse, and desalination while protecting public health and the environment.

The Foundation sponsors research on all aspects of water reuse, including emerging chemical contaminants, microbiological agents, treatment technologies, salinity management and desalination, public perception and acceptance, economics, and marketing. The Foundation's research informs the public of the safety of reclaimed water and provides water professionals with the tools and knowledge to meet their commitment of increasing reliability and quality.

The Foundation's funding partners include the Bureau of Reclamation, the California State Water Resources Control Board, the California Energy Commission, and the California Department of Water Resources. Funding is also provided by the Foundation's Subscribers, water and wastewater agencies, and other interested organizations

Potential Infectivity Assay for *Giardia lamblia* Cysts

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WateReuse Research Foundation
Alexandria, VA

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Acronyms

ADHE	aldehyde/alcohol dehydrogenase E
BSA	bovine serum albumin
Cy3	cyanine dye (570-nm emission wavelength)
Cy5	cyanine dye (670-nm emission wavelength)
cDNA	complementary DNA
DAPI	4′,6′-diamino-2-phenylindole
DAVID	Database for Annotation, Visualization and Integrated Discovery
DTT	dithiothreitol
EF1A	Elongation factor 1 α
GO	gene ontology
HI	heat-inactivated
HSP	heat shock protein
MeV	multi-experiment viewer
mRNA	messenger RNA
MIDAS	Microarray Data Analysis System
NIAID	National Institute of Allergy and Infectious Diseases
NIH	National Institutes of Health
ORF	open reading frame
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PFGRC	Pathogen Functional Genome Research Center
ppb	parts per billion
RNA-Seq	high-throughput sequencing of reverse-transcribed RNA samples
rRNA	ribosomal RNA
RT-PCR	reverse transcriptase PCR
SAGE	serial analysis of gene expression
SSC	saline sodium citrate
SD	standard deviation
SDS	sodium dodecyl sulfate
TIFF	Tagged Image File Format
TIGR	The Institute for Genome Research
TPI	triosephosphate isomerase
UMSS	Universal Microarray Standard Set
UV	ultraviolet
VSP	variant surface protein

Foreword

The WateReuse Research Foundation, a nonprofit corporation, sponsors research that advances the science of water reclamation, recycling, reuse, and desalination. The Foundation funds projects that meet the water reuse and desalination research needs of water and wastewater agencies and the public. The goal of the Foundation's research is to ensure that water reuse and desalination projects provide high-quality water, protect public health, and improve the environment.

An Operating Plan guides the Foundation's research program. Under the plan, a research agenda of high-priority topics is maintained. The agenda is developed in cooperation with the water reuse and desalination communities including water professionals, academics, and Foundation subscribers. The Foundation's research focuses on a broad range of water reuse research topics including:

- Definition and addressing of emerging contaminants
- Public perceptions of the benefits and risks of water reuse
- Management practices related to indirect potable reuse
- Groundwater recharge and aquifer storage and recovery
- Evaluation of and methods for managing salinity and desalination
- Economics and marketing of water reuse

The Operating Plan outlines the role of the Foundation's Research Advisory Committee (RAC), Project Advisory Committees (PACs), and Foundation staff. The RAC sets priorities, recommends projects for funding, and provides advice and recommendations on the Foundation's research agenda and other related efforts. PACs are convened for each project and provide technical review and oversight. The Foundation's RAC and PACs consist of experts in their fields and provide the Foundation with an independent review, which ensures the credibility of the Foundation's research results. The Foundation's Project Managers facilitate the efforts of the RAC and PACs and provide overall management of projects.

The Foundation's primary funding partners include the Bureau of Reclamation, California State Water Resources Control Board, Foundation subscribers, water and wastewater agencies, and other interested organizations. The Foundation leverages its financial and intellectual capital through these partnerships and other funding relationships.

Cysts of the protozoan parasite *Giardia lamblia* are found worldwide in surface water, in wastewater, and in treatment plant effluents. If ingested, infectious cysts release a trophozoite that can initiate an infection by colonizing the small intestine and dividing to high numbers. The goal of this project was to develop a molecular assay that can rapidly discriminate between infectious cysts and cysts unable to cause an infection.

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Executive Summary

BACKGROUND, OBJECTIVES, AND RATIONALE

Cysts of the protozoan parasite *Giardia lamblia* are found worldwide in surface water, in wastewater, and in treatment plant effluents. If ingested, infectious cysts release a trophozoite that can initiate an infection by colonizing the small intestine and dividing to high numbers. Immunofluorescence assays that are routinely used to microscopically detect cysts in water concentrates or fecal samples do not differentiate between infectious cysts and cysts that are unable to cause an infection. In experimental settings, laboratory rodents are needed to assess infectivity, but this approach is not practical for water monitoring.

The goal of this project was to develop a molecular assay that can rapidly discriminate between infectious cysts and cysts unable to cause an infection. Messenger RNA (mRNA) was evaluated as a molecular marker of infectivity. In living cells mRNA levels are regulated by the rate of synthesis (transcription) and decay. Upon cell death, most mRNA transcripts decay and become undetectable. Such mRNA transcripts can rapidly and specifically be monitored using established polymerase chain reaction (PCR) technology.

RESULTS

The mRNA population (transcriptome) of live cysts and of cysts inactivated by aging, heat, and ultraviolet (UV) irradiation was surveyed using oligonucleotide microarrays. For this analysis, mRNA is copied in bulk to complementary DNA (cDNA) and fluorescently labeled to generate a probe. Microarrays were hybridized with such probes to obtain a quantitative representation of the complete cyst transcriptome. Statistical analysis of replicate microarrays revealed that, as compared to trophozoites (the dividing stage in the parasite's life cycle), only about 1/20 of the mRNA transcripts was detected. The analysis of replicate microarray data set identified about 200 mRNA transcripts in live cysts. As an alternative approach, cysts were induced to excyst and the transcriptome of excysting cysts compared to that of control cysts. Selected transcripts expressed at a high level in infectious cysts and absent from inactivated (dead) cysts, or overexpressed in excysting cysts, were evaluated using reverse transcriptase PCR (RT-PCR). These analyses confirmed differential mRNA levels in live and inactivated cysts. These findings did not apply to cysts irradiated with a lethal dose of 254-nm UV light, a treatment that had little impact on the cyst transcriptome. This observation is consistent with the mode of action of UV irradiation, which does not act by killing microorganisms but by inducing mutations in the DNA.

CONCLUSIONS

The transcriptome of *G. lamblia* cyst comprises relatively few mRNA transcripts, some of which are present at a high level. In cysts that are inactivated by heat or by aging, most of the transcriptome is degraded. Many mRNA transcripts can thus serve as markers of cyst infectivity. Monitoring the infectivity of cysts that underwent UV irradiation may require the identification of different mRNA markers as the impact of UV light on the transcriptome is less apparent.

Additional work is needed to confirm some of the microarray analyses. High-throughput sequencing of mRNA generates more accurate profiles of the cyst transcriptome. This approach would be particularly suitable to identify genes that are differentially expressed following UV irradiation. Combined with infectivity tests in rodents, such analyses could confirm mRNA transcripts suitable for monitoring infectivity and reveal the extent of natural variation in the transcriptome of live cysts. Based on these data, optimized RT-PCR protocols for the specific and sensitive detection of selected mRNA will need to be developed.

Chapter 1

Introduction

1.1 *G. LAMBLIA* CYSTS IN WATER

Giardia lamblia (*G. duodenalis*) is a diplomonad parasite that causes approximately 20,000 reported cases of giardiasis a year in the United States alone (Yoder et al., 2010). The trophozoite stage of the parasite reproduces in the small intestine by binary fission. Environmentally resistant cysts differentiate in the intestine and are excreted with the feces. *G. lamblia* cysts are common in wastewater and in surface water (Garcia et al., 2002; Sulaiman et al., 2004; Robertson et al., 2006). Although cysts are routinely detected with immunofluorescence, this technique does not differentiate between infectious cysts and cysts that are unable to cause an infection, making it difficult to assess the health risk these cysts represent. The lack of an easy infectivity assay for waterborne protozoan cysts and oocysts has been noted (Dowd and Pillai, 1997; Thiriat et al., 1998). The limitations of vital dyes in estimating the infectivity of *G. lamblia* or *Cryptosporidium parvum* have been reported (Bukhari et al., 2000; Korich et al., 1990). Excystation has also been found to be an inaccurate indicator of infectivity with *Giardia muris* (Hayes et al., 2003), although Hoff et al. found the excystation of *G. muris* cysts to be an accurate surrogate of mouse infectivity (Hoff et al., 1985). The drawback of vital dyes and excystation methods is that they are unreliable for analyzing cysts and oocysts that have been exposed to chemical disinfectants or UV irradiation. These limitations apply to both *Giardia* cysts and *Cryptosporidium* oocysts. Propidium iodide, either used by itself (Sauch et al., 1991), or together with fluorescein diacetate (Labatiuk et al., 1991) or with 4′,6′-diamino-2-phenylindole (DAPI) (Jenkins et al., 1997), is the most commonly used dye. Such dyes typically underestimate the infectivity of cysts and *C. parvum* oocysts (Neumann et al., 2000a). The nucleic acid dyes SYTO-9 and SYTO-59 were found to correlate with *C. parvum* oocyst infectivity, but the threshold separating infectious from dead cysts varied in different batches of oocysts (Neumann et al., 2000b). Assessing the ability of cysts to exclude or take up vital dyes also requires cysts in sufficient number and of sufficient purity to enable direct microscopic observation.

