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Characterization and Immunolocalization of a *Cryptosporidium* Protein Containing Repeated Amino Acid Motifs

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The oocyst wall is one of the components that permits cryptosporidia both to survive in the environment and to retain infectivity. With the aim of identifying *Cryptosporidium* proteins specifically expressed at the oocyst stage, we screened λgt11 genomic libraries of *Cryptosporidium parvum* with both an oocyst antiserum and a specific genetic probe. We isolated, from distinct libraries, two overlapping clones containing an open reading frame encoding a 1,252-amino-acid polypeptide. The analysis of the deduced amino acid sequence revealed unusually high contents of cysteine, proline, and histidine. The sequence was also characterized by two distinct amino acid motifs, each repeated several times. The DNA sequences coding for the amino acid repeats showed a high frequency of synonymous mutations, a result suggesting that the repeated motifs may be functionally and/or structurally important to the parasite. Antisera and monoclonal antibodies developed against a recombinant polypeptide encompassing the first 786 amino acids revealed that the corresponding protein in *C. parvum* had an apparent molecular weight of 190,000. Moreover, confocal microscopy analysis with immunofluorescence indicated that the protein was localized on the oocyst wall as a uniform stain and within the oocyst itself as bright granules in close association with the residual body.

Cryptosporidia are protozoan parasites that multiply in the intestinal cells of the brush border of several animal species. During the last few years, the number of human infections began to rise dramatically (2, 3). Most of the patients were neonates, young children (13, 19, 23), or immunodeficient individuals, many of whom had developed AIDS (7). Immunocompromised patients often develop a severe, irreversible diarrhea that causes malnutrition and represents a significant contributing factor leading to death. Moreover, no effective therapeutic compounds against *Cryptosporidium* infection are available.

Despite great medical interest, little genetic information is available on cryptosporidia. The lack of an efficient in vitro culturing system for this parasite (5) still represents a serious obstacle to understanding the biology of the parasite and to studying the immunological and biochemical properties of *Cryptosporidium* antigens. In fact, until recently, few parasite proteins have been extensively characterized (1, 14–16) and very few genes have been cloned (4, 6, 9). There is an urgent need to collect more information on the biology of *Cryptosporidium* proteins. New control measures can be developed only by disclosure of the molecular mechanisms accounting for *Cryptosporidium*-host cell interactions and for parasite multiplication within intestinal cells. The identification and characterization of new parasite proteins may provide additional tools for studying the immune response against *Cryptosporidium* antigens.

We used an antiserum raised against parasite oocysts to identify in a λgt11 genomic expression library genes encoding proteins of the oocyst wall and antigens of sporozoites. We thought that such genes would be important both in understanding how the parasites survive and retain infectivity for several months in the external environment and in searching for the antigens that may be the targets of the immune response. To generate λgt11 expression libraries of cryptosporidia, we used either DNA extracted from purified oocysts or DNA derived from the gut cells of the brush border of a *Cryptosporidium parvum*-infected calf. For this purpose, we developed a method for collecting the infected cells of the superficial layer of the mucosa, thus increasing the parasite DNA/intestinal cell DNA ratio. On the basis of the reactivity with a *Cryptosporidium* oocyst antiserum, we selected from the library insert cpRL3, which was generated with the DNA extracted from infected gut cells. This insert encoded a partial sequence of 786 amino acids. The analysis of the deduced amino acid sequence suggested that the *Cryptosporidium* protein may play an important functional and/or structural role in the parasite. Using cpRL3 as a hybridization probe, we isolated from a library generated with shared DNA of *Cryptosporidium* oocysts an insert of 4,420 bp. This insert encompassed the entire sequence of cpRL3 and contained an open reading frame of 1,252 amino acids. The deduced amino acid sequence was characterized by a high content of cysteine and by the presence of two distinct repeated amino acid motifs. We developed sera and monoclonal antibodies against a recombinant polypeptide encompassing the first 786 amino acids to study the expression and localization of the protein in *C. parvum*.

MATERIALS AND METHODS

Development of a λgt11 library with DNA extracted from infected gut mucosa. To develop a *C. parvum* genomic

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expression library, we used DNA extracted from the intestinal mucosa of an infected calf. We developed a method for collecting the *Cryptosporidium*-infected cells of the superficial layer of the mucosa. A neonatal calf was infected with 6×10^8 oocysts of *C. parvum* MI ISS-1 (18). After 5 days, the gut of the calf was opened, cut into segments of 30 cm each, and washed in phosphate-buffered saline (PBS). Nitrocellulose filters (soaked in PBS) of the same size as the gut segments were applied to the mucosal side for a few seconds. The filters were progressively numbered and processed for DNA extraction. A small sample was removed from each filter and analyzed by microscopy to determine whether parasites had been removed. We used only the DNA extracted from the filters that were shown to have removed a large number of parasites. The DNA was digested with *Eco*RI and cloned in λgt11 by means of the *Eco*RI site, so that it was placed in the coding sequence of the β-galactosidase gene. Phage DNA with cloned inserts was packaged in vitro (Boehringer Mannheim in vitro packaging kit) to generate the library. The quality of the library was evaluated by analyzing the sizes of a number of inserts by the polymerase chain reaction (PCR) with oligonucleotides that corresponded to the flanking sequences of the *Eco*RI site in the β-galactosidase gene. The library had a complexity of 4.5×10^6 plaques and an estimated average insert size of 1,800 bp. The expression library was analyzed by use of a rabbit serum developed against purified oocysts of *C. parvum* MI ISS-1. The serum was used after removal of the background reactivity by several absorptions on filters soaked with bacterial and phage lysates. Specific antibodies bound to the filters were detected by use of a second antirabbit antibody conjugated to alkaline phosphatase.

Development of a λgt11 library with DNA extracted from purified oocysts. Total nucleic acids were isolated from 10^9 excysted oocysts after incubation at 50°C for 3 h in 10 ml of 50 mM Tris-HCl (pH 7.6)–100 mM NaCl–1% sodium dodecyl sulfate (SDS)–100 mM EDTA–0.5 mg of protease K per ml. After phenol extraction and ethanol precipitation, shared DNA was treated with T4 PolI and *Eco*RI methylase. *Eco*RI linkers were added, and the DNA was digested with *Eco*RI. The DNA preparation was separated on a 1% low-melting-temperature agarose gel. After removal of the region of the gel containing DNA molecules shorter than 300 bp, high-molecular-weight DNA was bound to DEAE filters during reverse electrophoresis. Subsequently, the DNA was eluted with 1.5 M NaCl and 10 mM Tris–1 mM EDTA (pH 8) (TE) buffer at 65°C. After ethanol precipitation, the DNA was resuspended and pooled in 50 ml of 1/4× TE buffer. Purified DNA was ligated with dephosphorylated *Eco*RI λgt11 arms in accordance with manufacturer instructions and incubated with in vitro packaging extracts. Recombinant phage particles were amplified by preparing plate lysates with *Escherichia coli* Y1090, yielding 100,000 plaques. The library was screened with a ^{35}S-labelled randomly primed cpRL3 insert. All enzymes and λgt11 arms were purchased from Boehringer Mannheim.

