A *Cryptosporidium parvum* Genomic Region Encoding Hemolytic Activity

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Successful parasitization by *Cryptosporidium parvum* requires multiple disruptions in both host and protozoan cell membranes as cryptosporidial sporozoites invade intestinal epithelial cells and subsequently develop into asexual and sexual life stages. To identify cryptosporidial proteins which may play a role in these membrane alterations, hemolytic activity was used as a marker to screen a *C. parvum* genomic expression library. A stable hemolytic clone (H4) containing a 5.5-kb cryptosporidial genomic fragment was identified. The hemolytic activity encoded on H4 was mapped to a 1-kb region that contained a complete 690-bp open reading frame (*hemA*) ending in a common stop codon. A 21-kDa plasmid-encoded recombinant protein was expressed in maxicells containing H4. Subclones of H4 which contained only a portion of *hemA* did not induce hemolysis on blood agar or promote expression of the recombinant protein in maxicells. Reverse transcriptase-mediated PCR analysis of total RNA isolated from excysted sporozoites and the intestines of infected adult mice with severe combined immunodeficiency demonstrated that *hemA* is actively transcribed during the cryptosporidial life cycle.

The coccidian protozoan *Cryptosporidium parvum* is now recognized as a common cause of diarrheal disease in humans worldwide (5). Individuals with AIDS are at particular risk for developing a debilitating protracted diarrheal illness that often persists until death (24). This intestinal pathogen has also been shown to play a significant role in the vicious diarrhea/malnutrition cycle experienced by young children in developing countries (9). Numerous outbreaks of cryptosporidiosis associated with person-to-person contact in day care centers and hospitals and waterborne spread from contaminated public water supplies have been reported in the United States (9, 23). Multiple chemotherapeutic agents have been tested in infected humans and in animal models of cryptosporidiosis, but none has been found to consistently eradicate the organism from the gastrointestinal tract.

Similar to other coccidia, *C. parvum* is an obligate intracellular parasite which invades by destabilizing the host enterocyte membrane and forming an intracellular parasitophorous vacuole containing the developing organism. Ultrastructural studies have demonstrated that a series of modifications and disruptions occur in the membranes of both the intestinal epithelial cell and the protozoan as the parasitophorous vacuole is established within the apical portion of the enterocyte, a membrane-derived feeder organelle is formed, and developing life stages break through the parasitic and host cell membranes to reenter the gut lumen (22, 25, 30). A better understanding of the cryptosporidial mechanisms involved in these membrane alterations could provide information that will lead to the development of more effective treatment strategies.

The lack of efficient laboratory culture methods to obtain large quantities of *C. parvum* has hampered efforts to directly isolate and characterize cryptosporidial proteins expressed

during the protozoan's life cycle. However, previous reports have demonstrated that it is possible to express recombinant proteins in *Escherichia coli* that are encoded on cloned cryptosporidial genomic fragments (11, 21, 27, 29). In order to identify cryptosporidial proteins with membrane-disrupting properties, a *C. parvum* genomic expression library was transformed into a nonpathogenic *Escherichia coli* strain and the transformants were screened for hemolytic activity on sheep blood agar. The cloning and characterization of a cryptosporidial genomic region which confers a hemolytic phenotype on *E. coli* XL1-Blue are described in this report.

MATERIALS AND METHODS

Library construction. Cryptosporidial oocysts were obtained from experimentally infected Holstein calves and purified as previously described (17). Approximately 2×10^9 oocysts were freeze-thawed eight times with liquid nitrogen and incubated overnight at 55°C in lysis buffer (120 mM NaCl, 10 mM EDTA, 25 mM Tris [pH 7.5], 1% *N*-lauroylsarcosine) containing 1 mg of proteinase K per ml. Nucleic acids isolated by phenol-chloroform extraction and ethanol precipitation were treated with RNase to remove coprecipitating RNA. *Eco*RI linkers were ligated to randomly sheared chromosomal DNA, and a library consisting of 6.3×10^6 primary recombinants was constructed in the *Eco*RI site of the lambda ZAP II expression vector (Stratagene, La Jolla, Calif.). The phage library was excised with R408 helper phage (32) to generate subclones in the pBluescript SK[−] phagemid vector (1).