Although rodent models are available for the sensitive detection of infectious cysts and oocysts (Labatiuk et al., 1991), animals are not convenient for routine monitoring because of their cost, regulatory requirements, and the need for specialized facilities. Moreover, when one is working with low-cyst doses, the animals need to be monitored for at least 2 weeks before a negative diagnosis can be made. These limitations have motivated the search for molecular markers of infectivity.

1.2 GOALS AND RATIONALE OF THE PROJECT

The primary goal of this proposal was to develop a molecular assay for discriminating between infectious *G. lamblia* cysts and cysts that are unable to infect. Because cyst survival in the environment is essential for parasite transmission, *Giardia* and other microbial pathogens relying on environmental dispersion have evolved long-lived, environmentally resistant forms. Depending on the species, these forms are called cysts, oocysts, or spores. Highly resistant cyst walls and low metabolic rates ensure the longevity of encysted stages. Low metabolic rates preclude the application of metabolic markers, such as respiration, to the development of infectivity tests. Moreover, metabolic activity lacks specificity. We

investigated the feasibility of using messenger RNA (mRNA) transcripts as a marker of cyst infectivity. mRNA has several properties that make it ideal for this application, namely:

1. The sequence of mRNA transcripts[1] is species-specific.
2. Highly sensitive and specific mRNA amplification methods based on reverse transcriptase PCR (RT-PCR) or direct isothermal amplification can be applied.
3. Transcription of certain mRNA species is upregulated in response to changes in environmental conditions. Upregulation in response to exposure of cysts to elevated temperature (37 °C) occurs only in live cysts, a property that can be exploited for a surrogate infectivity assay.
4. Levels of cellular mRNA are determined by the rate of transcription and decay. In dead cells, in the absence of de novo transcription, mRNA levels rapidly diminish. Postmortem degradation of mRNA can be used as a complementary marker of infectivity.

The properties listed here make RNA an attractive surrogate marker of cyst infectivity.

Certain *G. lamblia* transcripts, including those encoding heat shock protein 70 (HSP 70), β-giardin, elongation factor 1 α (EF1A), and aldehyde/alcohol dehydrogenase E (ADHE) have been investigated as potential markers of cyst infectivity (Abbaszadegan et al., 1997; Mahbubani et al., 1991; Bertrand et al., 2009; Lee et al., 2009). To date one comprehensive survey of the cyst mRNA population has been performed. This study used serial analysis of gene expression (SAGE [Velculescu et al., 1995]) to compare the transcriptome of different stages in the *G. lamblia* life cycle (Birkeland et al., 2010). Based on their relative abundance, mRNA transcripts were grouped according to their transcriptional profile during the life cycle. Instead of native cysts produced in animals, Birkeland et al. use cysts produced in vitro (Gillin et al., 1987).

Given that the *G. lamblia* genome expresses almost 6000 genes, we proposed to undertake a genome-wide survey of the transcriptome of infectious, inactivated, and excysting cysts. The goal was to identify mRNA transcripts that would be suitable markers of infectivity. Given the large number of mRNA transcripts that have not been investigated, we postulated that such a survey would identify new potential infectivity markers. For this purpose an ideal transcript would be one that is abundant in live cysts and rapidly decays postmortem. Alternatively, transcriptional upregulation would occur upon exposure to certain environmental conditions. Both types of transcript would enable the differentiation of infectious and uninfectious cysts using RT-PCR. To investigate the *G. lamblia* cyst transcriptome, we used *G. lamblia* oligonucleotide microarrays. Although for most applications microarrays have been superseded by high-throughput sequencing of reverse-transcribed RNA samples (RNA-Seq [Mortazavi et al., 2008]), this technology was not readily available when this project was initiated.

In this project we pursued two alternative methods. (1) We compared the transcriptome of live, infectious cysts with that of aged and heat-inactivated (HI) cysts to identify mRNA transcripts that are abundant in live cysts and decay rapidly postmortem. (2) We exposed live cysts to conditions that induced excystation and compared the induced transcriptome with that of control cysts. Both approaches were designed to identify mRNA transcripts that

[1] Transcription is the synthesis of RNA using DNA as a template. mRNA transcripts are the products of this activity.

decayed rapidly or were significantly upregulated. The original scope of the project also included a validation of microarray data with RT-PCR and a comparison of the RNA-based infectivity assay with infectivity in gerbils. This comparison was not performed. Instead, experiments to assess the impact of chlorination and UV irradiation were investigated.

Chapter 2

Materials and Methods

2.1 *G. LAMBLIA* CYSTS AND TROPHOZOITES

G. lamblia cysts of assemblage B isolate H3 were purchased from Waterborne (New Orleans, LA). To induce excystation, cysts were subjected to a two-step procedure (Bingham and Meyer, 1979) that was modified according to al-Tukhi et al. (1991). A suspension of 5×10^6 *G. lamblia* cysts was washed in phosphate-buffered saline (PBS), mixed with nine parts of 1 N HCl, and incubated at 37 °C for 1 h. The suspension was then centrifuged at 800 × *g* for 10 min, and the cyst pellet was washed once with warm PBS. The cysts were then resuspended in TYI-S-33 medium (Keister, 1983) prewarmed to 37 °C and were incubated at 37 °C for 4 h. The samples were processed for RNA extraction and microarray analysis. An identical number of cysts maintained at 4 °C and not acidified was processed in parallel. Trophozoites of assemblage A isolate WB were cultured in TYI-S-33 medium.

Cysts were aged by storing them at 4 °C for 4 months. Heat inactivation was achieved by incubating live cysts at 70 °C for 15 min followed by a 4-h incubation at room temperature. The percentage of dead cysts in inactivated and aged cyst suspensions was estimated by staining with Hoechst dye or propidium iodide. Using this approach, we estimated that 100% of the cysts were dead after heat inactivation and >90% were dead in the aged sample.

2.2 UV EXPOSURE AND CHLORINATION

UV irradiation was performed by Dr. Karl Linden at the University of Colorado in Boulder. A suspension of 10^7 cysts of *G. lamblia* was sent directly from Waterborne to the University of Colorado. Half of the suspension was exposed to UV light at 254 nm from four 15-W low-pressure mercury vapor lamps housed in a collimated beam apparatus. The other half served as a nonirradiated control. The incident irradiance was measured using a calibrated radiometer (IL1700 radiometer, SED240/W detector; International Light, Peabody, MA). The average irradiance in the completely mixed water sample was calculated using an integration of Beer's law over the sample depth, incorporating factors for the water absorbance and reflection, based on Bolton and Linden's published protocol (Bolton and Linden, 2003). The time of exposure to achieve the desired UV dose was calculated by dividing the target UV dose by the average irradiance. A dose of 15 mJ/cm^2 was delivered. This dose was expected to achieve 3.5-log inactivation based on the Environmental Protection Agency dose tables in the Long Term 2 Enhanced Surface Water Treatment Rule (U.S. EPA, 2010).

Cysts were exposed to chlorine (Cl) by Dr. Jeanine Plummer at the Worcester Polytechnic Institute (WPI). A suspension of 10^7 *G. lamblia* cysts was washed once in Cl demand-free PBS and transferred to WPI. The cyst suspension was split into two equal portions. One portion was exposed to a target concentration of 5mg of Cl/L in 120 ml of demand-free PBS. The other suspension was treated in the same way except that no Cl was added. The Cl concentration was measured after 90 min of exposure, and no demand was detected in the cyst suspension. Final Cl concentration readings of 5.2 and 4.0 mg/L were recorded. After 90 min, the reaction was quenched with 3% sodium thiosulfate. Following exposure to Cl, cysts

were transported to Tufts University and were stored frozen in Trizol. RNA was extracted as described in the following.

2.3 RNA EXTRACTION, PROCESSING, AND MICROARRAY HYBRIDIZATION

Total RNA for microarray analysis was isolated with Trizol (Sigma-Aldrich) from trophozoites and cysts following three cycles of freezing and thawing at -70 °C and 37 °C for 5 min each. DNA was removed by using the TurboDNase kit from Ambion (Austin, TX) and the RNA was extracted with Qiagen (Valencia, CA) RNeasy columns according to the RNA cleanup protocol. RNA was amplified in vitro with the WT-Ovation Pico RNA Amplification System (NuGEN, San Carlos, CA). For the amplification reaction up to 5 µL of total RNA sample (about 50 ng) was used as substrate. A total of 2 µg of complementary DNA (cDNA) produced by the amplification reaction was labeled by using a Genomic DNA Enzymatic Labeling Kit from Agilent (Santa Clara, CA). Preparation of the cDNA probes for hybridizing to microarrays included the following steps

- Reverse transcription and aminoallyl (aa)-UTP labeling of RNA
- RNA hydrolyzation to stop reverse transcription
- Removal of unincorporated aa-dUTP and free amines
- Coupling of aminoallyl-labeled cDNA to Cy dye (Cy3 or Cy5)
- Purification of labeled cDNA
- Prehybridization of slides
- Preparation of the probe for hybridization
- Hybridization, 16–20 h at 42 °C
- Posthybridization washes
- Slide scanning