Microscopic examination of nitrocellulose filters. After the nitrocellulose filters had been removed from the gut, a small sample from each filter was incubated with 1% glutaraldehyde in cacodylate buffer for 2 h. The filters were dehydrated through an increasing ethanol series, embedded in Epon, and cured at 60°C for 24 h. Sections were cut at a 0.2-μm thickness and stained with toluidine blue.

Expression of the cpRL3 sequence in *E. coli*. The DNA insert cpRL3 was cloned in the *Eco*RI site of plasmid pDS56/RBSII-E$^-$ 6×His (a pDS56/RBSII-derived plasmid containing an *Eco*RI site in the polylinker). The expression unit of this vector is under the control of an isopropyl-β-thiogalactopyranoside (IPTG)-inducible promoter and yields a fusion between a stretch of 6 histidines and the amino terminus of the inserted sequences (24). cpRL3 was expressed in *E. coli* M15 carrying the *lac* repressor-producing plasmid pUHA1. Induction was performed in LB medium for 4 h at 37°C; 1 mM IPTG was added when the cell density had reached an optical density at 600 nm of 0.6.

Purification of recombinant polypeptide 6×His-cpRL3. The expression product of the cpRL3 sequence (recombinant polypeptide 6×His-cpRL3) fused to an amino-terminal stretch of 6 histidines was purified, in a single-step procedure, by nickel chelate affinity chromatography (24). In brief, 1 liter of an induced culture of M15(pUHA1) cells carrying plasmid pDS56/RBSII-E$^-$ 6×His-cpRL3 was harvested and stirred for 3 h in 100 ml of 6 M guanidine hydrochloride–100 mM Na_2HPO_4 (pH 8). The suspension was centrifuged at 10,000 × *g*, and the supernatant was directly applied to a nickel column (NTA-resin; Diagen). After an equilibration step with 8 M urea–100 mM NaH_2PO_4–10 mM Tris (pH 8), 6×His-cpRL3 was eluted by lowering the pH of the urea solution stepwise to pH 4. From 1 liter of culture, 1 mg of 6×His-cpRL3 was obtained.

Immunoblotting. Parasite lysates were obtained from *Cryptosporidium* oocysts purified by Percoll gradient centrifugation (25). Parasites were lysed by incubation of a pellet of 2×10^7 oocysts with 0.2 ml of sample buffer (33 mM Tris-HCl [pH 6.8], 190 mM glycerol, 0.1% SDS). Bacterial lysates were obtained by treatment of 10^9 induced or noninduced *E. coli* cells with 1 ml of sample buffer. Proteins in total cell lysates were separated by SDS-polyacrylamide gel electrophoresis (PAGE) (11) and electroblotted onto nitrocellulose filters (blotting buffer, 25 mM Tris, 192 mM glycine, 20% methanol). Nonspecific adsorption of antibodies to the nitrocellulose was prevented by saturation of the filters with 1% bovine serum albumin in 2× TBST (20 mM Tris-HCl [pH 8], 300 mM NaCl, 0.1% Tween 20) for 2 h at room temperature. Nitrocellulose filters were incubated with antibodies for 2 h at room temperature. After extensive washing with 2× TBST, antibodies bound to the filters were detected by use of goat anti-mouse immunoglobulin (heavy and light chains) conjugated to alkaline phosphatase (Promega). Phosphatase activity was disclosed by incubation of the filters with 0.3 mg of Nitro Blue Tetrazolium and 0.15 mg of 5-bromo-4-chloro-3-indolyl phosphate per ml in 100 mM Tris-HCl (pH 9.5)–100 mM NaCl–5 mM $MgCl_2$.

Monoclonal antibody production. After purification by nickel chelate chromatography, recombinant polypeptide 6×His-cpRL3 was used as an immunogen to develop specific antisera and monoclonal antibodies. BALB/c mice were immunized three times with 50 μg of purified 6×His-cpRL3 polypeptide in complete (for the first immunization) or incomplete Freund's adjuvant. Five days after the last immunization, mouse spleen cells were fused with ×63 Ag 8653 myeloma cells and subsequently screened for antibody production (10). The supernatants of cultures from growing hybrids were tested in an enzyme-linked immunosorbent assay (ELISA) against 6×His-cpRL3.

Immunofluorescence microscopy. Purified parasite oocysts were air dried on a coverslip and fixed in cold acetone for 5 min. Nonspecific binding was prevented by preincubation of the samples in PBS containing 1% bovine albumin. The primary antibodies (culture supernatant) were allowed to react for 40 min at room temperature, and the secondary fluorescinated antibody (Becton Dickinson goat anti-mouse)

was allowed to react for 20 min. Observation of the samples was carried out with the confocal microscope apparatus developed by Bio-Rad Laboratories.

PCR. For all PCR experiments, the cpRL3 sequence was amplified in a standard 50-μl PCR reaction mixture (21, 22) for 35 cycles at 94.5°C (1 min), 58°C (30 s), and 72°C (1 min) with a Lab Line thermal cycler. The final concentration of $MgCl_2$ was 2 mM. The primers used were Cry-3 (5′-GTCCTACTGGATTCACTCTAC-3′; coding strand, nucleotides 722 to 742), Cry-5 (5′-CCAGGACATCATCATGGT CATTCTCATGGGC-3′; coding strand, 1099 to 1129), and Cry-6 (5′-CCGAATATGTAACACATTTATCCGC-3′; complementary strand, nucleotides 1828 to 1852). For amplification of the cpRL3 sequence from *Cryptosporidium* oocysts, samples were incubated for 5 min under reducing conditions and boiled for 10 min thereafter. *Taq*I polymerase was purchased from the Perkin-Elmer Corp.

Nucleotide sequence accession number. The EMBL accession number for the sequence of COWP-190 is Z 22537.

RESULTS

Identification of the cpRL3 and cpMM1 inserts. The infected gut contains parasites at all developmental stages; however, its use for purifying *Cryptosporidium* DNA and proteins has been hampered by the lack of a method for separating parasitized cells, situated exclusively on the brush border layer of the mucosa, from the majority of nonparasitized cells of the gut wall. We developed a method that allowed us to use DNA extracted from the gut mucosa of an infected calf to generate a genomic expression library. The DNA was extracted from nitrocellulose filters applied to the mucosal side of gut segments obtained from an infected calf. Microscopic analysis performed on thin sections revealed that the nitrocellulose filters had removed only the superficial layer of the mucosal cells, together with a large number of parasites (Fig. 1). The genes of protozoa, including cryptosporidia, contain very few introns, if any (4, 6, 9). Therefore, despite the presence of calf DNA, the *Cryptosporidium* coding sequences should be preferentially expressed. Using a rabbit antiserum raised against purified oocysts, we isolated insert cpRL3, which consisted of a 2,359-bp open reading frame encoding a polypeptide of 786 amino acids. The lack of both a start codon and a stop codon indicated that the sequence represents only part of the coding sequence of a parasite gene. To prove the *Cryptosporidium* origin of the cloned insert as well as to search for contiguous DNA sequences, we used labelled cpRL3 as a hybridization probe for screening a λgt11 library generated with shared DNA extracted from purified *C. parvum* oocysts. From this library, we isolated clone cpMM1, which consisted of 4,420 bp encompassing the entire cpRL3 sequence and providing an additional 2,061 bp at its 3′ end.

