Cloning of the entire COWP gene of *Cryptosporidium parvum* and ultrastructural localization of the protein during sexual parasite development

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SUMMARY

Molecular cloning and immunoelectron microscopy have been used to clone the full-length gene encoding *Cryptosporidium parvum* oocyst wall protein (COWP) and to analyse at the ultrastructural level the expression and localization of COWP during development in the gut. COWP is 1622 amino acids long, has a typical leader peptide and consists of 2 amino acidic domains each containing distinct repeated elements possibly originating from a common ancestral precursor. Electron microscopy localized COWP in a large cytoplasmic inclusion and in the wall-forming bodies of early and late macrogametes, respectively. Ultrastructural analysis of double-walled sporulating and mature oocysts indicated that COWP is selectively localized in the inner layer of the oocyst wall. This study provides the first localization at the ultrastructural level of a cloned coccidian oocyst wall protein.

Key words: *Cryptosporidium parvum*, COWP gene, immunolocalization, macrogametes, wall-forming bodies, oocyst wall.

INTRODUCTION

Cryptosporidium parvum is an Apicomplexan parasite that primarily infects the gastrointestinal tract of several animal species (Tzipori, 1988; Petersen, 1993; Martins & Guerrant, 1995; O'Donoghue, 1995). The sporulated oocyst of *C. parvum* is the infective stage that is transmitted from one host to the other by the faecal–oral route. The oocyst of *C. parvum* remains viable in water for relatively long periods of time after release from an infected host. As few as 30–100 oocysts initiate an infection in humans (Du Pont *et al*. 1995). *C. parvum* oocysts are resistant to several treatments including the addition of compounds releasing active iodine and chlorine (Korich *et al*. 1990). The ability of *Cryptosporidium* oocysts to survive in the environment and also in the presence of oxidative agents is a key feature of transmission and has been attributed to the protective function of the oocyst wall. The identification and the molecular characterization of the wall constituents would facilitate the understanding of how *Cryptosporidium* sporozoites retain their infectivity in a hostile environment. Electron microscopy observations indicate that the oocyst wall is a double-layered envelope, consisting of an irregular 10 nm layer separated from a thicker electron-dense inner layer by an electron-lucent space (Ungar, 1990). Although several anti-oocyst wall monoconal antibodies have been described (Bonnin, Dubremetz & Camerlynck, 1991; McDonald, McCrossan & Petry, 1995), little information is available concerning either the constituents of the oocyst wall or the molecular architecture of this parasite structure. Preliminary reports indicate that the wall of sporulated oocysts may contain up to 17 different proteins showing a wide range of molecular masses (Lumb, Lanser & O'Donoghue, 1988; Tilley *et al*. 1990). More recently, partial sequences of a *Cryptosporidium parvum* gene encoding the oocyst protein COWP have been described (Lally *et al*. 1992; Ranucci *et al*. 1993). Both the localization and the analysis of the deduced amino acid sequence suggest that COWP may play an important functional and/or structural role in *Cryptosporidium* oocysts (Lally *et al*. 1992; Ranucci *et al*. 1993). Among all cyst-forming coccidia COWP so far represents the only cloned protein involved in oocyst wall formation. For this reason we investigated further the structure of the COWP gene and studied, at the ultrastructural level, the expression of this parasite protein during the life-cycle of *C. parvum* in the intestinal epithelium of experimentally infected mice.

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MATERIALS AND METHODS

Parasites

Purified *Cryptosporidium parvum* oocysts were purchased from the Moredun Institute, Edinburgh.

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Construction of a genomic library in the phage vector λEMBL3

A λEMBL3 genomic library was developed using DNA extracted from the intestinal mucosa of an infected calf as already described (Müller *et al*. 1993; Ranucci *et al*. 1993). The DNA, consisting of a mixture of bovine and *C. parvum* DNA, was subjected to partial digestion using the restriction endonuclease *Sau*3AI. High molecular weight genomic fragments were then ligated to the λEMBL3 phage arms at their *Bam*HI sites. Recombinant phage DNA was packaged *in vitro* and a library with a complexity of $2{\cdot}2 \times 10^6$ pfu was obtained.

