Construction and validation of a polycompetitor construct (SWITCH) for use in competitive RT-PCR to assess tachyzoite-bradyzoite interconversion in *Toxoplasma gondii*

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SUMMARY

The obligate intracellular protozoan parasite, *Toxoplasma gondii* exists as 2 life-cycle forms in intermediate hosts. The rapidly dividing tachyzoites responsible for acute disease, present in the first 14 days of infection, give rise to slowly dividing bradyzoites that reside in tissue cysts. Reactivation of disease is associated with conversion of bradyzoites to tachyzoites. A sensitive method for detection and assessment of the number of each life-cycle stage would be useful for following these events. Herein we describe the construction and validation of a plasmid (pSWITCH) containing a polycompetitor construct (SWITCH) for use in competitive reverse transcriptase-PCR (cRT-PCR). pSWITCH contains competitors for SAG2A and LDH2 genes, which are exclusively expressed by tachyzoite and bradyzoite stages respectively, and for β-tubulin, a gene expressed by both stages. Using cRT-PCR, samples can first be accurately normalized for expression of the housekeeping gene, β-tubulin and then the relative levels of SAG2A and LDH2 expression compared to follow stage conversion. The abundance of transcripts for other genes of interest can then be followed during this process as demonstrated here for the SAG2-related family of genes. This technique offers a powerful tool for studying the processes involved in tachyzoite and bradyzoite interconversion.

Key words: *Toxoplasma gondii*, tachyzoites, bradyzoites, conversion, cRT-PCR, SAG2.

INTRODUCTION

Toxoplasma gondii is a protozoan parasite with a complex life-cycle. Whereas sexual reproduction only occurs in the intestine of felines (definitive host), asexual multiplication can occur in almost all warm-blooded animals infected as intermediate hosts (reviewed by Tenter, Heckeroth & Weiss, 2000). Following infection of an intermediate host, including humans, the parasite multiplies rapidly in almost every tissue as the tachyzoite form. This acute infection is generally self-limiting in an immunocompetent host and coinciding with the onset of immunity, the tachyzoites transform into bradyzoite stages. The bradyzoites are long-lived, slowly dividing and form cyst structures in which they reside. Their presence is not associated with active disease (reviewed by Dubey, 1998). It is, however, these cysts that give rise to disease reactivation should the host become immunocompromised. This can have fatal consequences as is evident in AIDS patients (Luft & Remington, 1992).

Infection during pregnancy can result in congenital transmission and in some cases abortion. Congenital infection can cause serious neurological and ocular lesions, but in a large number of cases is asymptomatic at birth (reviewed by Roberts and McLeod, 1999). However, even in these asymptomatic individuals, disease reactivation frequently occurs in later life resulting in ocular and neurological manifestations. This reactivation is again associated with the conversion of the latent bradyzoite to the rapidly dividing tachyzoite (reviewed by Boyer, 1996).

Understanding differences between the tachyzoite and bradyzoite as well as the process of stage conversion and the factors that influence it, may have implications for the development of novel antimicrobial agents. A method for monitoring stage conversion or assessing the relative abundance of each stage would provide a powerful tool for these studies. Furthermore, the ability to assess the relative abundance of each of these stages in various tissues of infected animals may provide information on disease progression in immunological studies.

Recent studies have identified a number of genes encoding proteins that are stage-specifically ex-

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pressed. Important tachyzoite-specific proteins include SAG1 (Kasper, Bradley & Pfefferkorn, 1984) and SAG2 (Prince *et al*. 1990). Since the discovery of a family of SAG2-related genes, the original SAG2 is now referred to as SAG2A (Lekutis, Ferguson & Boothroyd, 2000). Bradyzoite-specific proteins include BAG1 (Bohne *et al*. 1995) and LDH2 (Yang & Parmley, 1997). In contrast, β-tubulin is a housekeeping gene expressed in both tachyzoite and bradyzoite stages. Herein we describe the construction of a polycompetitor construct (pSWITCH) that contains competitors for β-tubulin, SAG2A and LDH2 genes. Using this construct in a cRT-PCR system we were able to normalize accurately samples for β-tubulin expression and follow the conversion of tachyzoites to bradyzoites *in vitro* by monitoring the abundance of SAG2 and LDH2 transcripts. The utility of this system was validated by demonstrating stage-specific transcription of the recently described SAG2 family of proteins (Lekutis *et al*. 2000).