Sequence analysis of cpMM1. Insert cpMM1 contained an open reading frame, not interrupted by introns, of 3,756 bp and encoding a polypeptide of 1,252 amino acids (Fig. 2). The DNA of the insert at the 3′ end of the coding sequence is characterized by several stop codons in all three frames and by an increase in the A+T content to nearly 85%. The lack of a start codon at the 5′ end indicated that part of the coding sequence was still missing. The deduced amino acid sequence revealed unusually high contents of cysteine, proline, and histidine. Analysis of the amino acid sequence of cpMM1 revealed the presence of two different repeated motifs, both characterized by the presence of cysteine residues at conserved positions. The type I amino acid motif

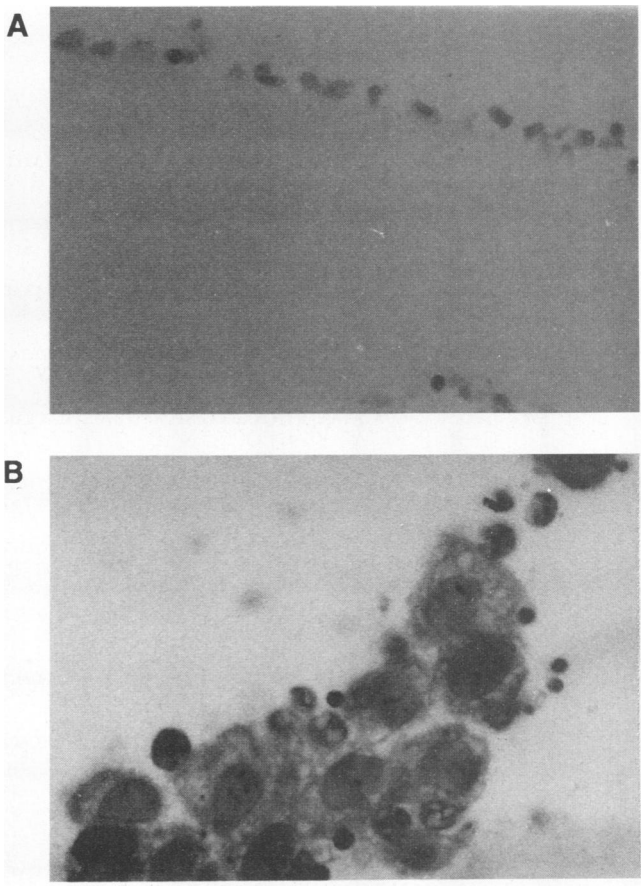

FIG. 1. Microphotographs of thin sections (0.2 μm) showing nitrocellulose filters that removed from the gut mucosa the cells of the brush border together with *C. parvum*. Magnifications: A, ×92; B, ×920.

was based around the consensus sequence CPPGF(Y)XL XGXQC and was repeated 18 times. The type II motif consisted of 49 amino acids with 6 cysteine residues, each connected to the next one by 11, 10, 7, 4, and 12 amino acids. This motif occurred eight times within the sequence encoded by cpMM1. On the basis of the structural organization of the repeated motifs, the amino acid sequence encoded by cpMM1 could be divided into two major domains. The amino-terminal domain encompassed the first 338 amino acids and was characterized by the presence of a cysteine residue every 10 to 12 amino acids. This region included 15 copies of the type I motif arranged in a regular array. The second domain, of 914 amino acids, mainly consisted of histidine-rich regions lacking cysteine residues, alternating with type II repeats. In both repeated motifs, constant amino acid residues were found in several conserved positions (Fig. 3 and 4). However, the corresponding codons showed a high frequency of nucleotide substitutions (both transversion and transition) in the third position (Fig. 3). The high frequency of synonymous mutations within the coding sequences of the repeats of cpRL3 indicated the presence of a pressure acting for the conservation of the amino acid sequences of the repeats. Therefore, these repeats may have an important functional and/or structural role for the parasite. When aligned with the sequences present in the GenBank and EMBL data bases, the DNA sequence of cpRL3 correspond-

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1    GAATTCGAATGCCCACCAGGTACAATTTTAAAAGATGATCAATGTCAATCGATAGAAAGAGTTGATACAATTTGTCCACCAGGGTTTGTAGATAATGGC
     E  F  E  C  P  P  G  T  I  L  K  D  D  Q  C  Q  S  I  E  R  V  D  T  I  C  P  P  G  F  V  D  N  G

99   GAAGATTGTGTCCAATTTTCTGCACCAGAGAAAATTTGCCCCCAAGGATTTTCTCTTTCCGGAAAACAATGTGTTAAAACAGAATCTGCTCCAAGATTA
     E  D  C  V  Q  F  S  A  P  E  K  I  C  P  Q  G  F  S  L  S  G  K  Q  C  V  K  T  E  S  A  P  R  L

198  ACAGAATGCCCACCAGGTACAACCTTGGAAAATAACAGTTGTATTTCATATGAACTAGAAGATGCCATTTGTCCACCTGGATATCTCGACAATGGATCA
     T  E  C  P  P  G  T  T  L  E  N  N  S  C  I  S  Y  E  L  E  D  A  I  C  P  P  G  Y  L  D  N  G  S

287  GACTGTGTTCAGTTTTCTCAACCAGAAAAGGAGTGTCCAACAGGTTTTGTATTAATTGGAAAACAATGTACCCAAACTACTCAAGCTCCACCACAACCA
     D  C  V  Q  F  S  Q  P  E  K  E  C  P  T  G  F  V  L  I  G  K  Q  C  T  Q  T  T  Q  A  P  P  Q  P

396  GAGTGTCCTCCAGGTACAAACCTGGTAAATGGACAATGCCAAAAAGTTGAAAGGATAAATATGGTATGTCCAACTGGTTTTATTGATAATGGTACAAAT
     E  C  P  P  G  T  N  L  V  N  G  Q  C  Q  K  V  E  R  I  N  M  V  C  P  T  G  F  I  D  N  G  T  N

495  TGTGCTTCTTTCTCCGCACCAAACAGAGAATGCCCACCTGGATATACACTTCTGGATCCCAATGCGAGCAAATAAAAGAAGCACCTCCTGTTTCAGAA
     C  A  S  F  S  A  P  N  R  E  C  P  P  G  Y  T  L  S  G  S  Q  C  E  Q  I  K  E  A  P  P  V  S  E

594  TGTCCACCAGGATATAAACTTCAAGGAAATCAATGTACTGCACTAAAAATGATCGATGCTATCTGCCCAGATGGATTTTTACCAAATGGAGACGATTGT
     C  P  P  G  Y  K  L  Q  G  N  Q  C  T  A  L  K  M  I  D  A  I  C  P  D  G  F  L  P  N  G  D  D  C

693  ATCCAATTTTCTCCTGCTTCAACTGTATGTCCTACTGGATTCACTCTACAAAATCAACAGTGTGTTCAAACAACTACCTCACCAAAAACACCAGAATGT
     I  Q  F  S  P  A  S  T  V  C  P  T  G  F  T  L  Q  N  Q  Q  C  V  Q  T  T  T  S  P  K  T  P  E  C