Subcloning and sequence analysis. Unidirectional deletion clones were generated with exonuclease III (Stratagene) according to the manufacturer's instructions. Exonuclease III-resistant sites were created by *Not*I digestion of the recombinant plasmid followed by end filling with thioderivatives, and sensitive sites were created by digestion with *Bam*HI. Nucleotide sequences were determined with ³²P-labeled primers with the *fmol* DNA Sequencing System (Promega, Madison, Wis.). Custom sequencing primers used to fill the gaps between deletion clones were synthesized by the Molecular Biology Resource Facility of St. Francis Hospital located at the University of Oklahoma Health Sciences Center.

Analysis of plasmid-encoded polypeptides. Proteins encoded on H4 and the H4 deletion clones were identified by expression in maxicells of *E. coli* CSR603 by using the procedure described by Sancar et al. (35). Maxicell lysates were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (20) on a 12.5% gel, and proteins were visualized by autoradiography on Kodak X-Omat AR film.

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Hemolytic assays. Overnight cultures of H4 and pBluescript XL1-Blue transformants were grown in 5 ml of terrific broth (prepared by adding a 100-ml sterile solution of 0.17 M KH_2PO_4 and 0.72 M K_2HPO_4 to a 900-ml sterile solution containing 12 g of Bacto tryptone, 24 g of Bacto yeast extract, and 4 ml of glycerol) (40) containing 100 μg of ampicillin per ml. After centrifugation, the culture supernatants were kept on ice until tested. The cell pellets were washed in sterile $1\times$ phosphate-buffered saline (PBS [pH 7.4]), resuspended in 1 ml of PBS, sonicated on ice, and centrifuged to remove insoluble membranes. Sheep erythrocytes were washed in PBS and brought up to a 5% (vol/vol) suspension in PBS. For each assay, 500 μl of the test sample was incubated with an equal volume of 5% sheep erythrocyte suspension for 4 h at 37°C. After centrifugation, the release of free hemoglobin was measured by reading the optical density of the supernatant at 540 nm. The values for 0 and 100% erythrocyte lysis were determined by the addition of PBS and 0.1% Triton X-100, respectively, to an equal volume of 5% sheep erythrocytes. Sonicates were tested directly and after dilution in either PBS alone, PBS with 10 mM MgCl_2 , PBS with 10 mM EDTA, 10 mM Tris-HCl (pH 7.2) with 150 mM NaCl (TBS), or TBS with 10 mM CaCl_2 . For assays in the presence of added Ca^{2+} , the sheep erythrocytes were resuspended in TBS, and sonicate dilutions were made with TBS. Sonicates were assayed after heat treatment at 60°C for 30 min and after incubation with 200 μg of proteinase K for 1 h at 37°C. Protein concentrations in the cell sonicates were determined by a bicinchoninic acid assay (38).

Pulsed-field gel electrophoresis. Cryptosporidial oocysts were prepared for electrophoresis by the method described by Mead et al. (26). Briefly, 10^8 purified oocysts were washed and resuspended in 50 μl of suspension buffer (75 mM NaPO_4 , 65 mM NaCl, 1% glucose) at pH 8.0. Fifty microliters of 1% low-gelling-temperature agarose (SeaPlaque GTG; FMC BioProducts, Rockland, Maine) previously melted in suspension buffer and cooled to 37°C was added, and 100- μl aliquots of the mixture were placed into molds on ice to gel. After solidification, each agarose block was incubated in 10 ml of lysis buffer (0.5 M EDTA, 1.0% sodium *N*-lauroylsarcosine, 2.0 mg of proteinase K per ml) at pH 9.5 at 50°C for 48 h. The blocks were then washed extensively and stored in 50 mM EDTA–10 mM Tris (pH 8.0) at 4°C. Agarose blocks were cut in half and loaded into the slots of a 0.7% agarose (pulse field certified agarose; Bio-Rad, Hercules, Calif.) gel in 22 mM Tris–22 mM boric acid–0.62 mM EDTA (pH 8.0). The DNA was fractionated on a CHEF-DR III pulsed-field electrophoresis system (Bio-Rad) alongside *Saccharomyces cerevisiae* chromosomal DNA size standards (Bio-Rad). Gels were run at 14°C for 40 h with a voltage of 6 V/cm, an angle of 120°, and a switch time from 60 to 120 s. After depurination in 0.25 M HCl, the gels were washed and blotted by capillary transfer to Zeta-Probe GT nylon membranes (Bio-Rad). The membranes were hybridized with a ^{32}P -labeled 5.5-kb fragment according to instructions supplied by the manufacturer and exposed to Kodak X-Omat AR film.