Screening of the genomic library

The whole non-amplified library was plated on the *E. coli* strain LE392 at a density of 50000 plaques/13 cm diameter plates. The screening was carried out using as a probe a DNA fragment of 4420 nucleotides corresponding to the insert of the phage clone cpMM1, encoding 1252 amino acids at the C-terminus of COWP (Ranucci *et al*. 1993). The DNA probe was labelled by random priming using [α^{35}S]dATP and hybridized to the nitrocellulose filters in 4 × SSC/5 × Denhardt's/0·5 % SDS at 65 °C. Washing was performed at 65 °C in 0·1 × SSC/0·5 % SDS.

Southern genomic analysis

DNA (1 μg) extracted from highly purified *C. parvum* oocysts, as well as genomic DNA from calf (10 μg) and *E. coli* (1 μg) was digested with the restriction endonuclease *Hinc*II and separated by gel electrophoresis (0·8 % agarose). The DNA was transferred onto nitrocellulose filters by capillary absorption. The filters were probed using the insert cpMM1 labelled by random priming with [α^{32}P]dCTP. Hybridization was carried out at 65 °C in 4 × SSC/10 × Denhardt's/0·5 % SDS in the presence of 30 μg/ml of salmon sperm DNA. Washing was performed at 65 °C in 0·1 × SSC/0·5 % SDS.

Expression of the recombinant polypeptide 6xHis-cpRL3 and development of the antiserum M10/01

The expression and the purification from *E. coli* of the recombinant polypeptide 6xHis-cpRL3, consisting of the amino acids spanning positions 371–1156 of the COWP precursor fused to a 6-histidines N-terminal tag, have been already described (Ranucci *et al*. 1993). Anti-COWP sera were raised in mice by intraperitoneal injection of the purified recombinant polypeptide 6xHis-cpRL3. Balb/c mice were immunized 3 times with 50 μg of purified 6xHis-cpRL3 polypeptide in complete (for the first immunization) or incomplete Freund's adjuvant. Individual sera were collected 2 weeks after the last immunization. The serum M10/01 was selected for the immunolocalization experiments on the basis of its high titre in ELISA against recombinant 6xHis-cpRL3 and for its specificity in immunoblot experiments against total oocyst lysates.

Immunoelectron microscopy

Purified *C. parvum* oocysts were incubated at room temperature for 2 h with 2 % formaldehyde and 0·8 % glutaraldehyde. After fixation the oocysts were washed 3 times with phosphate-buffered saline (PBS)/0·15 % glycine and pelleted in 10 % gelatine in PBS. The gelatine was allowed to solidify and small cubic blocks were cut at 4 °C and infused with 2·3 M sucrose in PBS for at least 2 h at 4 °C. The blocks were frozen in liquid nitrogen and ultrathin cryosections were prepared using an ultracryomicrotome. LR White-embedded ileum samples from *C. parvum*-infected mice were kindly provided by Dr B. Robert (Centre d'Economie Rurale, Marloie, Belgium). The procedures concerning the treatment of these samples have already been described in detail (Robert *et al*. 1994). Ultrathin sections of approximately 70 nm were cut using an ultramicrotome with a diamond knife. Free oocysts and ileum sections were collected on copper grids, washed with PBS/0·15 % glycine and successively incubated with 0·1 % bovine serum albumin (BSA) in PBS for 10 min and with the immune serum M10/01 (1:100 dilution in 1 % BSA) for 30 min. After several washings in PBS/BSA 0·1 % the sections were incubated with Protein A/gold for 30 min. Unbound Protein A/gold was removed by additional washing and the sections were incubated with 1 % glutaraldehyde to stabilize the Protein A/antibody/antigen complex. Control sections were processed in the same manner except that serum M10/01 was replaced by the mouse anti-serum T8, directed against an unrelated polypeptide produced in *E. coli* as a fusion with the same histidine tag of 6xHis-cpRL3. Cryosections were stained with 2 % uranyl oxalate and embedded in methyl cellulose/uranyl acetate, LR White sections were stained with 4 % uranyl acetate in water. All samples were analysed using a Hitachi H7000 electron microscope.