792  CCTCCAGGTTCTGCGTTGGATGGAGACTCGTGCACAAGACTTGTTCCCGGGGCTCTTCAATACGTTTGTCCTGTTGGTACTAGAGAGGGGGACGTTTGC
     P  P  G  S  A  L  D  G  D  S  C  T  R  L  V  P  G  A  L  Q  Y  V  C  P  V  G  T  R  E  G  D  V  C

891  GTAGAGAGATCGATTAGTTCGCCTGTTTTGGAATGCCCACCTGGTTATTCATTGGAAACAGGTAAACAATGTGTTAGAAGAAGCCAATATGACTGTTCA
     V  E  R  S  I  S  S  P  V  L  E  C  P  P  G  Y  S  L  E  T  G  K  Q  C  V  R  R  S  Q  Y  D  C  S

990  GTAACAACTTATGTTACAGAGTGTAAAACACCTGATGTTAAAGCACTAAGAAGATTAGCAGCTGCAAAAGAAACATCAACAGTTTATGAAACATCTGAG
     V  T  T  Y  V  T  E  C  K  T  P  D  V  K  A  L  R  R  L  A  A  A  K  E  T  S  T  V  Y  E  T  S  E

1089 ATACAAAATCCAGGACATCATCATGGTCATTCTCATGGGCATTCACATTCACAAGTTATACCAATTCAAACCCAGAATATACATACACAACATCATAAA
     I  Q  N  P  G  H  H  H  G  H  S  H  G  H  S  H  S  Q  V  I  P  I  Q  T  Q  N  I  H  T  Q  H  H  K

1188 GAGGCTCCAAGGCCAATTTGTGAAGATGTTCCAAAAATTACCCCAAAAACTTGTACAAAAGCTGATTCTGTCCCAGCTGTGCCTATTTGCGAGAACAAT
     E  A  P  R  P  I  C  E  D  V  P  K  I  T  P  K  T  C  T  K  A  D  S  V  P  A  V  P  I  C  E  N  N

1287 GCTGAACTTGTAGGAAAAGAATGTGTATTAACAAATTACTACCCATTAGAAGCAATTTGTCAAGATGGAACAAGATCAAAAGAGTGTGCTAAGTTTGTA
     A  E  L  V  G  K  E  C  V  L  T  N  Y  Y  P  L  E  A  I  C  Q  D  G  T  R  S  K  E  C  A  K  F  V

1386 AAAACTCCACCTACTTTAAAATGTCCGCCAGGTTCTGTAGATGTAGGATCTCAATGTCAAGTTAACAAATATTCACCATATGATCTTGCATGCCCTGCA
     K  T  P  P  T  L  K  C  P  P  G  S  V  D  V  G  S  Q  C  Q  V  N  K  Y  S  P  Y  D  L  A  C  P  A

1485 GGATATGCATTGGTTGGAGACAAATGCGCTACCACAAGAGAAAAAGTTTGCCCGAATGAAAGTTGCCAAAGAGTTGTAACTGCGCCTGTTTCTTTAACT
     G  Y  A  L  V  G  D  K  C  A  T  T  R  E  K  V  C  P  N  E  S  C  Q  R  V  V  T  A  P  V  S  L  T

1584 TGTCCCCCTGGATATCACCAAATAGATGAAGTTATGAATATTTCTGCTCATCCACACCACAGACACTTAGCTGGGGTTCAATCTACTTCTCAAAAGGGA
     C  P  P  G  Y  H  Q  I  D  E  V  M  N  I  S  A  H  P  H  H  R  H  L  A  G  V  Q  S  T  S  Q  K  G

1683 TATTCTCATGGACATAAATATACTCCTGTAATTTCTCAGCCACCACAACCAGTTCCAGTTGTTGCTCCTATTCAGCAAATGAAATGCATCCATGCAAAC
     Y  S  H  G  H  K  Y  T  P  V  I  S  Q  P  P  Q  P  V  P  V  V  A  P  I  Q  Q  M  K  C  I  H  A  N

1782 CATGCTCCATATAATCTTATCTGTCCTGTTGGATCAAGACTTGTAGCGGATAAATGTGTTACATATTCGGATAAAATATGTCCAAATGGTAATTGCGAG
     H  A  P  Y  N  L  I  C  P  V  G  S  R  L  V  A  D  K  C  V  T  Y  S  D  K  I  C  P  N  G  N  C  E

1881 CGTATATATAATGAGCCTGCTGAATTAGTATGCCCTCCAGGATTCTCATCATCTAAACCAATTCAGCCAATAAGCCATTCTCATATTAACCATCCAAAT
     R  I  Y  N  E  P  A  E  L  V  C  P  P  G  F  S  S  S  K  P  I  Q  P  I  S  H  S  H  I  N  H  P  N

1980 GTTTCTGTTCCCGTCCAACCACAAACTATTAACCAACCACAAGTAATTCAACAAAGACAAGTAAATTATCAGCCACAAGTAATTCATCAAACACAGGAA
     V  S  V  P  V  Q  P  Q  T  I  N  Q  P  Q  V  I  Q  Q  R  Q  V  N  Y  Q  P  Q  V  I  H  Q  T  Q  E

2079 ATTTTAACAACTTATCCAACTCCAGTTTACCAAACCGGCACAATTTATCAAGGACATCATCATCATCATCATCATCATCACAGAAATCTAGCTTCCCCT
     I  L  T  T  Y  P  T  P  V  Y  Q  T  G  T  I  Y  Q  G  H  H  H  H  H  H  H  H  H  R  N  L  A  S  P

2178 GAGTGCATTAAGACAATTTCAGTACCTTATATTTTAAAATGCGAATCTCCATTTATTTTAGATGGCGACAAATGTATCGAAAAAACAGAAAAAATTTGT
     E  C  I  K  T  I  S  V  P  Y  I  L  K  C  E  S  P  F  I  L  D  G  D  K  C  I  E  K  T  E  K  I  C

2277 CTACAAGGTGACTGCAGAAAACAAGTCGTCGTTCCACCAACTCTTTCATGTCCACAAGGTTACAGAAATGCCAACGGAATTCAAACAGCAATTTCAAGC
     L  Q  G  D  C  R  K  Q  V  V  V  P  P  T  L  S  C  P  Q  G  Y  R  N  A  N  G  I  Q  T  A  I  S  S

2376 AAGCATACCGCCGGAACACATCATTATTCAACACCTTCAGCAGAATGTGTTAGTTCTATTTTTGAAGAGTATAGTTTAGTATGTTCATCAGGGTTCGTG
     K  H  T  A  G  T  H  H  Y  S  T  P  S  A  E  C  V  S  S  I  F  E  E  Y  S  L  V  C  S  S  G  F  V

2475 TTATTAGGGGATAGATGTGCATTGTTTACAAATAAAATCTGTCCAAATGGTAACTGCGAGAGATTGATAAGTAAACCTGCCAATATGGTATGCCCACCT
     L  L  G  D  R  C  A  L  F  T  N  K  I  C  P  N  G  N  C  E  R  L  I  S  K  P  A  N  M  V  C  P  P
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FIG. 2. DNA sequence and deduced amino acid sequence of the cpMM1 insert. The sequence of the cpRL3 insert spans from bp 1 to 2359; cysteine residues are underlined.