RNA isolation. RNA was isolated from purified *C. parvum* sporozoites that were prepared as previously described (19). The sporozoites were frozen overnight at –70°C in guanidinium isothiocyanate solution and then ground exhaustively with a cold mortar and pestle. Total RNA was isolated by phenol-chloroform extraction and incubated with RNase-free DNase (Stratagene) at a concentration of 1 U/ μg for 1 h at 37°C to remove contaminating DNA.

Male and female 6-week-old C.B-17Icr scid/scid (SCID) mice were given intragastric inoculations of 2×10^6 purified cryptosporidial oocysts as previously described (17). Three infected mice were killed by CO_2 inhalation at 60 days postinoculation along with three uninfected age-matched control mice. The ascending colons were removed, opened longitudinally, and flushed to remove fecal debris. The tissues obtained from one infected SCID mouse and one uninfected SCID mouse were fixed in 10% buffered formalin for histopathologic analysis. Similar to previous results (17), severe histopathologic changes were present in the colonic tissue of the infected mouse, with cryptosporidial organisms identified in six or more sites per $\times 200$ field, and in groups of six or more organisms per site, with some sites containing sheets of organisms (18). No cryptosporidial organisms were observed in the normal-appearing ascending colon removed from the third uninfected mouse. The colonic segments from the remaining mice were stored immediately at –70°C for RNA isolation. The frozen intestinal tissues were ground exhaustively in guanidinium isothiocyanate solution, and total cellular RNA was isolated by phenol-chloroform extraction with incubation in RNase-free DNase as described above.

RT-PCR. For cDNA synthesis, 1 μg of sporozoite RNA or 3 μg of murine intestinal RNA was incubated for 1 h at 42°C in a 20- μl reaction mixture containing 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM MgCl_2 , 0.4 μg of random hexamers (Gibco, Gaithersburg, Md.), 10 mM (each) deoxynucleotide triphosphate, 20 U of placental ribonuclease inhibitor (Gibco), and 200 U of Moloney murine leukemia virus reverse transcriptase (RT [Gibco]). The samples were then heated at 94°C for 5 min, diluted to a final volume of 100 μl with 0.1% (wt/vol) diethylpyrocarbonate-treated water, and stored at –20°C. PCR was performed in a 50- μl volume reaction mixture containing 15 μl of the cDNA, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl_2 , 0.01% (wt/vol) gelatin, 50 pmol of each primer, 10 mM (each) deoxynucleotide triphosphate, and 5 U of *Taq* DNA polymerase. Twenty-five cycles consisting of 94°C for 1 min, 60°C for 2 min, and 72°C for 1 min were performed. Reaction products were visualized by electrophoresis of 35 μl of the amplified product in a 1.5% agarose gel containing 1 μg of ethidium bromide per ml. Amplification was performed in a Perkin-Elmer model 480 thermocycler (Perkin-Elmer Cetus, Norwalk, Conn.). Negative