DNA sequencing

DNA fragments obtained by endonuclease digestion of phage genomic inserts were subcloned in the plasmid *pBluescript* SK (Stratagene, CA) and sequenced using the dideoxy-termination method.

Computer sequence analysis

Nucleotide and amino acid sequences were analysed using the programs of the Genetic Computer Group

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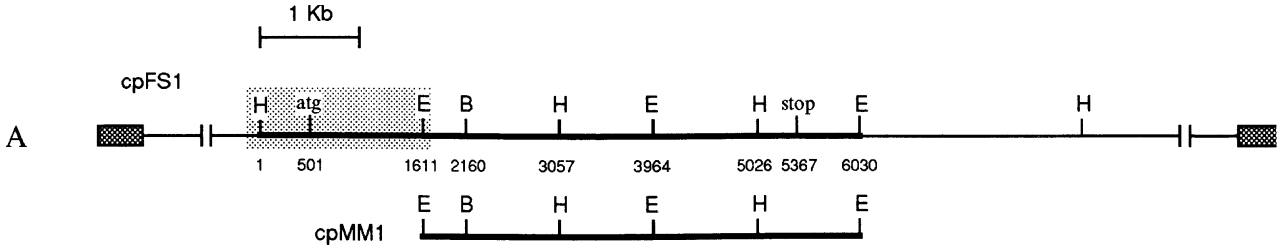

B

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 401 tcaatcaacaatttttttacctaataatttcaattactatttgaatatctgccctaatttaattattaggtcaatttcattaaatagtttttttcttttatt
     M  K  R  I  L  L  L  S  F  I  I  G  A  F  G ▼A  P  Q  V  K  P  N  I  P  G  V  A  S  P  L  P  H  T  K
 501 ATGAAACGTATTTTATTATTGTCGTTTATTATTGGTGCATTTGGTGCGCCTCAGGTTAAACCCAACATTCCTGGTGTAGCTTCCCCTTTGCCACACACAA
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      M  P  F  C  Q  E  P  A  I  Y  H  E  G  H  C  L  I  V  T  A  P  L  K  Q  C  P  P  G  Y  E  I  S  G
 701 TATGCCTTTCTGTCAGGAACCAGCAATCTATCACGAGGGGCATTGTTTAATAGTAACAGCACCACTAAAGCAATGTCCACCAGGATACGAAATATCAGGA
      K  Q  C  T  A  T  K  T  A  S  Q  Q  P  S  C  P  P  G  T  T  L  H  G  T  E  C  I  S  K  H  M  I  D  T
 801 AAGCAATGTACTGCTACTAAAACCGCTTCTCAACAACCATCTTGTCCTCCAGGTACTACATTACATGGAACAGAGTGTATCTCTAAACATATGATAGATA
       V  C  P  P  G  F  V  D  N  G  R  D  C  V  A  F  T  M  P  E  K  S  C  P  P  G  F  V  F  L  K  Q  C
 901 CAGTATGTCCCCCTGGTTTCGTAGATAATGGAAGAGATTGTGTTGCATTCACTATGCCTGAAAAATCATGTCCCCCAGGATTCGTTTTTCTGAAACAATG
    V  Q  S  D  T  A  P  P  N  P  E  C  P  P  G  T  I  L  E  N  G  T  C  K  L  I  Q  Q  I  D  T  V  C
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       P  E  S  A  E  L  E  S  T  C  P  P  N  S  I  F  E  N  G  K  C  K  V  I  K  N  I  D  M  V  C  P  P
1201 CACCAGAATCAGCTGAATTAGAATCGACATGCCCACCTAATTCAATATTTGAAAATGGAAAATGTAAAGTGATTAAAAATATTGATATGGTCTGCCCACC
      G  Y  T  D  S  G  D  D  C  V  L  Y  V  A  P  A  K  E  C  P  P  N  F  I  L  Q  G  L  Q  C  I  Q  T
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       L  D  N  G  K  D  C  V  K  Y  S  P  V  T  K  E  C  P  P  G  F  T  L  S  G  N  R  C  V  Q  M  V  N
1501 TTTTAGACAATGGAAAAGATTGTGTAAAATATTCTCCAGTCACAAAGGAATGCCCACCCGGGTTTACATTGAGTGGGAACAGGTGCGTTCAAATGGTAAA
      A  P  M  E  F
1601 TGCTCCCATGGAATTC
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Fig. 1. (A) Endonuclease restriction map of the genomic inserts cpFS1 and cpMM1. The thick line indicates the regions of the inserts that have been sequenced. The arms of the λEMBL3 vector are shown in dark grey. The numbers indicate the position of the endonuclease cleavage sites, of the putative translation start (ATG) and of the stop codon. The sequenced region of the cpFS1 insert upstream to the known sequence of the COWP gene is shadowed. H, *Hin*cII; B, *Bam*HI; E, *Eco*RI. (B) Nucleotide sequence of the cpFS1 insert contained between the 5′*Hin*cII and *Eco*RI cleavage sites (position 1–1611). The nucleotides upstream to the putative translation start site are shown in lower case letters. The sequence of the *Hin*cII and *Eco*RI sites is underlined. The deduced amino acid sequence of the N-terminal portion of COWP (in single letter code) is shown above the coding sequence. The arrowhead indicates the putative cleavage site of the signal peptide. The nucleotide sequence is available in the EMBL database with the accession number Z22537.