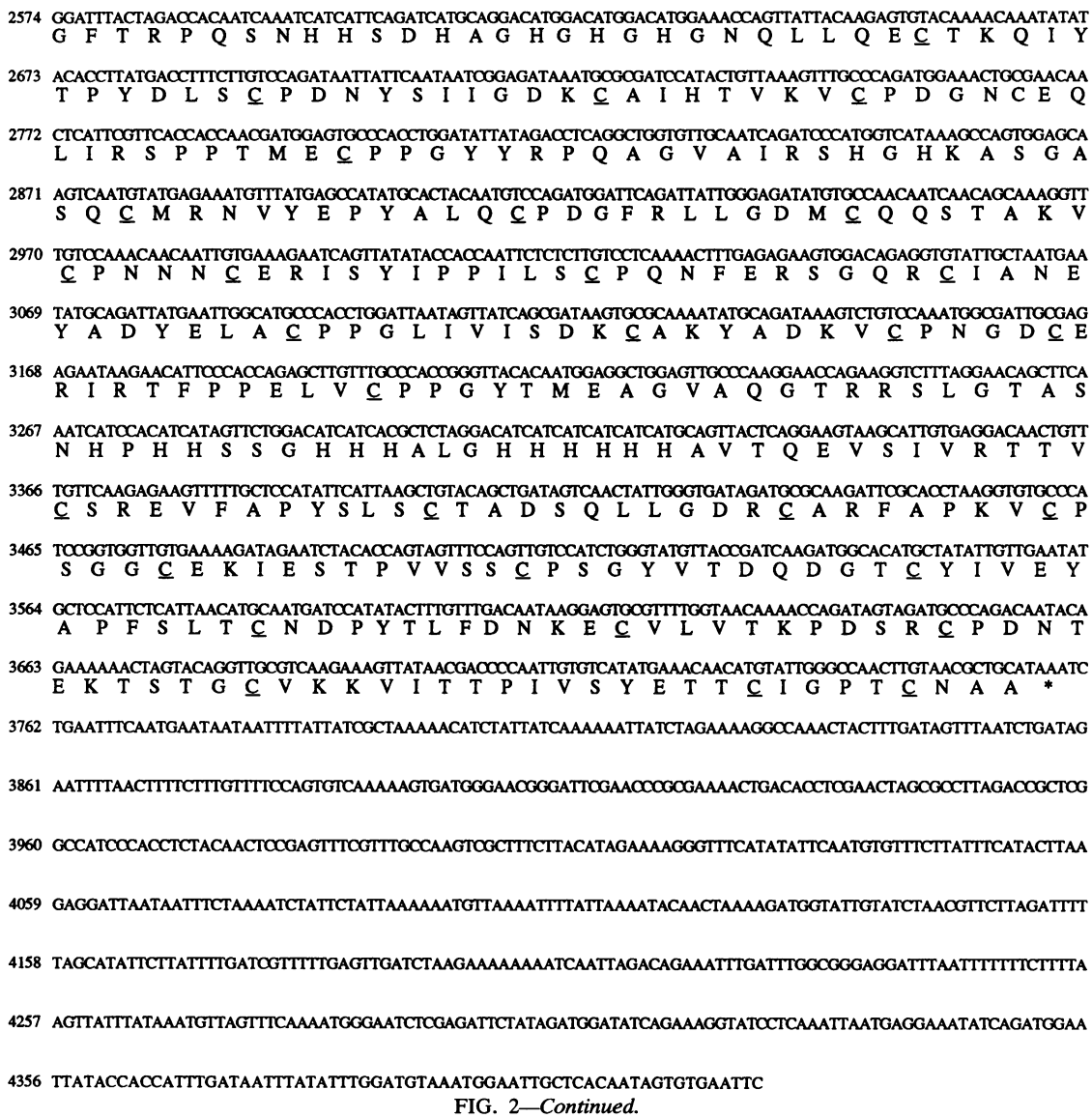

FIG. 2—*Continued.*

ing to the first 2,359 bp of cpMM1 was found to be identical to a *Cryptosporidium* DNA partial sequence (12) supposed to code for a component of *Cryptosporidium* oocysts. Neither the DNA nor the amino acid sequence showed significant homology to any other sequence present in the EMBL data base. The lack of conclusive information concerning the function and the localization of the parasite protein encoded by cpMM1 prompted us to study its expression in *C. parvum*. For this purpose, we expressed the cpRL3 sequence in *E. coli* and developed sera and monoclonal antibodies against the corresponding recombinant polypeptide.

Expression of cpRL3 in *E. coli*. The 2,359-bp cpRL3 insert was cloned in the *Eco*RI site of plasmid pDS56/RBSII-E$^-$ 6×His. The resulting plasmid, pDS56/RBSII-E$^-$ 6×His-cpRL3, contained a DNA sequence that added a stretch of 6 histidines at the amino terminus of the recombinant protein. The presence of the histidine stretch within the sequence conferred on the expressed protein an elevated affinity for nickel. The polypeptide encoded by cpRL3 (6×His-cpRL3) was expressed upon induction with IPTG and purified by nickel chelate chromatography. Purified 6×His-cpRL3 was used as an immunogen to develop both antisera and monoclonal antibodies.

Antibody specificity. After three immunizations with recombinant 6×His-cpRL3, the serum of mouse M10/01 showed a strong reactivity against the recombinant polypeptide in an ELISA at a dilution of $1/5 \times 10^5$. The serum and several monoclonal antibodies developed from the spleen of this mouse were analyzed for specificity and used to study the expression in the parasite of the amino acid sequence encoded by cpRL3. A 1/5,000 dilution of the M10/01 serum reacted in immunoblots against 6×His-cpRL3 expressed in *E. coli* both before and after purification on a nickel column (Fig. 5A). The specificity of this reaction was indicated by the total absence of reactivity against the *E. coli* proteins present in the lysate of noninduced bacteria (Fig. 5A). The M10/01 serum did not show any reactivity against TRAP (20), a recombinant *Plasmodium falciparum* protein that was expressed in *E. coli* by the transcription unit of plasmid pDS56/RBSII and therefore contained an amino acid stretch

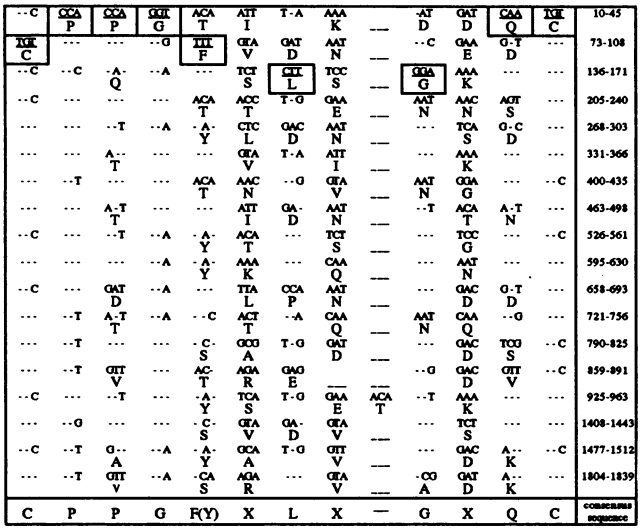

FIG. 3. Comparison of the nucleotide and derived amino acid substitutions in the sequences of the type I repeat of cpMM1. The numbers refer to the corresponding DNA coding sequences. The codons used most frequently are boxed. Identity to the coding sequence is shown by dashes. For DNA mutations generating an amino acid substitution, the amino acid is indicated below the corresponding codon.