FIG. 1. Physical map and deletion clones of the H4 cryptosporidial insert. Solid lines indicate *C. parvum* genomic regions. Dashed lines indicate pBluescript. T3 and T7 designate primer sites in the vector which flank the multiple cloning region. ORF indicates the open reading frame of *hemA*, and the arrow indicates the direction of transcription. The hemolytic activities of the deletion clones are indicated with a + or –. N, *NotI*; B, *BamHI*; E, *EcoRI*; Sp, *SphI*; K, *KpnI*; S, *SacI*.

control reactions which either omitted the reverse transcriptase (RT) or template were performed to ensure that the PCR products were not due to amplification of contaminating DNA. The *hemA* primers are depicted in Fig. 2. The cryptosporidial actin primers were designed on the basis of the reported sequence of a *C. parvum* actin gene (14) (GenBank accession number M69014). The forward primer covers positions 365 to 384, and the reverse primer covers positions 659 to 640 of the deposited nucleotide sequence. The mouse β -actin primers (8) flank an intron in the mouse β -actin gene (41) and amplify a 620-bp fragment from murine cDNA. The oligonucleotide primers were synthesized at the Molecular Biology Resource Facility, St. Francis Medical Research Institute, University of Oklahoma Health Science Center.

Nucleotide sequence accession number. The nucleotide sequence for *hemA* was deposited in GenBank under accession number U18120.

RESULTS

Identification of H4. A cryptosporidial genomic expression library in the pBluescript phagemid was transformed into *E. coli* XL1-Blue, and the transformants were plated on 5% sheep blood agar containing 100 μg of ampicillin per ml. After incubation at 37°C for 24 h, transformants with a hemolytic phenotype were identified by the development of a clear zone around the bacterial colonies. Transformants exhibiting hemolytic activity were restreaked for verification, and four stable hemolytic recombinant strains were identified. Dot blot hybridization studies and restriction mapping determined that two of these strains contained a recombinant plasmid with the identical 5.5-kb cryptosporidial genomic insert. These transformants exhibited the most striking hemolytic zone on blood agar plates compared with the other two hemolytic strains, and one, designated H4, was chosen for further analysis. To confirm that the hemolytic activity was encoded on the recombinant plasmid, fresh XL1-Blue cells transformed with the H4 recombinant plasmid, nonrecombinant pBluescript, and a recombinant plasmid from a nonhemolytic clone chosen randomly from the cryptosporidial genomic library were tested on blood agar. Transformants containing the H4 recombinant plasmid consistently caused hemolysis, while transformants containing pBluescript and the nonhemolytic recombinant plasmid remained nonhemolytic. Similar results occurred with transformation of two other nonpathogenic *E. coli* strains, HB101 and TOP10.

Analysis of the H4 cryptosporidial insert. A set of overlapping deletion clones of H4 were generated from the T3 promoter side of the 5.5-kb insert with exonuclease III as shown in Fig. 1. The subclones were transformed into XL1-Blue, and the sizes of the remaining cryptosporidial inserts were determined. Transformants containing various lengths of the insert were plated on sheep blood agar to determine their hemolytic phenotype. Hemolytic activity was still detectable after removal of 4.5 kb of the original insert. Nucleotide sequence analysis of the deletion clones demonstrated the presence of a complete 690-bp open reading frame (*hemA*) located within a 1-kb re-