Oligonucleotide microarrays were obtained from the NIAID Pathogen Functional Genomics Research Center (PFGRC). The arrays (*G. lamblia* microarray version 2) contain 19,230 elements consisting of duplicates of 70-mer oligomers derived from 9115 predicated open reading frames (ORFs) including the clearly identified 6470 ORFs of the genome of *G. lamblia* WB C6 as described in the *Giardia* database cryptoDB.org and of 500 *Arabidopsis thaliana* control oligomers. To prehybridize, slides were placed in a Coplin jar containing 50 mL of preheated prehybridization buffer (20× saline sodium citrate [SSC], 10% sodium dodecyl sulfate [SDS],and 0.5 g of bovine serum albumin) and their contents were incubated at 42 °C for 2 h. Slides were then washed by using distilled water filtered through a 0.2-µm-pore-size Millex filter (Millipore). Then the slides were washed in isopropyl alcohol for 2 min and dried by centrifugation in a regular benchtop centrifuge. To perform hybridization, dried Cy3/Cy5-labeled cDNA was dissolved in 50 µL of hybridization buffer (40% formamide, 5× SSC, 0.1% SDS, and 0.1 M dithiothreitol). In some experiments 2 µL of Universal Microarray Standard Set (UMSS) was added to the probe mixture and was denatured for 10 min at 95 °C. Then 50 µL of probe mixture was added to a microarray slide and covered with LifterSlip coverslips (Thermo Scientific). Slide contents were incubated in a 42 °C water bath for 16–20 h. For posthybridization wash, slides were first submerged in a low-stringency solution (2× SSC and 0.1% SDS) preheated to 55 °C and washed twice for 5 min each on a shaker. Slides were subsequently washed twice in medium-stringency solution (0.1× SSC and 0.1% SDS), followed by two more 5-min washes at high stringency (0.1×

SSC) at room temperature. Slides were dried in a benchtop centrifuge and scanned with an Agilent scanner.

The probes used for hybridization of microarrays 749 and 747 were spiked with a prelabeled control probe UMSS consisting of a mix of 1000 Cy-dye end-labeled 40-mer probes (500 Cy3 and 500 Cy5) complementary to 500 70-mer *A. thaliana* targets spotted on the *G. lamblia* microarrays. A 2-µL volume of the probe mix obtained from the PFGRC was spiked into the experimental probe just before hybridization to the microarray. This act generated control signals in both Cy-dye channels.

2.4 RT-PCR

cDNA obtained using the in vitro amplification method described earlier was diluted 100-fold, and 1 µL was amplified by PCR. PCR was performed in 20-µL capillary tubes using a LightCycler thermal cycler (Roche Diagnostics). Reaction mixtures contained 1× LC-Fast Start DNA master mix for SYBR Green I (Roche Diagnostics), 3 mM $MgCl_2$, 20 pmol each of forward and reverse primers, and 1 µL of cDNA template. The PCR program included a denaturation step of 10 min at 95 °C followed by 45 cycles of 1 s at 95 °C, annealing at the temperature determined for each primer pair for 8–9 s, and extension at 72 °C. Following amplification, the PCR products were subjected to melting curve analysis by raising the temperature from 45 to 95 °C at a rate of 0.05 °C/s. During the initial optimization phase, following melting curve analysis, PCR products were analyzed on agarose gels to ensure that products of the correct size were amplified. The sequences of the PCR primers, developed for this project except for the one noted, are shown in Table 2.1.

Table 2.1. PCR Primers

Gene_ID	Annotation	Primer Sequence[a]
100864	Vacuolar protein	F: GCGTTCACTTTGTGCAT R: GGACGACCGCATACTC
12059	Hypothetical	F: GCCTTATGCGCCTACC R: GTCTGCGGGAGTGATAC
113093	VSP	F: GACAAAAGACTACGCCG R: CGAAGCATTCTCCAGG
90672	Protein kinase	F: AGGACAGACAGGGAC R: CCAAGCGATTGGATTGA
121046	Histone H2B	F: CGCCTGATGAAGAAGACG R: GTGTTCCGCTTGCTGA
6430	14-3-3 multifunctional protein	F: GTGTTCCGCTTGCTGA R: GCTTGAGGATGTCGTTGC
17090	Trophozoite antigen	F: GCCCGTAGAGTTCTGG R: CGTCACTATCTCCCCG
7110	Ubiquitin	F: GTTGAGCCCACAGATACC R: GTTACCACCACGGAGG
13774	Hypothetical	F: ACTGAAGTTACCCGTGAC R: GCTAGGCGATACCGCA
93938	Triosephosphate isomerase	F: GTGGCCACCACICCCGTGCC R: CCCTTCATCGGIGGTAACTT
r0019-25	18S rRNA[b]	F: ATCCTGCCGGAGCGCGACG R: GGGGTGCAACCGTTGTCCT

[a]F, forward primer; R, reverse primer.

[b]As published (Ng et al., 2005). Other primers were developed for project.

Because trophozoites and cysts originated from assemblages A and B, respectively, we verified that the PCR results were not affected by the genotype. Equivalent amounts of DNA from assemblage A isolate WB and assemblage B isolate GS were amplified in parallel using primers specific for portion of the ubiquitin, histone H2B, and 14-3-3 protein shown in Table 2.1. No bias that could be linked to the genotype was observed by gel analysis.

2.5 MICROARRAY DATA ANALYSIS

Files in tagged image file format generated by the microarray scanner were imported into TIGR_Spotfinder software (Saeed et al., 2003). Spots were manually curated to exclude artifacts. Background cutoff was set at 5%. Cy3 and Cy5 fluorescence values output by Spotfinder were exported to Microsoft Excel. Fluorescence values from duplicate spots were averaged. The Database for Annotation, Visualization and Integrated Discovery (DAVID) suite of bioinformatics tools (Dennis et al., 2003) was used to identify functional annotations that are enriched as compared to the *G. lamblia* genome annotation. The program was accessed through the Web interface at http://david.abcc.ncifcrf.gov/tools.jsp.

To convert fluorescence values into heat maps, each column of Cy3 and Cy5 fluorescence output by Spotfinder (Saeed et al., 2006) was normalized within columns by subtracting the mean and dividing by the standard deviation. Empty spots and *A. thaliana* control spots were removed, and fluorescence from duplicate spots was averaged. Data were saved as a tab-delimited text file and imported into the Multi-Experiment Viewer of the TM4 microarray software suite (Saeed et al., 2006). The color option was reset such that black indicates normalized fluorescence values of >100,000 and that white indicates zero signal.

Chapter 3

Results

3.1 MICROARRAY DATA NORMALIZATION

We used two-color microarrays to compare Cy3- and Cy5-labeled cDNA originating from matched pairs of cyst or trophozoite samples. In addition, live cysts were compared to aged cysts, HI cysts, UV-irradiated cysts, and excysting cysts. The cyst transcriptome originating from the samples was compared to live (infectious) cysts taken from the same suspension. Except for the experimental treatment, treated and control cysts were subjected to the same manipulations. The transcriptomes of the two cyst populations being compared were expected to vary significantly, as were the cyst and trophozoite transcriptomes. Aged cysts were expected to lack any transcriptional activity. As a result of postmortem RNA decay, these cysts were expected to have less mRNA. In contrast, cysts exposed to excystation conditions were anticipated to exhibit a more diverse and perhaps more abundant transcriptome. We also assumed that, given the very different lives, dividing trophozoites would be transcriptionally more active than dormant cysts. Downstream microarray data-processing programs such as Microarray Data Analysis System (Quackenbush, 2002) were not used because such programs normalize Cy3 and Cy5 fluorescence based on the assumption that both samples compared on a two-color microarray contain equal amounts of total RNA. This is because these programs are designed to compare, say, a healthy and a diseased cell population or two cell populations originating from different tissues. Since we did not expect this assumption to apply, background-subtracted fluorescence values generated by Spotfinder were used without normalization. In a deviation from conventional microarray data processing, Cy3 and Cy5 fluorescence values obtained from each spot were not transformed into $\log_2$ ratios. Since a large proportion of transcripts was not detected in cysts, the exclusion of ratios with a numerator or denominator equal to zero would have removed biologically relevant information. To overcome this limitation, the analyses of Cy3 and Cy5 values are primarily based on the difference between fluorescences, rather than on the ratio. The difference is designated Δ fluorescence. The processing of the fluorescence values was limited to background subtraction.

Because conventional data normalization algorithms were not applied, the global Cy3 and Cy5 fluorescence intensities were compared in a dye swap experiment using RNA extracted from trophozoites in exponential (24-h culture) and stationary (72-h culture) phases of growth. This experiment was necessary to assess the magnitude of any dye effect and to ensure that subsequent analyses of the cyst transcriptome were not skewed by differences in labeling efficiency or by any bias in the data acquisition pipeline. RNA extracted from an exponential and a stationary trophozoite culture was split into two equal portions. cDNA from exponential and stationary cultures was labeled with Cy3 and Cy5, respectively, such that all four possible sample × dye combinations were obtained. Two microarrays were hybridized with each 24-h/72-h combination. Fluorescence values from these arrays were acquired as described earlier. Figure 3.1 shows a comparison of the distribution of Δ fluorescence values obtained from these two arrays. These data show that, overall, Cy5 fluorescence was more intense than Cy3's. The magnitude of the difference between Δ fluorescence distribution in these two trophozoite experiments represents the dye effect and is relevant to the interpretation of the cyst microarray data. The observed bias toward Cy5 fluorescence indicates that in this experiment Cy5 degradation was not an issue. Cy5

inactivation is frequently a concern because ground-level ozone concentration exceeding 5–10 ppb is known to inactivate this dye (Branham et al., 2007). In two other experiments (747, 749) we controlled for Cy5 inactivation by comparing fluorescence values of the UMSS signal. In these instances no Cy5 inactivation was observed.