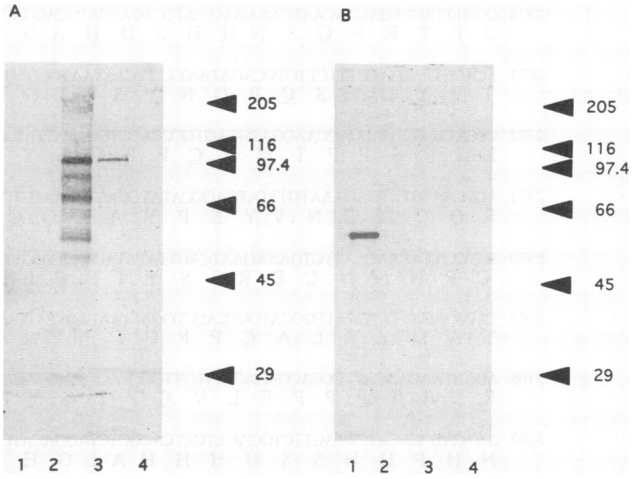

FIG. 5. Immunoblot analysis of mouse serum M10/01 (A) and control mouse anti-TRAP serum (B) against the expression product of control plasmid pDS56/RBSII-E$^-$ 6×His-TRAP and purified on a nickel column (lanes 1), the expression product of plasmid pDS56/RBSII-E$^-$ 6×His-cpRL3 and purified on a nickel column (lanes 2), a protein lysate of *E. coli* cells transformed with pDS56/RBSII-E$^-$ 6×His-cpRL3 and induced with IPTG (lanes 3), and a noninduced *E. coli* cell protein lysate (lanes 4). The molecular mass (kilodaltons) standards are indicated.

of histidines at its amino terminus (Fig. 5B). Furthermore, a serum from a control mouse immunized with recombinant TRAP did not react against the cpRL3 expression product. In the material purified on the nickel column, the immunoreactive bands migrating below 6×His-cpRL3 were interpreted to be degradation products of the purified recombinant polypeptide, since a reaction was not observed against the lysates of induced and noninduced *E. coli* (Fig. 5A). Recombinant polypeptide 6×His-cpRL3 migrated in SDS-PAGE with an apparent molecular weight of 105,000, although the amino acid composition predicted a molecular weight of 85,500, a result indicating that the particular amino acid composition may determine molecular weight migration artifacts in the corresponding parasite protein. Several monoclonal antibodies reacted against 6×His-cpRL3 in the ELISA. To study the expression of the protein in *C. parvum*, we used monoclonal antibody 11B2 [immunoglobulin G1(κ)], which showed in immunoblots against 6×His-cpRL3 a pattern of reactivity identical to that of M10/01. Monoclonal antibody 11B2 reacted against parasite-derived material both in immunoblots and in immunofluorescence tests.

Expression and immunolocalization of the protein in *C. parvum*. In immunoblots, both serum M10/01 and monoclonal antibody 11B2 decorated among the proteins from a lysate of *C. parvum* oocysts a band that showed, under reducing conditions in 10% SDS-PAGE, an apparent molecular weight of 190,000 (Fig. 6). When used under nonreducing conditions, the monoclonal antibody and the serum did not react against any of the *C. parvum* proteins, despite the fact that both reagents were able to recognize recombinant polypeptide 6×His-cpRL3 under reducing (in immunoblots) and nonreducing (in the ELISA) conditions. The high cysteine content of the amino acid sequence encoded by cpRL3 and cpMM1 could account for the lack of reactivity of both the serum M10/01 and the monoclonal antibody 11B2 under reducing conditions. Under nonreducing conditions, the parasite protein might exist in the form of a large molecular aggregate that would not enter SDS-polyacrylamide gels easily. Monoclonal antibody 11B2 was used in immunofluorescence tests of fixed *C. parvum* oocysts with the aim of localizing the cpRL3 and cpMM1 amino acid sequence in the parasite. To optimize the resolution, we performed the microscopic analysis with a confocal microscope. The surface of the oocysts was heavily decorated by monoclonal antibody 11B2, a result indicating that the parasite protein containing the amino acid sequence encoded by the DNA inserts cpRL3 and cpMM1 was a component of the oocyst wall (Fig. 7). The confocal microscope analysis showed that

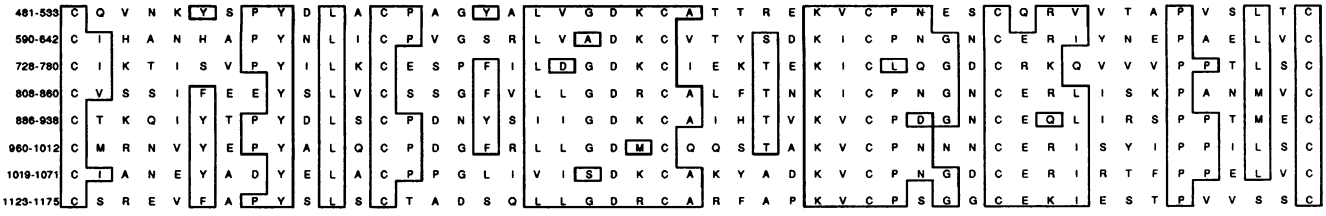

FIG. 4. Alignment of type II amino acid motifs in cpMM1. Regions of identity or high homology are boxed. The numbers refer to the amino acid positions encompassed by the sequences.

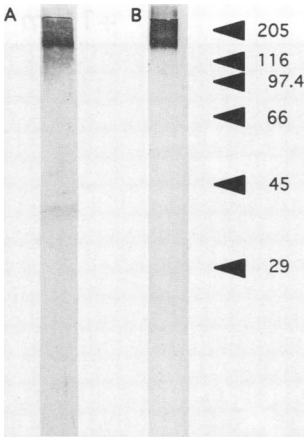

FIG. 6. Immunoblot analysis with mouse serum M10/01 (A) and monoclonal antibody 11B2 (B) against a protein lysate of *C. parvum* oocysts. The molecular mass (kilodaltons) standards are indicated.

the staining was uniformly distributed on the oocyst wall and that a granular reactive material was also present within the oocyst itself. The granules could be observed in both intact and excysted oocysts (Fig. 7) and appeared in close association with the residual body. On the basis of these results, the protein encoded by cpRL3 and cpMM1 will be referred to hereafter as *Cryptosporidium* oocyst wall protein of 190 kDa, or COWP-190.