MATERIALS AND METHODS

PCR

PCR reactions contained a final concentration of 1 × PCR buffer (50 mM KCl, 10 mM Tris–HCl, pH 9·0, 0·1 % Triton X–100), 1·5 mM $MgCl_2$, 200 μM of each dNTP, 0·5 μM of each primer and 0·25 Units *Taq* polymerase (Promega) in a total volume of 25 μl. Cycling conditions comprised an initial denaturation at 94 °C for 3 min followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 58 °C for 30 s (unless otherwise stated) and extension at 72 °C for 1 min. All reactions finished with a final extension period of 10 min at 72 °C.

Construct design (*pSWITCH*)

For purposes of competitive RT-PCR it was first necessary to introduce mutations into a selection of genes and to incorporate each into a plasmid. We chose to make deletion constructs using β-tubulin as a housekeeping gene (Nagel & Boothroyd, 1988), SAG2 as a tachyzoite-associated gene and LDH2 as a bradyzoite-associated gene. Previous research has utilized SAG2A for detection of tachyzoite-specific mRNA (Gazzinelli *et al*. 1993), whilst LDH2 was chosen because the mRNA of LDH2 has only been detected in the bradyzoite stage (Yang & Parmley, 1997).

To make a shortened competitor sequence for LDH2, a 162 bp region in the middle of the gene was deleted. Two fragments were amplified on either side of this region. A 170 bp long fragment was amplified upstream of the deletion using primers LDH2F (5′ATGACGGGTACCGTTAGC3′) and LDH2Arev (5′CTTGTCGCCTGTGAATCA3′), and a 100 bp fragment was amplified downstream using primers LDH2Afor (5′AGAGGTAGCGCAGGGAG3′) and LDH2R (5′GCTCGCTTCATGAAAGCA3′). Adenosine overhangs were removed from each fragment by Klenow treatment, after which they were simultaneously cloned into *Sma*I-digested phosphatase-treated pUC18 using the heat-shock transformation method of Cohen, Chang & Hsu (1972). To ensure that the fragments were cloned in the correct orientation, antibiotic-resistant clones containing inserts were screened by PCR. Specifically, PCR was performed using primers LDH2F and LDH2R to identify clones with a product of the expected 270 bp length.

For SAG2A, a similar approach was used to delete a 104 bp region. Primers used for the 153 bp 5′ fragment were SAG2 for (5′TGATGCATGCTCCAGTGGTTCCGTTGTCTTCC3′) and SAG2A-rev (5′GACCTTAGCTGTCAAGAC3′), and for the 154 bp 3′ fragment were SAG2Afor (5′CCGCTGGTCGAAATAATG3′) and SAG2rev (5′ACAAGCATGCGAGACCGGGAGCAAGAACC3′). Restriction sites for *Sph*I were engineered into primers SAG2for and SAG2rev (underlined). The PCR fragments were treated and cloned into the *Sma*I site in pUC18, and correctly orientated clones, containing a 307 bp insert identified, essentially as described for LDH2.

