INTESTINAL PATHOGENS

Cryptosporidium parvum (Apicomplexa) is a zoonotic pathogen that can cause severe diarrhoea in both humans and animals. Like other apicomplexans (e.g. Plasmodium, Toxoplasma and Eimeria) of medical and/or veterinary importance (Fayer et al., 1997; Okhuysen & Chappell, 2002; Tzipori & Widmer, 2000), the complex life cycle of C. parvum starts with the excystation of sporozoites from ingested oocysts within the host intestinal lumen. The free sporozoites will infect intestinal epithelial cells and develop by merogony into first and second generations of merozoites, which subsequently differentiate into micro- or macrogametocytes by gametogenesis. Fertilization results in zygotes that further develop into mature, sporulated oocysts that can either ‘auto-infect’ the same host or be excreted into the environment to become infectious to other human or animal hosts (Fayer et al., 1997).

Although C. parvum can be cultured in vitro by infecting various primary or transformed human or animal cell lines, the development of this parasite in vitro has been mostly limited to the asexual development (merogony) (Arrowood, 2002; Upton, 1997; Upton et al., 1995). Well-developed C. parvum can typically be observed in vitro after infecting host cells for 24 and 48 h, in which most parasites are in the stages of Type I and II meronts (with a few developing gametes), respectively. However, the growth of parasites apparently starts to decline after cultivation in vitro for 72 h or longer, in which many of them have typically developed into macrogametes or microgametocytes. Although limited numbers of C. parvum oocysts produced in vitro have been reported, most of them are not biologically viable (Hijjawi et al., 2001; Yang et al., 1996). As yet, it is unclear why most C. parvum can not complete sexual development under in vitro conditions.

C. parvum possesses a compact genome with short intergenic regions, and only a limited number of introns have been reported in the C. parvum genome (Bankier et al., 2003; Caccio et al., 1997; Deng et al., 2002). Since introns have to
be removed by splicing mature RNA, primers spanning the intron regions have been frequently used in RT-PCRs to distinguish RNA-originating amplicons from those amplified from DNA. Using this approach, however, the presence of intron-containing transcripts was unexpectedly observed in late developmental stages of \( C. \) parvum cultured in vitro, but not in those obtained from infected calves. These observations imply a possible abnormality in intron-processing by \( C. \) parvum during some in vitro life cycle stages, and these can be correlated with the dramatic arresting of growth and sexual development of parasites cultured in vitro.

**METHODS**

**Parasites.** Fresh oocysts of \( C. \) parvum (Iowa strain, less than 3 months old since harvest from infected calves) were purified from calf faecal samples by a gradient sucrose centrifugation method, sterilized for 5 min in 10% Clorox on ice, and washed intensively in water by centrifugation for five to eight times. Sterilized oocysts were further separated from a limited amount of debris, including some dead bacteria, by Percoll gradient centrifugation. Sporozoites were prepared by excystation of oocysts in PBS (pH 7.5) containing 0.25% trypsin and 0.75% taurodeoxycholic acid for 60–90 min at 37°C, washed with PBS (three to five times) and concentrated as described previously (Zhu & Keithly, 1997).

For cultivation of \( C. \) parvum in vitro, HCT-8 or Caco-2 cells (10^6 per well) were seeded in six-well plates and allowed to grow overnight or until they reached confluence. Free sporozoites were added to the monolayers at parasite-to-host cell ratios of either 1:5 (for infections lasting up to 24 h) or 1:10 (for infections lasting longer than 24 h), respectively. Free sporozoites were removed after 3 h infection by a medium replacement. After monolayers were infected with sporozoites at various time-points (e.g. 3, 6, 12, 24, 36, 48, 60 or 72 h), cells were washed three times with PBS and treated immediately with RNA later (Ambion) to preserve RNA integrity. Total RNA was then isolated from RNA later-treated monolayers using an RNeasy isolation kit (Qiagen). For the isolation of total RNA, uninfect host cells cultured for 24 h were similarly treated and used as one of the negative controls. In addition, total RNA was also isolated from the small intestinal epithelial cells from four calves (in vivo samples): two were uninfect and the other two were infected with \( C. \) parvum for 72 h. All RNA samples were further treated with RNase-free DNase and purified again. In some cases, the DNase treatment procedure was repeated up to five times to ensure no contamination of genomic DNA occurred in any samples.