Analysis of the COWP-190 sequence in *C. parvum* isolates and in other protozoa. Using oligonucleotides derived from the cpRL3 sequence, we developed a PCR assay (Fig. 8). Both oligonucleotide combinations Cry-3/Cry-6 and Cry-5/Cry-6 were able to amplify DNA segments of the expected molecular weights from the DNA of plasmid pDS56 RBSII-E$^-$ 6×His-cpRL3 and from the DNA of *C. parvum*. The amplification reaction done with the oligonucleotide combination Cry-3/Cry-6 had a detection limit of 40 parasite oocysts (Fig. 8C). Furthermore, the fragment amplified by Cry-3 and Cry-6 contained several unique restriction nuclease sites that could be used as markers to analyze the polymorphisms of cpRL3 in *C. parvum* isolates. The DNAs from three *C. parvum* isolates (FI ISS-2, TO ISS-3, and RM ISS-4) (18) originating from AIDS patients were amplified in the PCR with primers Cry-3 and Cry-6. The polymorphisms of the amplified sequences were analyzed with restriction endonucleases *Cla*I, *Dra*I, *Hin*cII, *Pst*I, and *Sma*I. For the cpRL3 sequence amplified from the human isolates and from bovine isolate MI ISS-1, we could not observe any restriction length polymorphisms with this collection of restriction enzymes, a result suggesting that the COWP-190 sequence tested may be conserved among bovine and human isolates. Furthermore, we examined by use of the PCR the presence of COWP-190-related sequences in other protozoa. Using both oligonucleotide combinations, we found that it was not possible to amplify any sequence from the DNA of *Giardia lamblia*, which is an intestinal protozoan, and from the DNAs of *P. falciparum* and a *Sarcocystis* sp., which are phylogenetically related (Sporozoa) to *C. parvum* (Fig. 8B and C).

DISCUSSION

Although recently improved, *Cryptosporidium* culture methods still do not provide sufficient quantities of parasite proteins and genetic material for studying the molecular and biochemical properties of parasite components. Alternatively, cryptosporidia can be obtained from the feces of infected animals by use of Percoll gradients. This method is time-consuming and permits the purification only of parasites at the oocyst stage. The intestinal mucosa may be regarded as a potential and abundant source of *C. parvum* parasites from which all developmental stages might be collected. If intracellular stages could be recovered from the brush border, then many of the limitations involved in the use of in vitro cultures might be overcome. We provided evidence that nitrocellulose filters applied to the mucosa of an infected gut could remove the intestinal cells of the brush border together with a large number of parasites. Furthermore, our data indicate that the DNA extracted from these filters could be used to generate a λgt11 expression library containing *C. parvum* DNA inserts. Of 2.5×10^6 plaques tested, 5 carried a DNA insert identical to cpRL3, a result suggesting that the parasite genome is represented several times in the library. We isolated from this expression library a DNA insert encoding a partial sequence of 786 amino acids. The possibility that the cpRL3 insert did not originate from *Cryptosporidium* DNA was ruled out by use of the sequence of the cpRL3 insert as a probe to search for related sequences in a genetic library generated with *C. parvum* DNA only. Insert cpMM1, which was found by this approach, encompassed the entire sequence of cpRL3 and contained an additional 2,061-nucleotide sequence at the 3′ end of the cpRL3 sequence. Insert cpMM1 contained an open reading frame of 3,756 bp and coding for 1,252 amino acids. The polypeptide encoded by cpMM1 probably represents 80% of the COWP-190 sequence. This prediction takes into account the expected molecular weight of the amino acid sequence encoded by cpMM1, the apparent molecular weight of COWP-190 in immunoblots, and the peculiar migration in SDS-PAGE of recombinant cpRL3. The deduced amino acid sequence is characterized by a high cysteine content and by the presence of two types of repeated amino acid motifs that identify two distinct domains. The presence of amino acid repeats is a common feature of many protozoan proteins, especially *Plasmodium* proteins (8). The function of the repeats is not completely understood; it has been suggested that in *Plasmodium* proteins, they represent decoy targets implicated in the evasion by the parasite of the immune response (8). It is not likely that the repeats in the parasite protein play such a role. Although the expression product of cpRL3 was very immunogenic (after three immunizations, we could detect in most immunized mice an antibody titer in an ELISA higher than 4×10^4), neither resistant individuals (five immunocompetent laboratory volunteers) nor five AIDS patients with a *Cryptosporidium* infection had a detectable level of antibodies against recombinant cpRL3 in an ELISA (data not shown).

Any hypothesis concerning the function of the protein encoded by cpRL3 and cpMM1 and its repeated amino acid motifs must take into account the localization of the protein in *C. parvum*. In a previous report, an oocyst localization of the parasite protein was suspected on the basis of an immunoblot analysis. A polyclonal antibody raised against a recombinant fusion protein encompassing the cpRL3 sequence recognized a 190,000-molecular-weight band more intensely in a protein lysate obtained from disrupted oocysts than in a lysate obtained from sporozoites (12). The results reported did not allow inference as to whether the parasite protein was localized on the oocyst wall, on the residual