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1  aaatactataaattaatcctatatatttttaatttataattgaaactaattaaataaaataaaggtagaatgagccaaaaatagatttgaatcaaggt
   E R T E S K C P E T L N E F S N M Y K H K K M P C K S A L N F G E N
101 gaaagaactgagtcacaaatgtccagaaacgccttaatgaattttctaatagtataagcataaaaaaatgccaatgaaatcagcgctgaactttggtgaaa
   -----
201 I I N M G A P S F I P E Y P T A Y Y S N P E V M N Q G I N S L V D
   atattataaatatgggagctccaagttttataaccagaatatccaacagcttattatttcaaccagaagttatgaatcaaggataaattcattagtgga
   T K Q L Y D Y S S P V G D L E K T I E H Y K M S H E I G W N A S N
301 tacaacaactatatgactattcatccctgtaggtgatcttgaaaaaaccattgaacattacaaaatgagtcagaaataggatggaatgcaagtaat
   S F T P T N S G S L E L F Q F E R R D S P V A V D T L K N Y P S F E
401 tctttcactcctacaaattctggttctttggaacttttccaatttgaagaagagatagtcctgtcgctgttgatactttaaaaaattaccatcttttg
   R M N S G F L G F G R K P S N S I F S I G Q K L E R L S N E I I
501 agagaatgaatagtggttttctggattcggagaaaaccttcaattcaatattttctattggacaaaaacttgaagactcttcttaataatgaat
   -----
601 N N I N T L P E I E N G I E N G I E N G I E N G D I P H V D L E Q
   caacaatatcaatacccttcctgaaattgaaaatggattgagaacggtattgaaaaatggaattgaaaatggagatatcctcacgttgatttggagcaa
   Y T Q L S Y F E K Y I N F N I D Q I K *
701 tatacacaattatcttactttgaaaaatatatcaatttttaacattgaccaaaatgaagtgaatccatttcttaaatataactactttttataacccaaaaat
801 gctatgaacaaatcatttttgcacaaatatattggaatattattagatttatgaatatcaagctgttcaaaagaatagcctgatactccacgtgaaaatt
901 tgtaatttgaagaagaatagtaactccagggaataatacaaa

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FIG. 2. Nucleotide sequence and deduced amino acid sequence of *hemA*. Nucleotides are numbered beginning 70 bases upstream of the putative initiation codon and ending 183 bases past the stop codon, which is marked with an asterisk. A possible acceptor splice site signal is underlined. A tetrapeptide repeat in the carboxy terminus of the predicted protein is overlined. The potential N-glycosylation site is indicated by ▼. The arrows indicate the forward and reverse primers used in RT-PCR analysis.

gion at the 3' end of the insert. XL1-Blue transformants containing subclones of the 5.5-kb insert, which included only a portion of *hemA*, did not demonstrate a hemolytic phenotype on sheep blood agar.

The nucleotide sequence of *hemA* is provided in Fig. 2. The first ATG (position 71) in the open reading frame appears to represent the putative initiation codon, since there are a purine at position -3 consistent with other eukaryotic translation initiation signals (16) and an in-frame stop codon that occurs immediately upstream. The high A+T content (69%) of *hemA* and the codon usage predicted from the primary amino acid sequence of the *hemA* gene product are similar to those described for other reported cryptosporidial genes (14). Computer analysis of the nucleotide sequence with the GRAIL program (13) and University of Wisconsin Genetics Computer Group sequence analysis package version 7 (10) predicted *hemA* to be a likely protein coding region. There were no identifiable polyadenylation signals in the remaining 183 bp of H4 insert downstream of the *hemA* stop codon. The 229-amino-acid protein predicted by the *hemA* nucleotide sequence is predominantly hydrophilic and has a calculated molecular mass of ca. 26 kDa. Asparagine residues make up 11% of the deduced primary amino acid sequence. One potential N-glycosylation site is present, and there are numerous serine and threonine residues that could serve as sites of O-linked glycosylation. No signal sequences or conserved peptide motifs were evident, but a tetrapeptide, IENG, is repeated four consecutive times in the carboxy terminus of the predicted protein. Analysis of this repeat region by HELICALWHEEL (10) indicates that it may form a membrane-spanning amphipathic helix. A BLAST (2) search of the current DNA and protein databanks did not reveal other gene products with a strong similarity to the protein predicted from the *hemA* nucleotide sequence. It is interesting, however, that the peptide sequences with the highest similarity scores (sum *P* value, 0.004) were from an *E. coli* O157 plasmid-encoded hemolysin (37) that is a member of the pore-forming RTX toxin family (42). There is 24.5% identity over a 53-amino-acid span between residues 71 and 124 of the predicted cryptosporidial protein and residues 566 and 619 of the *E. coli* toxin. When gaps are introduced to maximize alignment, there are 20% identity and 42% similarity between the complete *hemA*-encoded protein sequence and residues 500 to 750 of the bacterial toxin. Recent studies have shown that this

region of the bacterial hemolysin contains residues that are necessary for toxin activation in vivo (39). The significance of this similarity is questionable, however, since no other RTX toxins were identified in the database searches.