The β-tubulin construct containing a 130 bp deletion was generated using the PCR-based approach called gene splicing by overlap extension (SOE) (Horton *et al*. 1989). A 243 bp 5′ fragment was generated using primers TubA2Bam (5′TAGGATCCTTGGGAGGTCATCTC3′) and TubBrev.mod (5′*CACGCAATAACCTTCACACTCCAAATTTATAAC*TGTCAGGGCGGAAGAGCT3′) at an annealing temperature of 52 °C. A 208 bp 3′ fragment was generated using primers TubCfor.mod (5′*GTTATAAATTTGGAGTGTGAAGGTTATTGCGTG*GTTTCCAGATCACCCACAGT3′) and TubrevDBam (5′ATGGATCCAGAGCGTTGCGTTGTACG3′). Restriction sites for *Bam*HI were engineered into primers TubA2Bam and TubrevDBam (underlined). Primers TubBrev.mod and TubCfor.mod contained overlapping complementary sequences (shown in italics) so that when the products were mixed, denatured and reannealed, the strands having the matching sequences at their 3′ ends reannealed and acted as primers to each other. Extension of this overlap by PCR at an annealing temperature of 50 °C produced the expected 412 bp β-tubulin deletion construct. This product was purified and cloned into *Sma*I-digested phosphatase-treated pUC18 again as described for LDH2.

The LDH2, β-tubulin and SAG2A deletion constructs were excised from their respective pUC18 vectors using the appropriate restriction enzymes and sequentially cloned into pUC18 at restriction sites *Sma*I, *Bam*HI and *Sph*I respectively (GenBank accession number AF339037). This produced the

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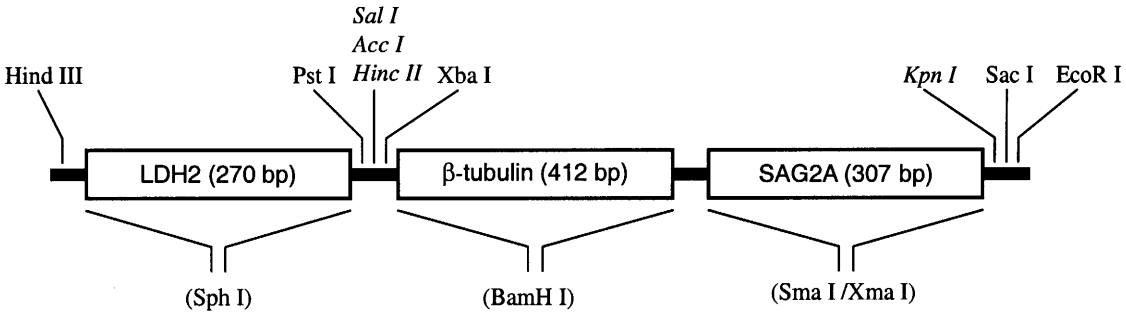

Fig. 1. Restriction enzyme map of the pUC18 modified multi-cloning site (MCS) from pSWITCH (Genbank accession number AF 339037). Competitor templates for LDH2, β-tubulin and SAG2A were cloned into the *Sph*I, *Bam*HI and *Sma*I/*Xma*I sites respectively. The remaining restriction enzyme sites in the MCS, with the exception of the *Kpn*I and the *Sal*I/*Acc*I/*Hin*cII sites (italics) are preserved for future use.

multiple-specific competitor plasmid pSWITCH (Fig. 1). All other restriction enzymes within the multi-cloning site (MCS) of pUC18 are available for future cloning with the exception of *Hin*cII and *Kpn*I which occur within the construct sequence. The new tri-competitor insert (SWITCH) was then excised from the plasmid by digestion with 2 remaining unique restriction enzymes and gel purified. Sequential dilutions of this insert were prepared and stored in aliquots for later use.

In vitro *tachyzoite–bradyzoite differentiation*

To induce tachyzoites to differentiate to bradyzoites *in vitro*, a modification of the high pH method of Soete *et al.* (1993) was chosen. Six 25 cm² flasks containing confluent monolayers of human foetal lung fibroblast (MRC-5) were grown at 37 °C under 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) with 10% foetal calf serum (FCS) at pH 7·2. These monolayers were infected with Beverley strain tachyzoites from a recently lysed culture at a multiplicity of infection of 0·1 (i.e. 1 parasite/10 host cells). These were grown in normal conditions for 4 h to allow invasion and initial growth. The medium was then replaced with inducing medium (RPMI/HEPES pH 8·1, 5% FCS) and the flasks placed in an air incubator at 37 °C. Inducing medium was replaced every 2 days. Starting 24 h post-infection, 1 flask was harvested every day. Host cells and parasites were pelleted by centrifugation at 2000 ***g*** for 5 min. The parasite/host pellets were stored at −70 °C.