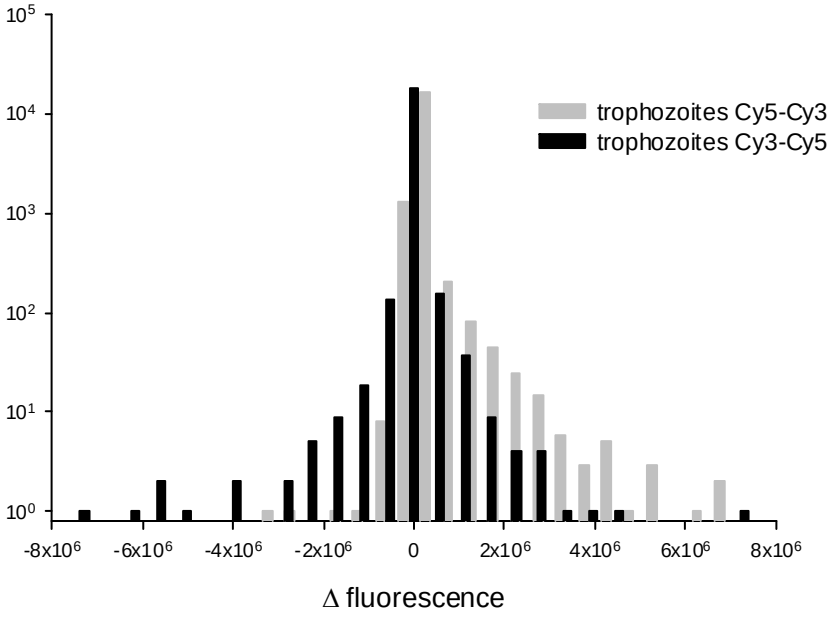

Figure 3.1. Distribution of raw Δ fluorescence values from dye-swapped microarrays 062 and 063. Fluorescence from stationary trophozoite sample was subtracted from exponential sample. Grey bars show Cy5-Cy3 Δ fluorescence, whereas black bars show the distribution of Δ fluorescence for the inverse experiment. The difference between the two distributions is a measure of the dye effect.

3.2 ANALYSIS OF THE TRANSCRIPTOME OF INFECTIOUS CYSTS

We used two-color microarrays to compare Cy3- and Cy5-labeled cDNA originating from matched pairs of cyst samples. The microarray experiments are summarized in Table 3.1. Arrays 062 and 063 were used for the dye swap experiment described earlier. The transcriptional response of cysts to five conditions—aging, heat inactivation, excystation, UV irradiation, and chlorination—was investigated. Two replicate arrays were used for comparing live with HI cysts and live with aged cysts.

Table 3.1. Summary of Microarray Experiments

Microarray No.	Trophozoite or cyst used for:		Comment
	Cy3-labeled cDNA	Cy5-labeled cDNA	
062	Exponential trophozoites	Stationary trophozoites	Dye swap
063	Stationary trophozoites	Exponential trophozoites	
750	Exponential trophozoites	Stationary trophozoites	
065	Live cysts	HI cysts	Replicates
069	Live cysts	HI cysts	
070	Live cysts	Aged cysts	Replicates
128	Live cysts	Aged cysts	
747	Live cysts	UV-irradiated cysts	
749	Live cysts	Excysted cysts	

The Cy3 data from six microarrays hybridized with cDNA originating from independent samples of infectious cysts were used to analyze the cyst transcriptome. These fluorescence data were compared to three microarrays hybridized with trophozoite cDNA (Table 3.1). Figure 3.2 illustrates the mRNA abundance and diversity in cysts as compared to those of dividing trophozoites. Whereas 5412 of 6912 (78%) unique trophozoite transcripts were detected (mean fluorescence $\neq 0$), the proportion of cyst transcripts detected was 67% (4652 of 6912). More meaningful is to exclude any fluorescence value of $<10^4$, a threshold chosen based on the typical fluorescence of empty microarray spots, to conservatively differentiate between signal and background. These values are found in Figure 3.2 by intersecting the trophozoite and cyst curve with $y = 10^4$. A total of 4599 (66%) trophozoite transcripts met the more stringent definition, as opposed to only 215 (3%) cyst transcripts.

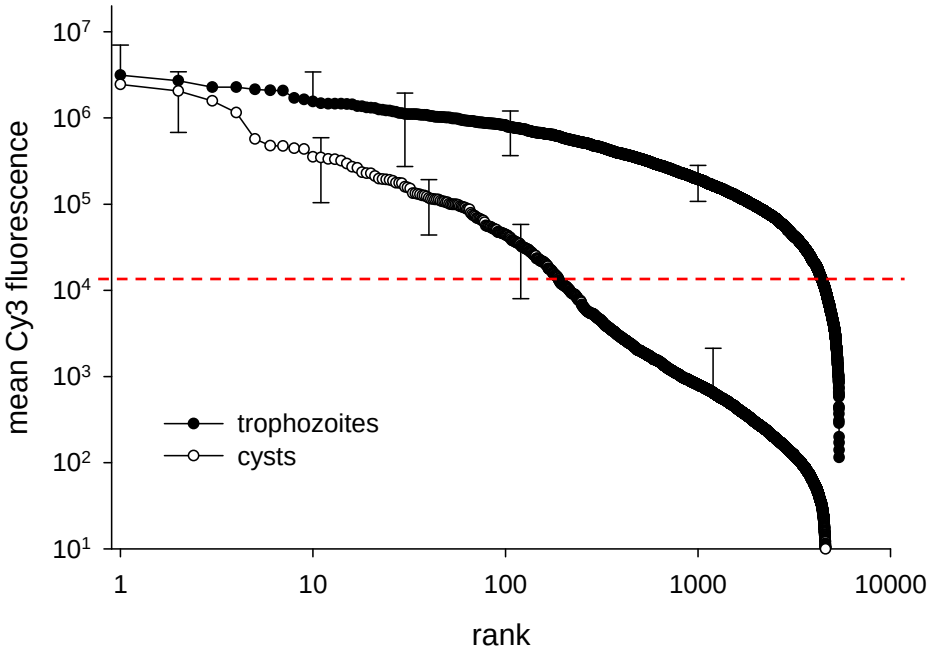

Figure 3.2. Comparison of cyst and trophozoite transcriptomes. Mean Cy3 fluorescence values from trophozoite (*n* = 3) microarrays and cyst (*n* = 6) microarrays are ranked from left to right in decreasing order. Illustrating the difference in mRNA abundance between trophozoite and cyst, over 4500 trophozoite genes exceeded 10,000 units of fluorescence, but only 215 cyst genes met this definition. Because fluorescence values are ranked, vertically aligned data points may not originate from the same gene. Error bars show standard deviation.

An alternative view of the microarray data is the heat map shown in Figure 3.3. Cy5 and Cy3 fluorescence values were normalized as described in section 2.5. The dye used for each sample is shown in Table 3.1. Each row represents one fluorescence channel. The complete heat map includes 6912 columns, one for each gene; only the first few hundred columns are shown. On the map, the genes are displayed in the order they are arrayed. The order has no functional significance. Dark shading indicates brighter fluorescence, whereas white indicates that the transcript was not detected. The predominance of white fields in the heat map illustrates the large number of spots that were not detected, indicative of an impoverished cyst transcriptome. Also visible is the correlation among replicate arrays, which causes the appearance of vertical lines extending across several rows. Typically those lines are interrupted in rows 7–10. These rows correspond to the aged and HI cysts, which have a particularly impoverished transcriptome. The reappearance of the same lines in the two bottom rows (UV irradiated and excysted) illustrates the resemblance among the transcriptomes of infectious, UV-treated, and excysted cysts. Some variation among data from infectious cysts (rows 1–6) is also apparent. This difference reflects biological differences among cyst samples or technical variation.