In vitro expression of the cryptosporidial protein. [³⁵S]methionine-labeled plasmid-encoded proteins of H4 and the H4 deletion clones were expressed in *E. coli* CSR603 maxicells to identify the cryptosporidial proteins encoded on the recombinant plasmids. A ca. 21-kDa protein was apparent on autoradiographs of maxicell lysates from strains transformed with H4 and H4 deletion clones containing the complete *hemA* coding region but not in those of lysates from transformants containing nonrecombinant pBluescript or an H4 deletion clone containing only a portion of *hemA* (Fig. 3).

Hemolytic activity of H4. To further characterize the hemolytic activity of the H4 transformants, hemolytic assays were performed with culture supernatants and cell sonicates of H4 and pBluescript transformants. The H4 culture supernatants

FIG. 3. Autoradiograph of [³⁵S]methionine-labeled plasmid-encoded proteins expressed in maxicells. Lanes: 1, nonrecombinant pBluescript; 2, H4; 3, H4.2; 4, H4.3; 5, H4.4. The 29-kDa (kD) protein is the product of the β -lactamase gene encoded on pBluescript.

TABLE 1. Localization of hemolytic activity in cell cultures of H4 transformants

Test sample	% Hemolysis ^a	
	H4	pBluescript
Culture supernatant	4.10 ± 2.1	3.48 ± 0.5
Cell sonicate		
Undiluted	85.33 ± 2.9	1.18 ± 0.6
Diluted ^b	42.58 ± 2.4	1.88 ± 0.6

^a Percentage of sheep erythrocytes lysed after 4 h at 37°C. Values represent the mean ± standard deviation of four experiments.

^b Diluted in PBS to ~280 µg of protein per ml.

did not exhibit significant hemolytic activity when added to sheep erythrocytes, indicating that the hemolytic activity encoded on H4 was not actively secreted into the culture media. However, sonicates of H4-transformed cells displayed strong hemolytic activity compared with sonicates of pBluescript-transformed cells and a 0.1% Triton X-100-positive control (Table 1). Incubation of erythrocytes with undiluted H4 cell sonicates caused more than 85% erythrocyte lysis after 4 h. Dilution of the H4 sonicates with PBS to a protein concentration of ~280 µg/µl prior to incubation with the erythrocytes resulted in approximately 40% hemolysis. The effects of various conditions on the hemolytic activity of the diluted sonicates were examined (Table 2). Proteinase K digestion and heat treatment of H4 sonicate dilutions prior to the assay markedly decreased their hemolytic activity. The addition of Ca²⁺ or Mg²⁺ had no effect on the assay results, while the addition of EDTA caused a very modest yet significant decrease in hemolytic activity. Assays performed at 4°C and room temperature resulted in little hemolysis. Extension of the 37°C incubation to 24 h increased hemolysis from 40% to 60%, while incubations up to 24 h at room temperature or 4°C did not result in appreciable hemolysis compared with that of a pBluescript control (data not shown). Storage of the sonicates at 4°C for 1 week did not diminish their hemolytic activity.

Karyotypic Southern blot analysis. To confirm that the

TABLE 2. Effects of various assay conditions on hemolytic activity of H4 cell sonicates

Treatment condition	% Hemolysis ^a	
	H4	pBluescript
Control	40.83 ± 2.3	1.43 ± 0.6
Mg ²⁺ ^b	38.13 ± 2.5	0.95 ± 0.6
EDTA ^b	36.88 ± 1.9 ^c	1.09 ± 0.7
Ca ²⁺ ^b	40.83 ± 1.1	1.17 ± 0.6
Heat ^d	0.81 ± 0.7 ^c	1.34 ± 0.8
Proteinase K ^f	1.24 ± 0.8 ^c	1.16 ± 0.9
Incubation		
4°C	2.00 ± 1.3 ^c	1.67 ± 0.6
25°C	4.30 ± 1.0 ^c	1.81 ± 0.5

^a Percentage of sheep erythrocytes lysed after 4 h with an incubation temperature of 37°C except where indicated. Values represent the mean ± standard deviation of four experiments. Sonicates were diluted with PBS to ~280 µg of protein per ml.