RNA extraction

Pellets containing host and parasite cells were homogenized in 1 ml of TRIZOL® reagent (GibcoBRL Life Technologies, Paisley, UK). Total RNA was isolated following a protocol based upon the single-step acid guanidinium thiocyanate–phenol–chloroform RNA isolation method (Chomczynski & Sacchi, 1987) in accordance with the manufacturer's instructions.

Preparation of genomic DNA from T. gondii

Genomic DNA was extracted from tachyzoites of *T. gondii* RH strain by alkaline lysis (Johnson, Dubey & Dame, 1986).

Reverse transcription PCR (RT-PCR)

Complementary DNA (cDNA) was produced from total RNA using M-MLV reverse transcriptase (GibcoBRL Life Technologies). In a 90 μl reaction volume, 7 μg of RNA was combined with 18 μl of 5× first stand buffer (250 mM Tris–HCl, pH 8·3, 375 mM KCl, 15 mM $MgCl_2$), 18 μl of deoxynucleoside triphosphate mix (10 mM), 9 μl of 0·1 M dithiothreitol, 80 units of RNAsin ribonuclease inhibitor (Promega), 500 ng of random hexamer primers (Promega, Southampton, UK) and 1200 units of M-MLV reverse transcriptase. Following a 10 min pre-incubation at 27 °C, the mixture was incubated at 42 °C for 60 min, followed by reaction termination by heating at 95 °C for 5 min. All cDNA was stored at −20 °C until used in PCR.

Competitive RT-PCR (cRT-PCR) for parasite transcripts

The levels of parasite transcripts were assessed by cRT-PCR using the multi-specific competitor SWITCH. The concept of cRT-PCR has been described previously (Reiner *et al.* 1993; Bouaboula *et al.* 1992; Roberts *et al.* 2000). To ensure that equal quantities of parasite cDNA were used in PCR reactions for each sample, levels of transcript were first normalized against the *T. gondii* β-tubulin transcript. To achieve this, PCR was first performed for β-tubulin in the absence of competitor and samples adjusted until similar band intensities were observed. To complete normalization, PCR for β-tubulin was then performed in the presence of a constant amount of competitor. Where necessary cDNA samples were adjusted until amplification resulted in equal intensities of specific and competitor products for all the samples. For all further

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experiments, the amounts of cDNA for each day were maintained. Primer combinations used for cRT-PCR were as follows: for β-tubulin, TubA3for (5′GACGAACACGGAATTGATC3′) and TubDrev (5′AGAGCGTTGCGTTGTACG3′), for SAG2, SAG2newfor (5′CTTCCAAT GTGGGGATAAAC3′) and SAG2revbio (5′ACAAGCATGCGAGACCGGG AGCAAG AACC3′), and for LDH2, LDH2forbio (5′TGACGGGTACCGTTAGC3′) and LDH2revbio (5′GCTCGCTTCATGAAAGA3′).

Evaluation of transcription of other genes

To evaluate the usefulness of this system in determining relative levels of transcription of other genes through the stage conversion process, the family of developmentally regulated SAG2-related genes recently identified by Lekutis *et al*. (2000) was used. Primers were designed to amplify fragments of SAG2B [2B.for (5′AGCTCTTCAGACACGACA3′) and 2B.rev (5′GGTCACCCCACTCTCGA3′)], SAG2C [2C.for (5′CAGTAATGCATCGCCTGT3′) and 2C.rev (5′GTTTCGGGGACAGTCAC3′)] and SAG2D [2D.for (5′AGTTTCGTGCAGCAGTCG3′) and 2D.rev (5′CAACCCGCCCAGCCGTC3′)].