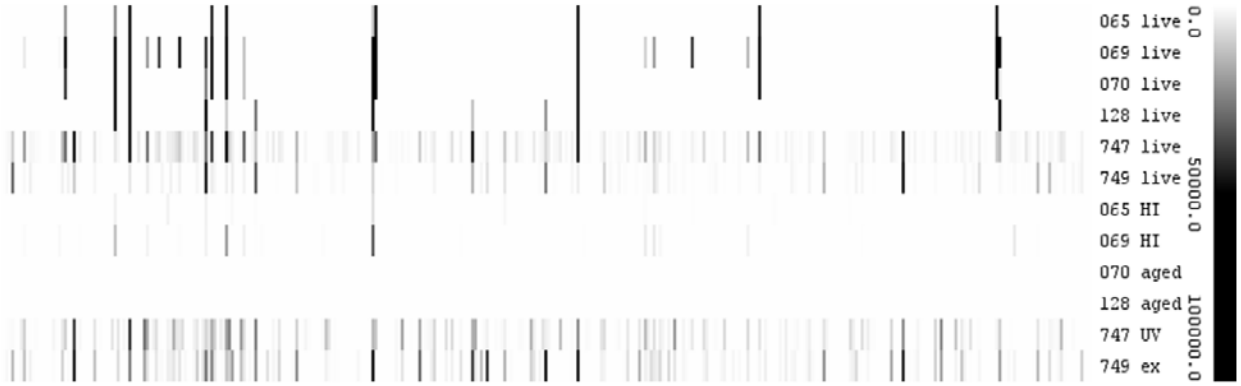

Figure 3.3. Heat map of fluorescence data from six microarrays hybridized with *G. lamblia* cyst cDNA. Each vertical line represents one gene. The darkness indicates the arithmetic mean of two replicate oligonucleotide spots present on the array. The scale is shown rightmost. White is for undetected transcripts, and black is for fluorescence values of >100,000. The same array code indicates that the fluorescence values originate from the same microarray. The predominance of white fields illustrates the small number of genes transcribed in cysts. The legend for each row shows the three-digit array code and the treatment (see Table 3.1). Treatments are abbreviated as follows: HI, heat-inactivated; aged, cysts aged 120 days at 4 °C; UV, exposed to UV irradiation; ex, induced to excyst; live, untreated and infectious. *A. thaliana* control spots and empty spots were removed.

Further insight into the cyst transcriptome was gained by analyzing the annotated function of 215 genes that generated mean fluorescence of >10,000. This cutoff was chosen based on the range of fluorescence emitted by empty spots. This number corresponds to the rank obtained by intersecting the cyst curve with the horizontal line $y = 10^4$ in Figure 3.2. For this analysis we used the program DAVID (Dennis et al., 2003). DAVID identifies enriched Gene Ontology (GO) terms. GO is a hierarchical classification of genes according to biological function (Ashburner et al., 2000). DAVID identified gene functions that are overrepresented in a list of genes as compared to all genes in the genome. In the list of highly expressed cyst genes, the following terms were significantly enriched: "structural constituents of ribosomes" ($p = 3.15 \times 10^{-28}$), as well as other cellular constituents and biological processes related to ribosomes ($p = 1.03 \times 10^{-20}$) and to ribonucleoprotein complex ($p = 3.13 \times 10^{-16}$). Note the very low probabilities (high significance level) for these three GO categories. These functions are not specific of cysts, as similar GO categories were identified among the 215 highest-ranking trophozoite transcripts. "Structural constituents of ribosomes" was again the top-ranking molecular function ($p = 2.34 \times 10^{-33}$), followed by "ribonucleoprotein complex" ($p = 2.73 \times 10^{-19}$) and "non-membrane bound organelle" ($p = 5.75 \times 10^{-14}$). The GO analysis indicates that the main difference between cyst and trophozoite transcriptomes is quantitative and that the functional profile in both life cycle stages is similarly enriched for ribosomal function. The 10 genes with the highest expression in cysts are listed in Table 3.2.

Table 3.2. Ten Most Highly Expressed Genes in Cysts[a]

Gene_ID	Annotation	Mean Cy3 Fluorescence	SD[b]
GL50803_7110	Ubiquitin	2,447,247.5	1,668,739
GL50803_135002	Histone H4	2,057,058.3	1,376,160
GL50803_121046	Histone H2B	1,577,324.2	1,090,907
GL50803_9848	Dynein light chain	1,150,244.9	739,418
GL50803_32146	α-Tubulin	569,155.4	437,609
GL50803_135231	Histone H3	477,104.8	254,294
GL50803_6430	14-3-3 protein	473,506.6	323,959
GL50803_4812	β-Giardin	444,643.3	322,613
GL50803_16114	Ribosomal protein L36-1	434,308.3	226,251
GL50803_19182	VSP	353,785.3	179,690

[a]Based on 6 replicate microarray experiments.

[b]Standard deviation.

3.3 TRANSCRIPTIONAL RESPONSE OF CYSTS TO AGING, HEAT INACTIVATION, EXCYSTATION, AND UV IRRADIATION

To gain insight into the response of the *G. lamblia* cyst transcriptome to different conditions and identify mRNA transcripts that can serve as markers of infectivity, cysts were subjected to five different treatments. RNA was extracted in parallel from treated and control cysts, amplified, converted to cDNA, labeled with Cy3 or Cy5, and hybridized to two-color microarrays as described earlier. Cysts subjected to the following treatments were analyzed:

- aging
- heat inactivation
- excystation
- UV irradiation
- chlorination

The microarray data were summarized using the Δ fluorescence metric described previously, where cDNA from untreated (infectious) cysts was labeled with Cy3 and cDNA from treated cysts was labeled with Cy5 (see Table 3.1).

Two replicate microarray experiments were performed with aged cysts. These analyses showed that aged cysts were essentially devoid of detectable levels of mRNA. None of the Cy5 fluorescence values averaged over two independent microarrays surpassed the background threshold of 10,000. These results are represented in Figure 3.3 and as a frequency distribution in Figure 3.4. Reflecting the dramatic mRNA depletion during aging, mean Δ fluorescence values for all genes were positive. As expected, no transcript was expressed at a higher level in aged cysts than in live ones. When overlaid onto

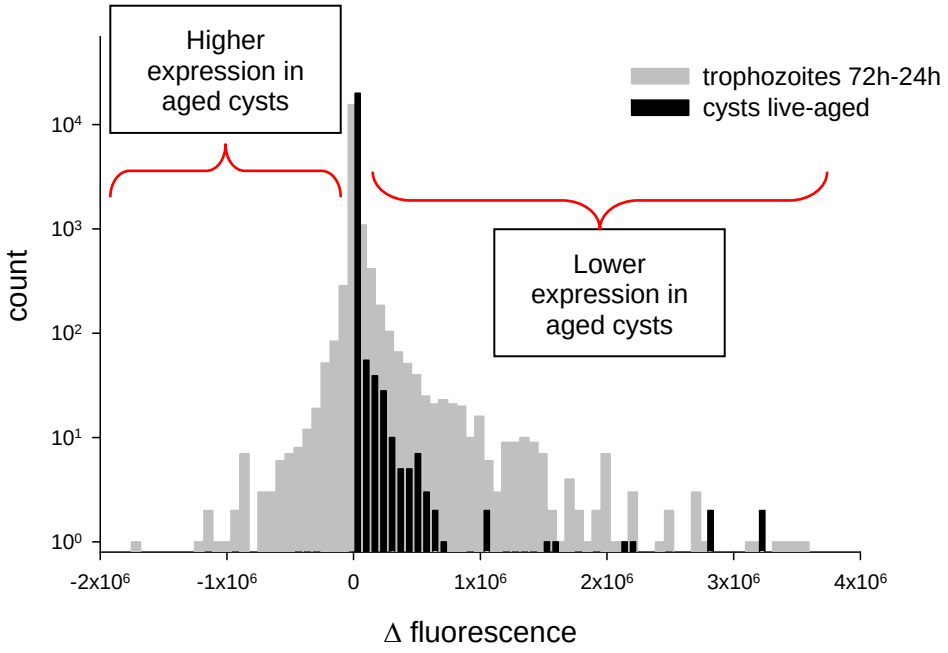

Figure 3.4. Frequency distribution of Δ fluorescence in aged cysts. Cyst data (black bars) are overlaid onto mean Δ fluorescence data of a pair of dye swap trophozoite microarrays (062, 063) shown in Figure 3.1.

the distribution of trophozoite Δ fluorescence (grey bars), the relative small number of detectable mRNA transcripts in live cysts is clearly apparent. As part of quality control, we confirmed that these results were not caused by degradation of Cy5. These controls included a pair of hybridizations with exponential and stationary trophozoites labeled with reversed dyes (Figure 3.1) and the inclusion in four microarray experiments of UMSS control probe described in section 2.3. None of these controls showed evidence of differential labeling or degradation of the Cy5 signal.

An analogous microarray analysis was performed with cysts exposed to conditions which trigger excystation. As in the previous experiment, Cy3 was used to label cDNA from live (untreated) cysts and Cy5 for labelling cDNA from the experimental cysts. Δ fluorescence was determined and the distribution of these values overlaid onto that of the trophozoite benchmark (Figure 3.5). In contrast to the experiment with aged cysts, the vast majority of Δ fluorescence values were negative. This finding indicates that, with few exceptions, mRNA transcript concentration in excysting cysts exceeded that of control cysts. This observation indicates that excystation conditions stimulated transcription of some genes and that transcriptional downregulation was limited to very few genes. Consistent with these cysts being in the initial phase of differentiation into trophozoites, the analysis of overrepresented

GO terms among the 578 genes with fluorescence values of >10,000 gave results similar to those for cysts and trophozoites. Functions associated with ribosomes were again significantly overrepresented. The molecular function "structural constituents of ribosomes" had the most significant *p* value (1.57×10^{-13}).