^b Diluted with PBS containing 10 mM Mg²⁺, 10 mM EDTA, or 10 mM Ca²⁺.

^c *P* < 0.05 compared with H4 control (Tukey honestly significant difference multiple comparisons test).

^d Heated at 60°C for 30 min prior to incubation with erythrocytes.

^e *P* < 0.001 compared with H4 control (Tukey honestly significant difference multiple comparisons test).

^f Pretreated with proteinase K at 37°C for 1 h prior to incubation with erythrocytes.

FIG. 4. Chromosomal mapping of the H4 cryptosporidial insert. (Left) Fractionation of chromosomal DNA from 5×10^7 *C. parvum* oocysts by pulsed-field gel electrophoresis on a 0.7% agarose gel. (Right) Southern blot analysis of the gel by using the ³²P-labeled 5.5-kb H4 insert as a hybridization probe. Lanes: 1, *S. cerevisiae* chromosomal molecular weight markers; 2, *C. parvum* chromosomal DNA.

5.5-kb insert of H4 was cryptosporidial in origin, chromosomal DNA isolated from *C. parvum* oocysts was fractionated by pulsed-field gel electrophoresis, capillary blotted to a nylon membrane, and hybridized with a ³²P-labeled 5.5-kb fragment. Similar to findings in previous reports (14, 26), five distinct chromosomal DNA bands were identified on ethidium bromide-stained agarose gels. Hybridization studies mapped the H4 cryptosporidial genomic region to the second smallest chromosome (Fig. 4).

Transcriptional analysis of *hemA*. In order to determine whether *hemA* is actively transcribed during the sporozoite life stage, RT-PCR analysis was performed with total RNA isolated from excysted sporozoites by using primers designed to amplify a 369-bp fragment internal to *hemA*. Positive control reactions were performed with primers designed to amplify a 295-bp region within a *C. parvum* actin gene (14). Agarose gel electrophoresis of the RT-PCR mixtures yielded the expected gene fragments for each primer set (Fig. 5). All amplification products were verified by Southern blot hybridization studies with radiolabeled PCR products that had been previously confirmed by nucleotide sequence analysis to match the expected regions of *hemA* or the cryptosporidial actin gene as probes (data not shown). Amplification with the *hemA* primers gave an additional band of 270 bp that did not hybridize to the *hemA* probe. Negative control reactions which omitted either the RT or template did not yield a PCR product.

Total RNAs isolated from the intestinal tissues of adult SCID mice were examined by RT-PCR to determine whether *hemA* is actively transcribed during cryptosporidial infection of mammalian hosts. The 369-bp *hemA* amplification product was present in agarose gels of reactions performed with RNA isolated from the ascending colons of infected mice but not with RNA isolated from the ascending colons of uninfected mice (Fig. 6). Mouse β-actin primers were included in the RT-PCR mixtures as a positive control to verify that intact RNA had been isolated from the uninfected mice. Similar results were obtained when the cDNAs generated from the infected and control mice were tested with primers to the *C. parvum* actin

FIG. 5. RT-PCR amplification of a 369-bp *hemA* fragment and a 295-bp cryptosporidial actin gene fragment from sporozoite RNA. Random hexamers were used to prime cDNA synthesis. The RT was omitted in the negative control reactions. Lanes: 1, ϕ X174, *Hae*III-digested molecular weight markers; 2 and 4, negative controls for the lane 3 and 5 reactions, respectively; 3, RT-PCR with the *hemA* primer pair; 5, RT-PCR with the actin primer pair.

gene. Negative control reactions and Southern blot verification of the amplified products were performed as described above.