RESULTS

Specificity of PCR

The specificity of PCR primers for tachyzoite and/or bradyzoite cDNA was assessed using *T. gondii* cDNA isolated from tachyzoites or bradyzoites. As expected the SAG2A transcripts were abundant in the tachyzoite stage but absent in the bradyzoite stage, whereas LDH2 transcripts were absent in tachyzoites but abundant in bradyzoites (Fig. 2). In contrast, β-tubulin transcripts were found in both stages. Using the same primer pairs, the appropriate competitor products could be easily amplified from pSWITCH and differentiated from the authentic product by their size differences. As the PCR primers for β-tubulin and LDH2 were designed on either side of an intron, amplification of genomic DNA (gDNA) results in larger products than the expected size for cDNA (Fig. 2). This provides a means to detect gDNA contamination in samples. This design feature was not possible for SAG2A as it does not have any introns. No PCR products were amplified from host cell-derived cDNA using any of the primer pairs (data not shown).

Sensitivity of PCR

To evaluate the sensitivity of the primers chosen for amplification of β-tubulin, SAG2 and LDH2, cDNA was obtained from a known number of tachyzoites and bradyzoites (determined by microscopy) and amounts of cDNA corresponding to 1000, 100, 10, 1 and 0·1 parasites were added to PCR reactions and amplified. Using the primers TubA3for and TubDrev for β-tubulin, it was possible to detect product from as few as 10 parasites. Similarly, using primers LDH2forbio and LDH2revbio for LDH2, it was possible to detect product from as few as 10 parasites. Using the original primer combination SAG2for and SAG2revbio, it was only possible to detect 100 parasites. Utilizing a new primer SAG2newfor with SAG2revbio, it was possible to increase the sensitivity to detect 10 parasites. This new combination of primers resulted in the PCR products from both parasite cDNA and the competitor cDNA being shortened by 27 bp, but did not affect cRT-PCR. This primer pair was used in all further SAG2A analyses. With all 3 primer pairs, the PCR product intensity was observed to increase with increasing cDNA template.

Monitoring tachyzoite to bradyzoite conversion in vitro

T. gondii β-tubulin cDNA was amplified from all samples and, as expected, the intensity of product increased over the 6 day period reflecting proliferation of parasite. To allow comparison of the relative proportion of tachyzoites and bradyzoites at each time-point, the amount of parasite cDNA added to each reaction was normalized for β-tubulin transcripts. This ensured that the total amount of parasite cDNA added to further PCR reactions was consistent between samples.

To achieve this the amount of cDNA added from each sample was adjusted until β-tubulin product intensities were similar to product amplified from the day 5 sample. A quantity of SWITCH that gave a similar band intensity was chosen as an appropriate dilution for cRT-PCR. When this constant amount of competitor cDNA was added to each sample and PCR performed, variability in band intensity was observed. This demonstrated that parasite cDNA levels were not yet accurately normalized and highlights the importance of the competitor for accurately equalizing levels for each sample. Samples were further adjusted so that the larger cDNA product and smaller competitor product were of similar intensity (Fig. 3) and this amount of cDNA was used in all subsequent PCR reactions.