software package (Devereux, Haeberli & Smithies, 1984).

RESULTS

Identification of the complete sequence of the COWP gene

Partial sequence information on the COWP gene was initially inferred from the analysis of phage inserts isolated from genomic λgt11 expression libraries (Lally *et al*. 1992; Ranucci *et al*. 1993). The insert cpMM1 (Fig. 1A) contains an open reading frame of 3756 bp encoding a polypeptide of 1252 amino acids. The DNA of the insert at the 3′ end of the coding sequence is characterized by the presence of several stop codons in all 3 frames. The lack of a translation start codon at the 5′ end indicates that part of the gene was still missing. In order to clone

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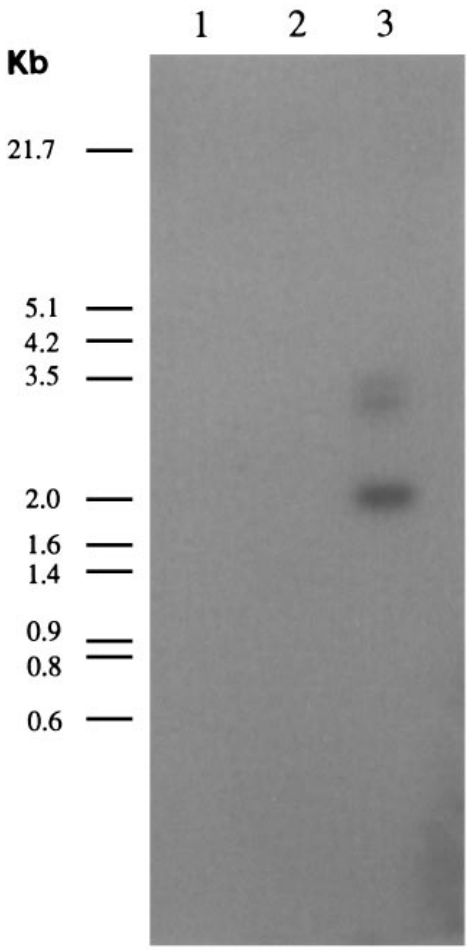

Fig. 2. Autoradiography of Southern blot performed on *Hinc*II-digested DNA extracted from bovine liver (10 μg), lane 1, *E. coli* M15 cells (1 μg), lane 2 and *C. parvum* oocysts (1 μg), lane 3. The DNA was hybridized using as a probe the ^{32}P-radiolabelled cpMM1 insert. The migration pattern of DNA molecular weight standards ($\times$1 kb) is indicated.