DISCUSSION

Hemolytic activity has been used as a marker to detect important virulence factors from a variety of intracellular bacterial pathogens. Some of these hemolysins are necessary for host cell invasion, while others function to disrupt phagocytic vacuolar membranes and to allow for intracellular replication of the organisms (12, 15, 28, 36, 42). Similarly functioning proteins are also produced during infection by protozoan pathogens. A trypsin-sensitive secreted hemolytic factor has been identified in supernatants of cultures containing *Trypanosoma cruzi* (3). Preliminary studies indicate that this protein disrupts intracellular phagolysosomes containing the parasite by forming large membrane pores in these structures, thus allowing escape of the parasite into the cytosol of the host cell (4). There is also evidence that *Toxoplasma gondii* secretes a phospholipase for use in host cell penetration (33, 34). Potential roles for membrane-altering proteins in cryptosporidial infection include dissolution of the invaginated host cell membrane during maturation of the parasitophorous vacuole, disruption of parasite and host cell membranes as different stages of the parasite are released into the intestinal lumen, and formation of protein pores in the feeder organelle to allow nutrients to cross from the host cell cytoplasm into the parasitic vacuole.

This is the first report of a cryptosporidial protein that has membrane-disrupting capabilities. The loss of hemolytic activity in subclones containing only a portion of *hemA* and after heat and protease treatment of the H4 sonicates strongly suggests that the membranolytic activity is caused by the *hemA* gene product. Furthermore, the RT-PCR data demonstrate that *hemA* is actively transcribed in the infectious sporozoite life stage and very likely in other stages present during the protozoan life cycle. The possibility that a portion of *hemA* represents one exon of a larger gene cannot be completely excluded, since a consensus eukaryotic acceptor splice signal, (Y)_nXYAGRT (7), is located within the coding region between positions 326 and 337 (Fig. 2). However, sequence analysis of the 2-kb region upstream of *hemA* does not reveal any

FIG. 6. RT-PCR amplification of a 369-bp *hemA* fragment (A) and a 295-bp cryptosporidial actin gene (B) fragment from total RNA isolated from the ascending colons of infected SCID mice. cDNA synthesis and negative control reactions were performed as described in the legend to Fig. 5. Primers which flank an intron in the mouse β -actin gene and amplify a 620-bp fragment from mouse cDNA were present in every reaction. Lanes: 1, ϕ X174, *Hae*III-digested molecular weight markers; 2, negative control for lane 3 reaction; 3, RNA from uninfected mice; 4, negative control for lane 5 reaction; 5, RNA from mice infected with *C. parvum* for 60 days.

other plausible protein coding regions or potential donor splice sites, and the limited data derived from currently described cryptosporidial genes suggest that as with other protozoa, *C. parvum* is not likely to contain many introns.

The size of the recombinant protein expressed in maxicells is slightly smaller than the calculated molecular weight on the basis of the predicted amino acid composition of the *hemA*-encoded protein. This discrepancy may be due to the preferential initiation of translation in *E. coli* at one of several other possible in-frame ATG codons that are present downstream of the putative translational start site at position 71 or to aberrant migration of the protein in SDS-PAGE gels as has been described with other protozoal proteins (6). The significance of the similarity between the *hemA*-encoded protein and the *E. coli* O157 RTX toxin and the mechanism by which the cryptosporidial protein causes hemolysis are unknown. It is likely, however, that the C-terminal tetrapeptide repeat plays a significant role in the hemolytic process. Repeat regions are commonly detected within protozoal proteins, but the tetrapeptide in the *hemA*-encoded protein has no similarity to those previously reported, including the amino acid repeat present in a cryptosporidial oocyst wall protein (29) and the conserved tetrapeptide found in proteins from the erythrocytic life stages of *Plasmodium falciparum* (31). Efforts are currently under way to purify the recombinant protein encoded by *hemA* in order to generate antibodies for use in studies to localize the protein

within the various cryptosporidial life stages and to further define its function.

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