**RT-PCR.** Since total RNA isolated from infected host cells or intestines might contain different ratios of parasite 18S RNA, the concentration of parasite total RNA in all samples was normalized using a semi-quantitative RT-PCR method described previously (Abrahamsen & Schroeder, 1999). Briefly, using an AccessQuick RT-PCR kit (Promega) and a pair of 18S RNA-specific primers (995F, 5′-TAG AGA TTG GAG GTT GTT CCT-3′; 1206R, 5′-CTC CAC CAA CTA AGA AGC GCC-3′), the amplifications were performed for 22 thermal cycles to ensure the amounts of RT-PCR products were within the linear range. The amplicons (10 µl each) were separated in 2% agarose gels, intensities were measured and then were used as a guide for normalizing parasite total RNA in the various diluted samples. These adjusted total RNA samples were subject to a second or third round semi-quantitative RT-PCR and the concentrations were further adjusted until the intensities of the amplicons were comparable. Therefore, although the concentrations of total RNA (a mixture of host and parasite RNA) might vary among different samples, those of parasite RNA in different samples were relatively similar (if not identical) and suitable to serve as templates for comparative amplification of parasite transcripts.

The intron-containing β-tubulin gene from \( C. \) parvum has been characterized previously (Caccio et al., 1997). In this study, the following two primers flanking the 85 bp intron were used for detecting \( C. \) parvum β-tubulin transcripts by RT-PCR: CpBTUB-F137 (5′-ATG TTC ATG GAG GAC AAT GTG-3′) and CpBTUB-R611 (5′-GAG TGA GTG ATT TGG AAA CCC-3′). (Note: the two underlined nucleotides may represent sites of variations between isolates based on the recent deposit of alternative sequences in GenBank, e.g. Y12615 vs BX538353). Each 25 µl reaction contained 50 ng each of the primers, 2.5 U reverse transcriptase (RTase), 2.5 U DNA polymerase, 1 µl normalized total RNA and other reagents as specified by manufacturer’s instructions (Promega). The synthesis of first strand cDNA was performed at 48°C for 45 min, followed by a heat-inactivation of RTase at 95°C for 2 min and subsequent 28–32 thermal cycles of amplification (95°C × 30 s, 50°C × 30 s and 68°C × 60 s for each cycle). All amplicons were analysed by 2% agarose gel electrophoresis.

**RESULTS AND DISCUSSION**

Introns are commonly present in eukaryotic organisms, but have to be removed from mature gene transcripts by
various complex intron-splicing mechanisms. Unlike other Apicomplexa (Toxoplasma gondii, Plasmodium falciparum and Eimeria tenella), introns are much less frequent in C. parvum genes. Among them, the β-tubulin gene contains only a single intron close to its 5' end (Caccio et al., 1997). Since β-tubulin is a house-keeping gene, its mRNA has been used as a reference in our RT-PCR analyses of other C. parvum transcripts using a pair of primers flanking the intron region. Theoretically, this should distinguish amplicons of normal cDNA (without introns) from those of genomic DNA in parasites infecting HCT-8 cells for 12–72 h (Fig. 1a). However, the co-existence of amplicons derived from intron-containing β-tubulin mRNA was unexpectedly observed in parasites cultured for 48–72 h (Fig. 1a). The intron-containing transcripts were also detected in late developmental stages of parasites cultured within Caco-2 cells (Fig. 1b), suggesting that this might be characteristic of all C. parvum cultured in vitro. The intron-containing amplicons were not derived from contaminated genomic DNA, since identical products were consistently amplified from RNA samples that had been extensively treated with DNase (Fig. 2a). On the other hand, no amplicons (with or without introns) were amplified from samples treated with RNase (Fig. 2b), indicating that all amplicons were indeed derived from C. parvum β-tubulin transcripts. Both RT-PCR amplicons were also cloned and sequenced to confirm that both types of amplicons were indeed derived from β-tubulin mRNA: one with and the other without the intron sequence. To test whether the intron was normally spliced in parasites growing in vivo, RT-PCR was performed using total RNA isolated from either uninfected or 72 h C. parvum-infected calf small intestinal epithelial cells. These data indicated that only normally processed β-tubulin transcripts (i.e. without an intron sequence) were present in in vivo samples (Fig. 3). These observations are consistent with the idea that intron-containing β-tubulin transcripts are unique to in vitro-cultured parasites.

**Fig. 1.** Agarose gel analysis of C. parvum β-tubulin amplicons amplified by RT-PCR from total RNA isolated from intracellular parasites infecting HCT-8 (a) and Caco-2 (b) cells for 12–72 h. M, Lanes loaded with molecular markers. Solid or open arrowheads indicate amplicons with or without introns, respectively.