The levels of SAG2A and LDH2 transcripts were assessed using cRT-PCR over the time-course of the stage switch experiment. In the absence of competitor, no reduction in the intensity of the SAG2A PCR product was evident on visualization of the ethidium bromide-stained gel. However, when PCR reactions were performed in the presence of an appropriate amount of competitor, the intensity of the authentic SAG2A product decreased relative to the competitor product over the time-course of the

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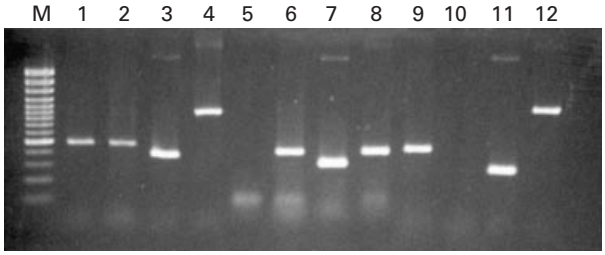

Fig. 2. Evaluation of PCR product size and primer specificity. Lanes 1–4 contain products amplified using β-tubulin specific primers TubA3for and TubDrev. Lanes 1 and 2 were amplified using cDNA from bradyzoite (Beverley strain) and tachyzoite (RH strain) respectively, Lane 3 was amplified from the pSWITCH plasmid, and Lane 4 from RH strain genomic DNA. Lanes 5–8 demonstrate products amplified by the SAG2A-specific primers SAG2newfor and SAG2revbio from bradyzoite, tachyzoite, pSWITCH and gDNA respectively. Lanes 9–12 demonstrate products amplified by the LDH2-specific primers LDH2forbio and LDH2revbio from bradyzoite, tachyzoite, pSWITCH and gDNA respectively. The large bands in lanes 3, 7 and 11 correspond with the expected size of the pSWITCH plasmid used as template in these reactions. Marker (M) is GeneRuler™ 100 bp ladder plus (MBI Fermentas).

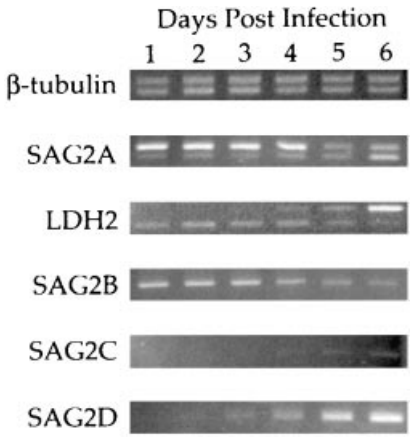

Fig. 3. Monitoring of tachyzoite–bradyzoite interconversion *in vitro* over a 6 day time-course and evaluation of transcription of the SAG2-related gene family. Samples were first normalized for β-tubulin expression (note equal intensities of upper bands (specific product) with lower bands (competitor product)). The quantity of sample used to achieve this normalization was used in all subsequent reactions. SAG2 expression declined over the 6 day time-course (note the decrease in the intensity of specific product (upper band) relative to competitor (lower band) with time). LDH2 expression increased over this time [note increase in intensity of specific product (upper band) relative to competitor product (lower band) with time]. Expression of SAG2B decreased with time (note decrease in band intensity with time), whereas SAG2C and SAG2D increased with time.

experiment (Fig. 3B). In contrast, an increase in the intensity of LDH2 PCR product was observed with time. This was best illustrated by an increase in the authentic product relative to competitor product (Fig. 3C). Together these results illustrate an increase in the proportion of bradyzoites with a concomitant decrease in tachyzoites relative to total parasite numbers.

Evaluation of transcription of other genes

To evaluate the usefulness of the pSWITCH system for investigating transcription of other genes, the levels of transcript for members of the recently described SAG2-related gene family were assessed. The β-tubulin normalized samples were subjected to PCR using primers designed to amplify portions of each SAG2 related gene. SAG2B transcript was observed to decrease over the 6 day period (Fig. 3D), while both SAG2C and SAG2D transcript increased over this period (Fig. 3E and F). These results demonstrate that the transcription of these genes is regulated in *T. gondii* during stage switch.