the entire coding sequence of the COWP gene, we used insert cpMM1 as a probe to screen a mixed *Cryptosporidium*/bovine genomic library developed in the phage vector λEMBL3, that can accommodate very large DNA fragments (> 12000 bp). On the basis of the hybridization signal given by the probe we isolated from this library several phage clones. Restriction mapping and Southern analysis indicate that the recombinant phage clone cpFS1 (Fig. 1A) was likely to contain an insert encompassing the entire COWP gene. This clone was selected for further characterization. An *Hinc*II–*Bam*HI restriction fragment of 2·16 kb (position 1–2160), encompassing the 5′ end of the cpMM1 insert and its upstream flanking region, subcloned in the plasmid *pBluescript* SK and subjected to sequence analysis, contained 1610 nucleotides upstream to the 5′ end of cpMM1. This additional sequence information allowed us to deduce the full-length amino acid sequence of the parasite protein encoded by the COWP gene (Fig. 1B). The open reading frame of COWP extends for further 1128 nucleotides upstream to cpMM1 and encodes a putative signal peptide followed by 5 tandemly arrayed amino acid repeats. The application of the -1/-3 rule (von Heijne, 1986) to the N-terminus of COWP predicts a high score putative cleavage site after Gly 15, suggesting that the ATG at position 501 may function as translation initiation codon. The presence of a signal peptide is consistent with the notion that COWP has an extracellular localization. The COWP gene consists of an open reading frame of 4866 nucleotides coding for a polypeptide of 1622 amino acids containing 9 potential N-linked glycosylation sites. The coding region has an A+T content of 60%, whereas in the non-coding upstream region (nucleotides 1–500) the A+T content reaches 74%. Analysis of the available upstream sequence did not allow identification of any known transcription regulatory element.

Genomic Southern analysis

Both cpMM1 and cpFS1 were isolated from phage libraries generated using DNA extracted from *Cryptosporidium*-infected gut mucosa. To rule out the possibility that the cpMM1 and cpFS1 inserts or parts of them originated from bovine or *E. coli* genomes, DNA extracted from bovine mucosa, *E. coli* and *C. parvum* oocysts was analysed in Southern blot experiments. The 3 DNA species were digested with the endonuclease *Hinc*II and probed at high stringency with the labelled cpMM1 insert. The probe hybridized with 3 bands of 3·2, 3·0 and 2·0 kb respectively among *C. parvum* DNA digestion products (Fig. 2). The probe did not show any reactivity against bovine and bacterial DNA (Fig. 2). The observed hybridization pattern correlated perfectly with the deduced restriction map of the clone cpFS1. The intensity of the hybridization signals of the 3 *Cryptosporidium Hinc*II digestion products reflects their content of DNA sequences encompassed by the cpMM1 probe. Both the pattern and the specificity of the Southern blot experiment indicate that the cpMM1 and cpFS1 inserts originate from *C. parvum* DNA and COWP represents a single copy parasite gene.

Structural organization of COWP

Like many other proteins of protozoan parasites, COWP showed a repetitive structure characterized by the presence of 2 distinct amino acid motifs: Type I and Type II repeats (Fig. 3). Both repeats contained 6 cysteine residues at conserved and regularly spaced positions and a high proportion of proline and glycine residues. The arrangement of the repeated motifs in the protein sequence suggests that COWP may consist of 2 major structural domains (Fig. 3). An amino-terminal domain that en-