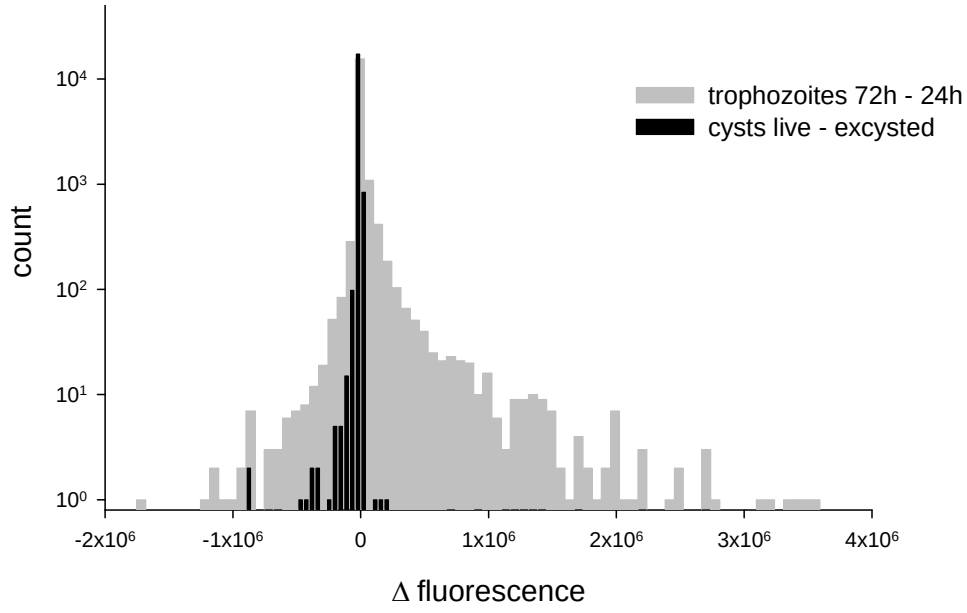

Figure 3.5. Frequency distribution of Δ fluorescence for excysted cysts. Cyst data (black bars) are overlaid onto mean Δ fluorescence data of two replicate trophozoite microarrays (062, 063) used as reference.

The effect of UV irradiation on the cyst transcriptome was also examined. Exposure to UV light inactivates cells by modifying the DNA without affecting viability. Consistent with this model, Δ fluorescence values showed no clear trend (Figure 3.6). A total of 177 genes had Δ fluorescence values of >10,000, and 206 genes had Δ fluorescence of <-10,000. DAVID analysis of overrepresented GO terms in those two groups revealed a similar enrichment of ribosomal functions among genes overexpressed in unexposed cysts. In contrast, among the genes in the negative Δ fluorescence range, namely, genes that are overexpressed in UV-treated cysts, different GO terms such as "catabolic process" and "protein complex" were slightly overrepresented. No GO term with significant *p* values was identified among the UV-induced genes. The lack of a clear impact of UV irradiation on the transcriptome is consistent with observations by Campbell and Wallis (2002). Whereas these authors found that cysts exposed to UV were no longer infectious to gerbils, irradiation did not affect the exclusion and uptake, respectively, of the vital dyes propidium iodide (PI) and DAPI (Campbell and Wallis, 2002).

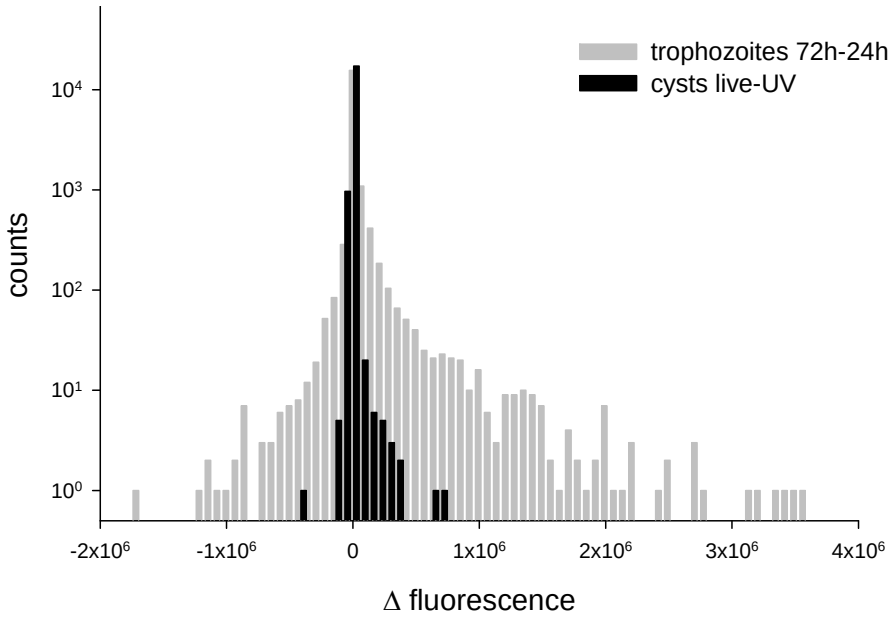

Figure 3.6. Comparison of live and UV-irradiated cysts. See legend to Figures 3.4 and 3.5 for details.

Two microarray hybridizations were also performed to compare the transcriptomes of control cysts and of cysts exposed to chlorine. Those experiments were not successful because the hybridization signal was weak. We first assumed that a delay in scanning the microarray caused by a breakdown of the scanner led to the inactivation of Cy5 (Branham et al., 2007). A second set of probes was synthesized from the same cDNA, but the results were similar. We concluded that the cysts used in this experiment had little RNA to begin with or that the RNA extraction procedure was inefficient. The assumption that the mRNA content varies among batches of cysts is speculative but should be considered in future research.

3.4 VALIDATION OF MICROARRAY RESULTS WITH RT-PCR

The concentration of selected mRNA transcripts was validated with RT-PCR. Table 3.3 summarizes the results from the PCR experiments. The primer sequences are shown in Table 2.1. The metric used in quantitative PCR is the Crossing Point, which is the number of PCR cycles required to generate a signal exceeding background. The Crossing Point and PCR template concentration are inversely correlated. Table 3.3 also shows $\log_2$ ratios of Live/Treated cysts, where "Live" indicates untreated, infectious cysts. A negative log indicates a concentration ratio smaller than 1, namely, a lower mRNA concentration in the treated sample. Crossing Points shown on the same row were acquired in the same experiment and are directly comparable. To assess the actual magnitude of the difference, one would need to convert Crossing Points into a relative concentration using a standard curve, an analysis we did not perform.

The selection of transcripts for RT-PCR analysis was based on the aggregated microarray data (Table 3.2). Out of 215 genes detected in cysts, GL50803_7110 (ubiquitin) was the gene showing the highest average expression in six biological replicates. GL50803_121046 (histone H2B) ranked third, GL50803_6430 (14-3-3 protein) seventh, and GL50803_17090 (trophozoite antigen GTA-1) 16th. GL50803_7110, GL50803_121046 and GL50803_6430 ranked first, third and eighth, respectively, in the live/aged Δ fluorescence ranking. GL50803_7110 showed the smallest Δ fluorescence (most negative) value ranking on position 6912. Thus, this transcript was expected to be among those that are most upregulated during excystation. The detection of only modest upregulation by RT-PCR analysis of GL50803_7110 mRNA is likely related to only one microarray analysis of excystation being performed. Data from single-microarray experiments are likely to be noisy. The other three transcripts tested by RT-PCR were selected on the basis of average fluorescence from two to six replicates and are thus expected to be more reliable.

The mean of the ratios shown on the last row of Table 3.3 is consistent with the microarray data. Log ratios for the HI and aged cysts were negative, as expected from the highly rarefied transcriptome in cysts subjected to these treatments. In contrast, the comparison of live to excysted cysts gave one positive, one negative, and two zero ratios, which is consistent with a transcriptome that globally was only slightly affected by the treatment. This property is also apparent in Figure 3.5, which shows a narrow distribution of Δ fluorescence values when live and excysted cysts are compared. A plot of the Crossing Point against the corresponding microarray fluorescence values is shown in Figure 3.7. The graph displays the expected inverse correlation, where high Crossing Points generally correspond to low fluorescence and vice versa. This correlation was apparent with cyst and trophozoite data.

ble 3.3. Summary of RT-PCR Quantification Experiments

_ID[a]	Annotation	Crossing Point									Live/ HI[i]	Live/ exc[i]	Live/ UV[i]	L A
		Neg[b]	No RT[c]	24 h[d]	72 h[e]	Live[f]	HI[g]	Exc[h]	Aged	UV				
	Vacuolar protein	>41		21.3	19.9	35.2		28.2				0.3		
864						35.0		34.7				0		
		>41		19.4	19.0	32.0	35.4		36.0		-0.1			
959	Hypothetical	>41		23.9	21.8	36.5		29.5						
						36.0		41.0						
		>41		23.2	21.8	36.6	41.0		37.0		-0.2			
093	VSP	>41		23.5	21.4	34.3	37.1		40.0		-0.1			
572	Protein kinase			20.7	20.1	36.7	41.0		41.0		-0.2			
046	Histone H2B			17.8	16.4	18.5	23.0		28.0		-0.3			
						24.1		23.9				0		
30	14-3-3 Prot	>41		17.6	15.6	20.9	26.1		29.8		-0.3			
990	Troph antigen			24.0	22.1	38.4	41.0		41.0		-0.1			
10	Ubiquitin			17.1	14.7	13.8	21.2		25.0		-0.6			
						17.3		18.7				-0.1		
		>41		17.9		18.8				17.2			0.1	
774	Hypothetical	39.2	38.1	25.9										
938	TPI	>41		33.0		29.3								
9-25	18S rRNA	>41												
		38.4		21.8		27.5	32.0				-0.2			
12	β-Giardin	38.2		22.0		29.2	34.1				-0.2			
		37.3		22.1		29.6	35.0				-0.2			
										mean ratio	-0.23	0.05	0.1	

complete gene_ID format is GL50803_XXXXXX.
ative PCR control (no added DNA template).
RT control; no RT added.
trophozoite culture.
trophozoite culture.