DISCUSSION

Herein we describe a method for monitoring *T. gondii* tachyzoite–bradyzoite interconversion based on the assessment of levels of stage specific transcripts by cRT-PCR. This method relies on the polycompetitor plasmid pSWITCH that contains the competitor sequences for *T. gondii* β-tubulin, SAG2 and LDH2. β-tubulin was chosen as a classical house-keeping gene for the precise normalization of parasite-derived cDNA, and SAG2A and LDH2 chosen as tachyzoite and bradyzoite-specific genes respectively (Nagel & Boothroyd, 1988; Kasper *et al.* 1984; Yang & Parmley, 1997). Both have previously been demonstrated to be developmentally regulated at the level of transcription, a trait necessary for the success of this technique. All competitor constructs were designed by deletion of a small region within the coding sequence of each gene. Alternative approaches that involve insertion of additional DNA between the primer sites or construction of completely synthetic competitors have also been widely used by other workers. However, the strategy we employed resulted in competitor cDNA templates that closely resemble that of WTcDNAs, which is most likely to ensure faithful mimicry of the relative efficiency of PCR amplification (Gilliland *et al.* 1990). For convenience all 3 competitor cDNAs were cloned into a single vector (pSWITCH) so that only 1 molecule is required for all determinations. Excision of the molecule (SWITCH) from pSWITCH is recommended before cRT-PCR, as efficiencies of amplification can be affected if the competitors are contained within circular DNA unlike the authentic cDNA that is linear. pSWITCH was designed to preserve other cloning sites available in pUC18 so that those remaining can be utilized to introduce competitors for other developmentally regulated genes as and when required. For synthesis

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of deletion constructs in the future, the method of SOE has proven to be a powerful non-random tool that we would encourage others to utilize (Horton *et al*. 1989).

An essential aspect of the procedure we describe is the careful normalization of samples against the house keeping gene β-tubulin using cRT-PCR. Stage conversion is then followed by the changing levels of SAG2A and LDH2 transcripts, also determined by cRT-PCR. The careful normalization of samples allows comparison of the expression of other genes of interest. To examine the potential of this system we investigated the levels of mRNA of 3 SAG2-related genes during tachyzoite to bradyzoite conversion. This family of SAG2-related genes has recently been characterized by Lekutis *et al*. (2000) and consists of at least 3 members that are developmentally regulated in *T. gondii*. The gene SAG2B encodes a protein present in tachyzoites only, while genes SAG2C and SAG2D encode proteins expressed exclusively on the surface of bradyzoites. In the present study we found the pattern of transcription to change during conversion consistent with the previous observations for protein expression. Together these results would indicate that regulation is at the level of transcription rather than post-transcriptional.

Competitive RT-PCR is a powerful method and has proven to have some significant advantages over other techniques including the use of stage-specific antibodies, bioassay and Northern blotting. One advantage of this technique over others available, such as the use of stage-specific antibodies, is the limited amount of starting material required. Another is that it is holistic in nature and allows an entire sample to be examined. The process of cDNA synthesis and template optimization can be carried out relatively quickly and requires no blotting, radioactivity or special densitometry equipment.

This method has not been designed as a quantitative technique, but rather as a semi-quantitative, illustrative tool for the rapid evaluation of expression levels of genes that may be stage-specifically regulated. There would be inherent danger in concluding that concentrations of WTcDNA and competitor cDNA correlate when band intensities are identical. Firstly, the relative molarity for a given concentration of DNA would differ between WT and competitor cDNA. Furthermore, as WTcDNA begins as a single strand whereas the competitor cDNA begins as double-stranded, the relative efficiency of amplification between the 2 species may vary.

Despite these precautions, cRT-PCR using SWITCH has been demonstrated in these studies to be a sensitive method for the detection of small numbers of parasites undergoing stage conversion *in vitro*. This method could also be utilized for monitoring gene expression during antisense experiments, promoter analysis or for evaluating transcription in a variety of mutant organisms. The method described herein could be easily adapted to assess total parasite number or the number of tachyzoites and bradyzoites in the tissue of infected animals. In this scenario cDNA would be normalized to a host house-keeping gene such as HPRT or β-actin using cRT-PCR and one of the constructs previously described such as pQRS (Reiner *et al*. 1993).

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