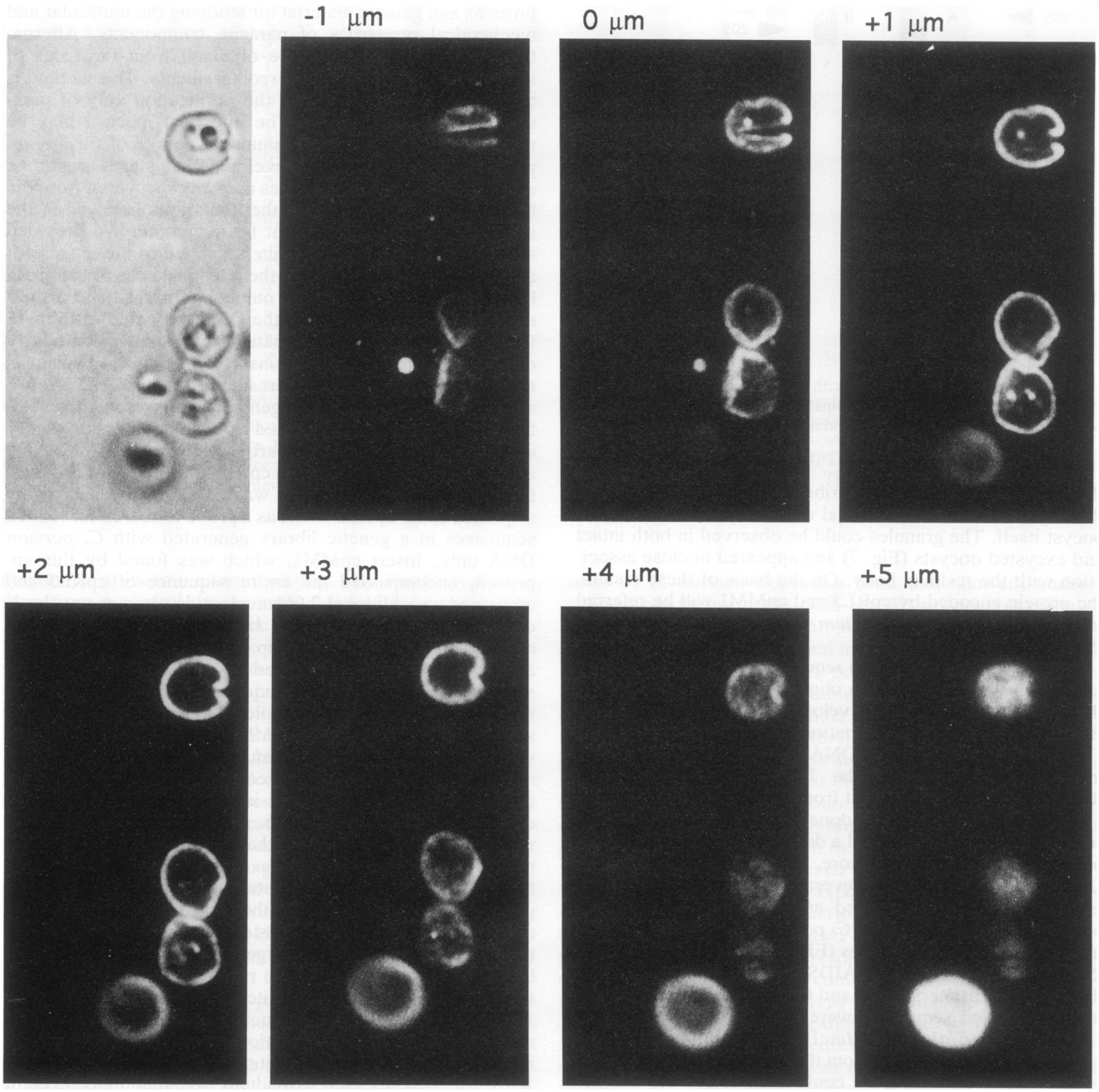

FIG. 7. Microphotographs of progressive focal planes (−1, 0, +1, +2, +3, +4, and +5 μm) of *C. parvum* oocysts stained by immunofluorescence with monoclonal antibody 11B2.

body, or on both. Moreover, the possibility was not ruled out that the sporozoites also expressed the protein at a low level (12). We have shown here, using monoclonal antibody 11B2 and immunofluorescence, that the *Cryptosporidium* protein encoded by cpRL3 and cpMM1 is a component of the oocyst wall. The localization on the oocyst wall is in agreement with the analysis of the amino acid sequence structure which, on the basis of the high cysteine content, was predictive of an extracellular localization of the protein. The immunolocalization on the oocyst wall may suggest an explanation for the function of the protein and for the role of the amino acid repeats in COWP-190. These may provide periodic spacing and appropriate conformation to permit a large number of disulfide bridges between the cysteine residues. Accordingly, the cysteines would allow the protein to constitute an intramolecular and intermolecular net of chemical bonds that might confer structural stability to the oocyst wall. The high frequency of synonymous mutations indicated the existence of a negative pressure for amino acid substitutions in the sequences of the repeats. Substitutions are not tolerated, possibly because they would impair the structural stability of the oocyst. In addition to the localization on the oocyst wall, we observed that monoclonal antibody 11B2 reacted with material localized within the

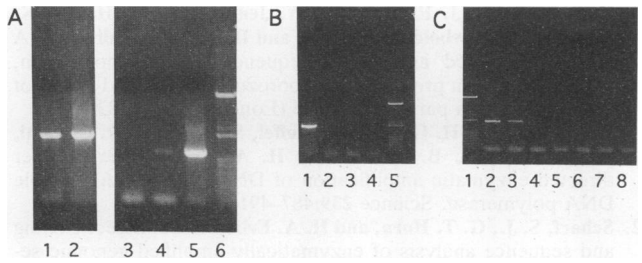

FIG. 8. (A) Electrophoresis of PCR products amplified from *C. parvum* DNA (lanes 1 and 4) and cpRL3-containing plasmid pDS56/RBSII-E$^-$ 6×His DNA (lanes 2 and 5) with the primer combinations Cry-3/Cry-6 (lanes 1 and 2) and Cry-5/Cry-6 (lanes 3 to 5). Lane 6, molecular standards. As a control, the PCR was done without template DNA (lane 3). (B) Electrophoresis of PCR products amplified from DNAs of several parasite species with the primer combination Cry-3/Cry-6. DNA was extracted from *C. parvum* (lane 1), a *Sarcocystis* sp. (lane 2), *G. lamblia* (lane 3), and *P. falciparum* (lane 4). Lane 5, molecular standards. (C) Electrophoresis of PCR products amplified from the DNA of progressively diluted *C. parvum* oocysts with the primer combination Cry-3/Cry-6. Lanes: 1, molecular standards; 2, 160 oocysts; 3, 80 oocysts; 4, 40 oocysts; 5, 20 oocysts; 6, 10 oocysts. As a control, the PCR was done without template DNA (lane 7) or in the presence of *P. falciparum* DNA (lane 8). In panels A, B, and C, lanes 6, 5, and 1, respectively, the DNA markers were 3,611, 1,166, 606, 517, 396, 318, and 263 bp.

oocyst itself. Because of the high fluorescence intensity emitted by the oocyst wall, it was difficult to establish whether the antibody reactivity within the oocyst was associated with a parasite structure. Only by examination of the immunofluorescence with a confocal microscope was it possible to identify the immunoreactive material as fluorescent granules within the oocysts. These granules varied in intensity, number, position, and dimension. We interpreted this staining pattern to be indicative of monoclonal antibody 11B2 localization on the residual body of the oocyst. This conclusion is also supported by the observation that the fluorescent granules could be detected in close association with parasite-derived material within disrupted oocysts. The possibility exists that monoclonal antibody 11B2 recognizes different proteins on the oocyst wall and on the residual body. This possibility is not very likely, because monoclonal antibody 11B2 was shown to be highly specific and, in immunoblots, recognized only one band in a lysate containing proteins from intact oocysts. Furthermore, it was shown by immunoelectron microscopy that a component of the oocyst wall could also be localized in the granules of the residual body (1). The protein constituents of the oocyst wall were studied in earlier works, and several immunoblot patterns were reported (14–16). On the basis of the apparent molecular weight in immunoblots, it is difficult to speculate whether the parasite protein encoded by cpMM1 corresponds to one of the bands described before as protein components of the oocyst wall. Differences in oocyst storage, lysate preparation, and polyacrylamide gel composition may account for discrepancies in the apparent molecular weight.

The DNA sequence of cpRL3 apparently is not very polymorphic among *C. parvum* isolates because (i) the PCR products amplified from the DNAs of one human and three bovine isolates did not show any restriction fragment length polymorphisms and (ii) a *C. parvum* DNA insert independently isolated from another laboratory had a DNA sequence identical to that of cpRL3 (12). Although genetic differences have been reported (17), the absence of polymorphisms in the DNA sequences of cpRL3 indicates that bovine and human isolates of *C. parvum* are closely related. The detection by PCR of the cpRL3 sequence seems to be restricted to the presence of *Cryptosporidium* DNA. Sequences identical or highly homologous to cpRL3 apparently are not present in protozoa phylogenetically related to cryptosporidia. PCR done with the primer combination Cry-3/Cry-6 was very sensitive; in fact, the amplification product of 40 oocysts could be demonstrated by ethidium bromide staining of an agarose gel. The amplification of the cpRL3 sequence from feces and other biological samples may represent a useful tool for the diagnosis of *C. parvum*.

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