[f]Untreated cysts.
[g]HI cysts.
[h]Cysts induced to excyst.
[i] Log_2 (Crossing Point ratio).

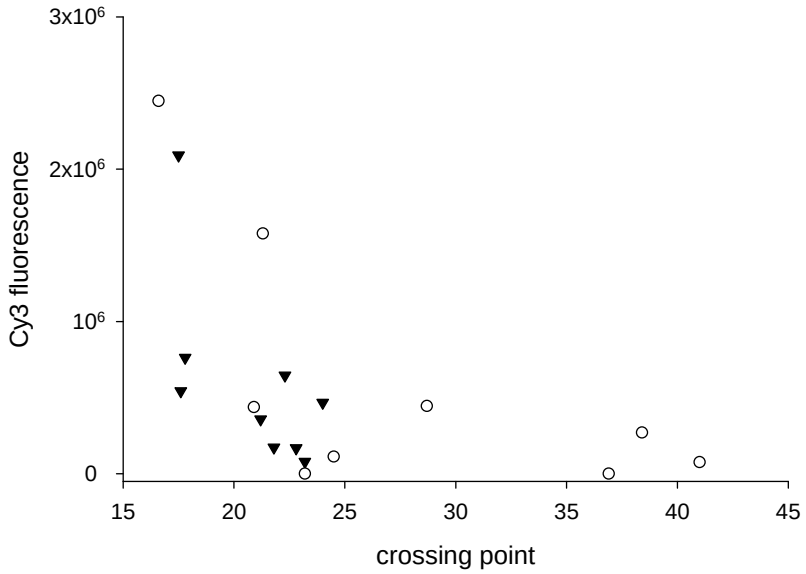

Figure 3.7. Validation of microarray data with quantitative RT-PCR. Mean Cy3 fluorescence was plotted against RT-PCR Crossing Point for live cysts (6 microarrays) and 24-h trophozoites (3 microarrays). The plot shows the expected inverse correlation between the two variables. Where the same gene was analyzed in replicate PCR analyses (see Table 3.3), the mean was used. Triangles, trophozoites; circles, cysts.

3.5 COMPARISON OF MICROARRAY DATA WITH PUBLISHED RT-PCR RESULTS

Several mRNA transcripts have been proposed as markers of *G. lamblia* cyst infectivity. Such transcripts were selected because they encode proteins found only in *Giardia* or for a reason not specified by the authors. The microarray data enable us to evaluate how the proposed transcripts compare to the cyst transcriptome in terms of abundance, postmortem decay, and differential transcription during excystation. The presence of β-giardin mRNA in cysts was investigated following inactivation by different treatments (Mahbubani et al., 1991). According to the mean level of expression in six replicate microarrays, β-giardin ranked eighth out of 215 significantly expressed genes and is therefore one of the most highly expressed genes. With respect to Δ fluorescence, β-giardin ranks sixth when live and aged cysts were compared. In a comparison of live to excysted cysts, the same transcript is the seventh most highly upregulated. Although the comparison of unexcysted and excysted cysts needs to be interpreted with caution because it is based on a single microarray, these results indicate that β-giardin mRNA is potentially an excellent marker of infectivity. If we extend the analysis of β-giardin mRNA to UV-irradiated cysts, we find that this transcript also ranks highly (fifth) in this comparison, although the magnitude of Δ fluorescence is less than half of what was found in the live-to-aged comparison. Again, the analysis of UV-treated cysts is based on a single microarray. Given that Mahbubani et al. had no knowledge of the *G. lamblia* transcriptome, their focus on β-giardin mRNA turned out to be an excellent choice.

HSP 70 mRNA was studied by two laboratories that assessed the upregulation of this gene in cysts exposed to 42 °C (Abbaszadegan et al., 1997; Lee et al., 2009). We did not heat-shock cysts, but we found that in live cysts HSP 70 (GL50803_17432) was not detected. In cysts that were induced to excyst, a procedure that included incubation at 37 °C, HSP 70 mRNA remained below microarray detection level. In contrast to β-giardin, from the point of view of this study, HSP 70 is likely to be an inferior marker, mainly because of its low baseline expression.

Bertrand et al. investigated three mRNA transcripts, including the previously analyzed β-giardin mRNA, EF1A, and ADHE, for a similar purpose (Bertrand et al., 2009). In our analyses EF1A mRNA (GL50803_112304) was below detection in live cysts and in cysts induced to excyst. ADHE (GL50803_93358) also did not feature among the transcripts we detected in live cysts. However, in cysts exposed to excystation conditions, mRNA encoded by ADHE generated 9757 fluorescence units, which is close to the threshold of 10,000. Subject to the limitation of the single-microarray experiment, it appears that the ADHE gene is induced during excystation. However, the absence of detectable levels of ADHE mRNA in live cysts makes this transcript a poor choice.

In summary, a comparison of published RT-PCR experiments focused on selected *G. lamblia* transcripts with the microarray data confirmed that β-giardin is an excellent candidate marker. Several other transcripts selected on the basis of their upregulation during heat shock, or selected for unspecified reasons (Bertrand et al., 2009), were either not detected or were present in cysts at a low level.

3.6 COMPARISON OF SAGE AND MICROARRAY DATA

In SAGE 15-bp fragments of cDNA are randomly derived from the transcriptome, concatenated, and sequenced. Each 15-bp sequence, often dubbed a tag, is computationally assigned to an mRNA transcript. The frequency with which each transcript is identified provided a measure of the relative abundance of each transcript (Velculescu et al., 1995). We compared our microarray data with the first comprehensive published analysis of the *G. lamblia* transcriptome (Birkeland et al., 2010). SAGE found 71 genes significantly upregulated during excystation. This group of genes was compared with our list of 543 genes upregulated during excystation with Δ fluorescence of 10,000 or more. Only seven genes were shared by both lists. On a Cy5-versus-Cy3 plot generated from our data, Birkeland's hits showed no differential expression (not shown). The three genes with the highest percentage of SAGE tags 60 min postexcystation were not among those detected in our study. Comparing the SAGE and microarray data from cysts also showed little correlation. For this comparison we included the 124 genes with 0.1% or more SAGE tags in the cyst library and compared this list to 215 genes with a mean (n = 6) cyst microarray fluorescence over 10,000 (Figure 3.8). This comparison revealed 19 matches, equivalent to only 15% (19/124) of the genes with at least 0.1% of SAGE tags. As an illustration, ubiquitin, one of our top-ranking genes, was not represented among the cyst SAGE tags, and histone H4, which ranks second in our classification, was not detected either. Somewhat better correlated was the expression of histone H2B (microarray rank, 3rd; SAGE rank, 37th) and dynein light chain (microarray, 4th; SAGE, 26th). The overall lack of correlation between cyst data sets could have several reasons, including experimental differences between the two studies. For instance, the excystation procedure we applied included a 4-h incubation period, whereas the SAGE study used 1 h of incubation. However, experimental differences are less likely to play a role in the comparison of nonexcysted cyst. The fact that the cysts used in our study were obtained from

gerbils, whereas Birkeland and colleagues produced cysts in vitro (Gillin et al., 1987), should be considered as a cause of poor correlation between cyst data sets. To investigate this possibility, we compared SAGE and microarray data sets from trophozoites. Because the

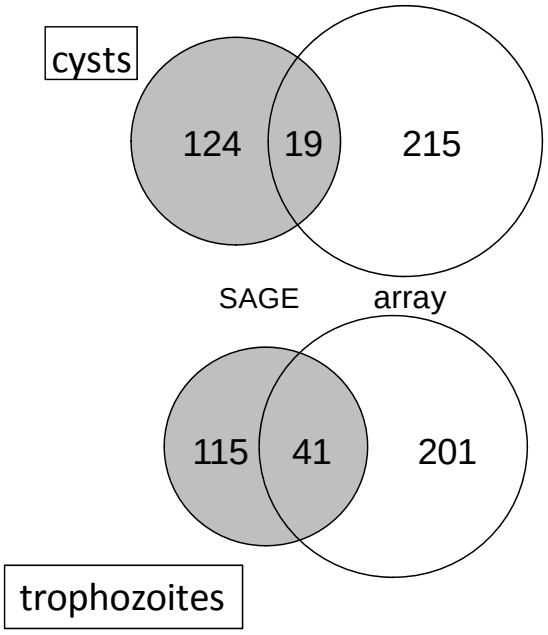

Figure 3.8. Venn diagram showing the number of highly expressed transcripts in SAGE and microarray analyses. Genes representing ≥0.1% of SAGE tags were included. Areas in each diagram are proportional to the number of genes. Grey, SAGE (Birkeland et al., 2010); white, microarray data from this study. Note the higher proportion of common genes in the trophozoite analyses, suggesting that the low correlation between cyst data sets might originate from biological differences between cysts generated in culture and cysts extracted from feces.

culture conditions used in both studies were similar, one would expect to find a better overlap among trophozoite data sets than observed with cysts. As for the comparison of the cyst data, we considered genes contributing at least 0.1% of trophozoite SAGE tags (n = 115, 3.8% of detected genes) and 201 genes with the highest microarray fluorescence value. When one includes only 201 genes from the microarray data, the ratio of SAGE to microarray genes is the same for the cyst and trophozoite comparison (1:1.75). In the trophozoite data comparison 36% (41/115) of SAGE genes were present in the microarray gene list. Thus, the percentage of matches among trophozoite data sets was more than twice that found in the cyst comparison. We interpret these results as an indication that cysts produced in vitro and cysts originating from an infection may differ in fundamental ways. Such differences may have contributed to the poor correlation between SAGE and microarray data.