<table>
<thead>
<tr>
<th>P.i. (h)</th>
<th>60</th>
<th>72</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNase</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>RTase</td>
<td>+</td>
<td>−</td>
</tr>
</tbody>
</table>

**Fig. 2.** (a) C. parvum β-tubulin transcripts containing or lacking introns could be detected by RT-PCR from RNA samples repeatedly treated with DNase (five times) (lanes 1 and 3). In negative controls, no amplicons were obtained by regular PCR without reverse transcriptase (RTase) (lanes 2 and 4). (b) No products could be amplified by RT-PCR from the RNA samples treated with RNase (lanes 1 and 4), suggesting that both amplicons were indeed derived from RNA. In the same experiment, both positive and negative controls (lanes 2, 3, 5 and 6) produced the desired results. M, Lanes loaded with molecular markers. P.i., post-infection. Solid or open arrowheads indicate amplicons with or without introns, respectively.
Using various TAMRA- or FAM-labelled probes, FISH was also performed to validate the RT-PCR data. In this experiment, no or small fluorescence signals were detected using the 'Intron-sense' (negative control) probe from parasites cultured for 36 and 60 h, respectively, suggesting that there was no undesired hybridization between probes.
and genomic DNA (data not shown). On the other hand, signals were readily observed from all parasites cultured in vitro using the ‘Exon-antisense’ (positive control) probe (Fig. 4, panels labelled with ‘Anti-exon’), confirming the presence of β-tubulin mRNA in parasites. However, when the ‘Intron-antisense’ probe was used, a small hybridization signal was seen in parasites cultured for 36 h, whereas a significant amount of fluorescence was detected in those cultured for 60 h (Fig. 4, panels labelled with ‘Anti-intron’). The intron-containing transcripts were present in the majority of intracellular parasites, most of which are developing macrogametes or microgametocytes. In a few parasites, discrete strong signals were also observed. These observations again confirmed the presence of unprocessed intron in β-tubulin transcripts in late life cycle stages.

Although *C. parvum* possesses a relatively small number of intron-containing genes and the actual intron-splicing mechanism is as yet unstudied in this parasite, data-mining the complete *C. parvum* genome project (http://cryptodb.org/) revealed the presence of intron-splicing factors (data not shown). Intron-splicing is one of the basic, and vital, cellular functions that ensures the ultimate fidelity of mature transcripts used for translating encoded proteins. In the *C. parvum* β-tubulin gene, which encodes one of the cytoskeleton proteins, a single intron is present close to the 5′ end of the gene (Caccio *et al.*, 1997). Therefore, translation from an intron-containing mRNA would produce a prematurely terminated small peptide that could not be utilized by the parasite for cytoskeleton remodelling. Our observations suggest that *C. parvum* cultured for 48 h or longer either may not be able to correctly process the intron of the β-tubulin transcripts or that the process has been significantly slowed. However, currently one can not rule out the possibility that the decay of intron process might simply be due to the onset of parasite death. Nonetheless, it is unclear whether this is limited to tubulin transcripts or is a general phenomenon for all intron-containing transcripts in *C. parvum* cultured in vitro. In either case, this is an intriguing question that warrants further investigation. In fact, our preliminary studies on the transcription of a putative phosphopantethein transferase gene (*CpPPT1*) in *C. parvum* have already detected its intron-containing transcripts by RT-PCR in parasites cultured in vitro for 60–72 h, but not in those cultured for less than 36 h (data not shown).

The study of *C. parvum* biology is greatly limited by the inability of the parasite to complete its life cycle in vitro to produce vital oocysts. This parasite can grow after infecting host cells, but optimal growth is mostly limited to the asexual development (merogony) that occurs within the first 48 h (Upton, 1997). The appearance of an unprocessed β-tubulin intron in *C. parvum* correlates well with the decline or arrest of growth of this parasite under in vitro conditions, implying that an abnormality in intron-splicing might be associated with difficulties in culturing this parasite. If this is true, investigations on the factors contributing to the intron-splicing in cultured *C. parvum* may provide important information for overcoming the limitations in culturing this parasite. Furthermore, the co-existence of processed and unprocessed introns, at least in β-tubulin transcripts in vitro, may also be utilized as a potential model to study the intron-splicing factors in this parasite.

**ACKNOWLEDGEMENTS**

This study was funded in part by grants from the National Institute of Infectious and Allergic Diseases (NIAID), National Institutes of Health (NIH), USA (R21 AI055278 and R01 AI044394 to G.Z. and U01 AI046397 to M.S.A.), and the Department of Veterinary Pathobiology, College of Veterinary Medicine, Texas A&M University (new faculty start-up funds to G.Z.).

**REFERENCES**