Chapter 4

Discussion and Conclusions

4.1 DISCUSSION

In this project we surveyed the global mRNA population of *G. lamblia* cysts with the goal of identifying mRNA transcripts that can serve as markers of cyst infectivity. We hypothesized that the identification of such mRNA markers requires a genome-wide analysis of the cyst mRNA population to identify transcripts that are sufficiently abundant in cysts and rapidly decay following inactivation or are significantly upregulated during excystation. This approach was motivated by a review of the literature that showed that the transcriptional regulation of only a few *G. lamblia* mRNA transcripts has been evaluated.

The research presented here used the trophozoite transcriptome as a benchmark. Trophozoite mRNA can readily be obtained from cultures, and most genes are expected to be expressed in this stage. In contrast to trophozoites, we found that the cyst transcriptome expresses fewer genes or that many genes are expressed below microarray detection level. The analysis of mRNA from cysts that were inactivated by exposure to heat or lost infectivity as a consequence of aging revealed major changes. The transcriptome of inactivated cysts was further reduced as compared to that of infectious cysts. This finding is consistent with what is known about the normal process of postmortem mRNA decay in mammalian tissues (Johnston et al., 1997; Catts et al., 2005; Bauer et al., 2003) and in protozoan oocysts (Widmer et al., 1999). In contrast, UV irradiation with a lethal dose had little effect on mRNA stability. This observation is consistent with the mode of action of UV irradiation. Irradiated cysts are not dead, but their genome is damaged and cannot replicate. Therefore, the effect of UV irradiation is not apparent until irradiated cysts are ingested and fail to replicate. The apparent lack of an UV irradiation effect on RNA integrity limits the application of the proposed RNA-based assay and indicates that mRNA markers for cyst inactivation by UV irradiation may not exist or that their identification requires more-sensitive methods. Replicate analyses of UV-irradiated cysts would have to be performed to assess whether differences in transcript abundance, such as observed with β-giardin, are reproducible and sufficiently large to enable this transcript to be used as a universal infectivity marker.

This project benefitted significantly from resources supported by the National Institutes of Health, including the *G. lamblia* oligonucleotide microarrays, the microarray data analysis software TM4 (Saeed et al., 2006), and DAVID (Dennis et al., 2003). The use of DNA microarrays to compare the transcriptomes of potentially very dissimilar mRNA populations required data analysis methods different from those normally used to analyze two-color arrays. The absence of a fluorescence signal for a majority of spots on arrays hybridized with cyst cDNA indicated that the cyst transcriptome is less diverse and less abundant than that of trophozoites. The use of conventional data analysis based on log (Cy3/Cy5) ratios would have resulted in the elimination of zero fluorescence values and the loss of biologically relevant information. Instead of ratios, we calculated the difference between Cy3 fluorescence and Cy5 fluorescence. Normalization procedures typically used for microarray data correct for a difference between mean Cy3 fluorescence and mean Cy5 fluorescence across the entire array (Quackenbush, 2002) on the assumption that mean Cy3 fluorescence

and mean Cy5 fluorescence are equal for the two samples being compared. Because cysts are assumed to have little metabolic activity, the failure to detect many transcripts was not surprising. To ensure that these differences were not artifacts, caused for instance by differential cDNA labeling or by Cy5 degradation upon exposure to atmospheric ozone (Branham et al., 2007), a dye swap experiment was conducted with trophozoite RNA. The comparison of the frequency distribution of Cy3 and Cy5 fluorescence signals showed that the observed differences among cyst transcriptomes exceeded any dye effect. In some experiments, prelabeled UMSS probe was added to the experimental cDNA probe. In addition, as shown in Table 3.3, extensive RT-PCR validation was performed using the same cDNA samples used for microarray hybridization. These controls and validations are consistent with the interpretation of the microarray data.

The cysts used in this study originated from gerbils experimentally infected with *G. lamblia* assemblage B. The scope of the project included a validation with assemblage A cysts, but two attempts to infect gerbils with assemblage A trophozoites were not successful. We did not pursue the possibility of using cysts of assemblage A produced in vitro (Gillin et al., 1987) because it is unclear to what extent such cysts are valid surrogates of native cysts. The comparison of the microarray results with the SAGE analysis of Birkeland et al. (2010) is consistent with the view that cysts produced in an infected host and in culture are biologically different.

Except for experiments requiring aged cysts, we treated and processed the cysts within a few days of shipping. There was little delay between purification and shipping of cysts. We noticed that in spite of the rapid turnaround, certain lots of cysts generated low microarray signals, even for untreated controls. This finding was the case for several experiments, including the chlorination experiment. For this experiment we used a dye swap design with two arrays and found that a low control signal was unrelated to the dye. Moreover, two microarrays hybridized in parallel with trophozoite cDNA did not present this problem. These observations point to the cyst as a possible cause of low microarray fluorescence and raise the possibility that the mRNA content of live cysts can vary. In lambs experimentally infected with *C. parvum*, oocyst viability decreases as the infection progresses (Bukhari and Smith, 1997), but we found no reference in the literature to such a phenomenon in *Giardia*. Inspection of the dates of the propagation of each cyst lot does not reveal any difference that could explain our observation. Microarrays hybridized with cDNA originating from such cysts did not generate useful data as far as the effect of chlorination is concerned. More important than the loss of data is the relevance of these observations to the goal of this project and to the feasibility of developing robust mRNA markers of infectivity. If hosts infected with *G. lamblia* excrete cysts with different mRNA content, it would be important to assess whether such cysts are infectious. The occurrence of infectious cysts with various amounts of mRNA could generate false negatives if tested using an mRNA marker. Alternatively, it is conceivable that low microarray fluorescence resulted from a low mRNA yield or perhaps mRNA degradation. These explanations do not seem very plausible as RNA concentration was monitored by measuring absorption at 260 nm and 280 nm and as only samples with the expected total RNA concentration were used.

The list of genes that are highly expressed in cysts (Table 3.2) includes β-giardin, which has been the focus of previous research (Mahbubani et al., 1991). The remaining genes have not been investigated with respect to transcript abundance. Genes that were the focus of other studies don't feature on our list. The transcripts we have identified as highly expressed are

potential infectivity markers, regardless of the protein they encode. Bioinformatics will be needed to identify in these transcripts sequences diagnostic for *G. lamblia* assemblages A and B. Primers and/or probes targeting such sequences will need to be checked for specificity to ensure that the sequences are not found in other organisms. The final choice of transcript will also be influenced by the detection method. If the method is PCR based and requires a fluorescent probe such as in the TaqMan method, the ideal sequence is likely to be different from those for methods that are not based on RT-PCR.

4.2 CONCLUSIONS

The main findings of this project can be summarized as follows:

- The *G. lamblia* cyst transcriptome is significantly reduced in complexity and abundance as compared to that of the trophozoites.
- In cysts that are inactivated by heat or have aged for several weeks, a large proportion of mRNA transcripts is no longer detectable.
- Excystation induces some transcriptional activation, but the difference between the control and the excysted transcriptome is relatively small. Therefore, transcripts that decay postmortem offer an easier target for a molecular infectivity assay than do genes that are induced during excystation.
- Based on the significant difference between the transcriptomes of infectious and dead cysts, many mRNA species can potentially serve as markers of cyst infectivity. Individual preferences and the assay platform will dictate the choice.
- The effect of UV irradiation on the transcriptome is very different from that of treatments that kill the cysts. No global impact on the transcriptome was observed in irradiated cysts.
- The comparison of the transcriptomes of cysts produced in culture and cysts originating from an infected host revealed significant differences.

Additional work is needed to replicate some of the microarray analyses performed during this project. Microarrays have largely been superseded by RNA-Seq for gene expression analysis (Mortazavi et al., 2008), and *Giardia* microarrays are no longer produced by the PFGRC. RNA-Seq analysis will refine the microarray results particularly for those treatments for which replicate microarray data sets were not obtained. RNA-Seq combined with flow cytometry could also be used to investigate the apparent variability of mRNA abundance in live cysts. Combined with infectivity tests in rodents, such analyses could reveal the extent to which the transcriptome can vary in infectious cysts. In future research, cysts obtained from infected rodents should be used rather than cysts produced in culture.

Before mRNA transcripts can be used to identify infectious waterborne *G. lamblia* cysts, it will be necessary to further investigate the transcriptional response of cysts to UV irradiation. Given the mode of action of UV irradiation, it is conceivable that infectious cysts cannot be differentiated by using mRNA markers from cysts subjected to lethal UV irradiation. In contrast to cysts exposed to chemical disinfectants or cysts that are no longer infectious as a result of aging, UV-irradiated cysts are viable but are unable to multiply because their DNA is damaged. Whether alternative markers that can be universally applied and do not require culturing or infecting laboratory rodents can be found remains an open question.

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