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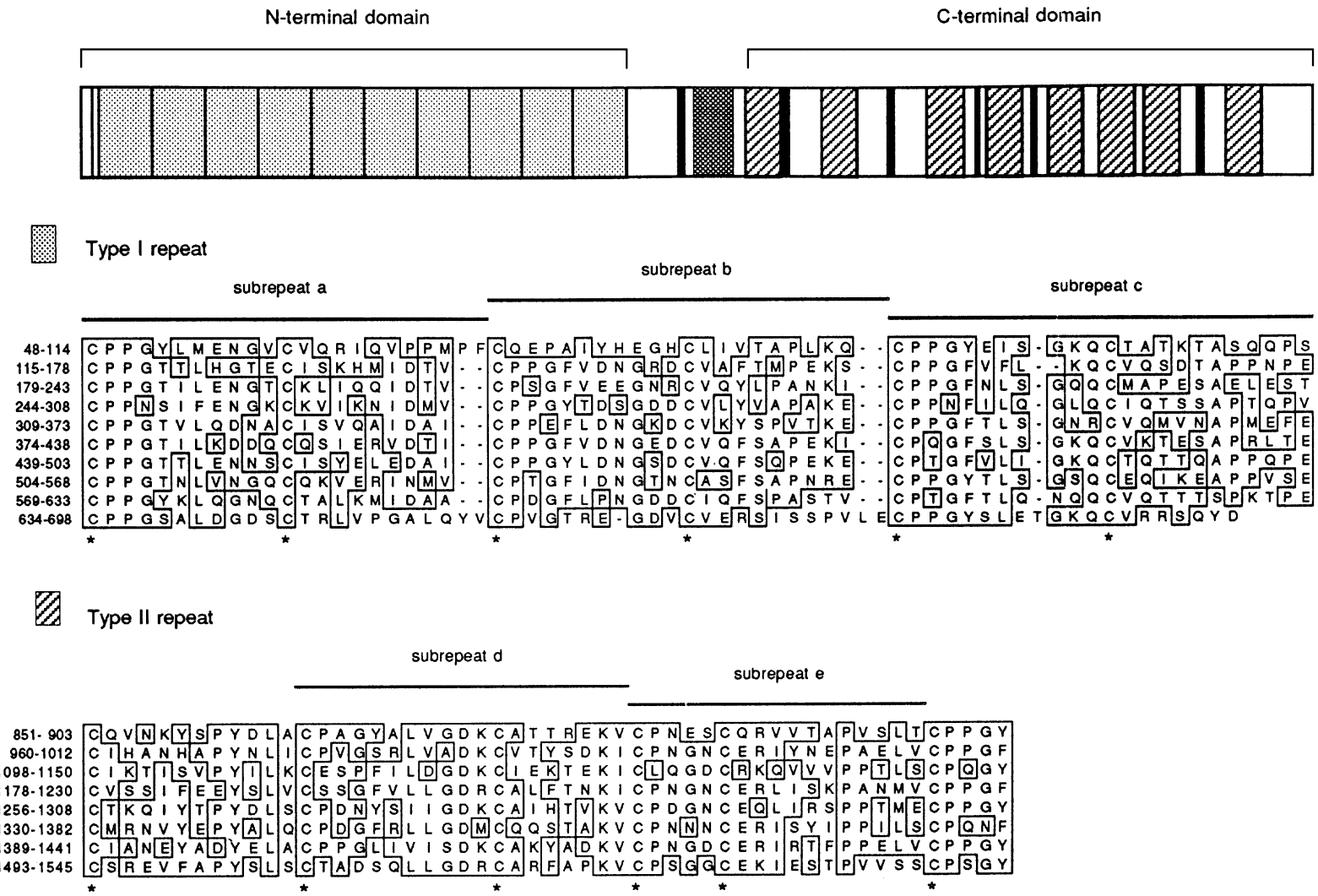

Fig. 3. Structural organization of the deduced amino acid sequence of COWP. The full length protein is schematically represented by a long rectangular box in the upper part of the figure. Histidine-rich stretches, Type I, Type II and Type I-like repeats are indicated as black, light grey, hatched and dark grey boxes, respectively. The lower part of the figure shows the alignments of the amino acid sequences of all Type I and Type II repeats. Regions of identity or high homology are boxed. The numbers refer to the position of each amino acid repeat within the sequence of COWP. Subrepeats a–e are indicated by thick horizontal bars above the corresponding sequences. Conserved cysteine residues are indicated by an asterisk.

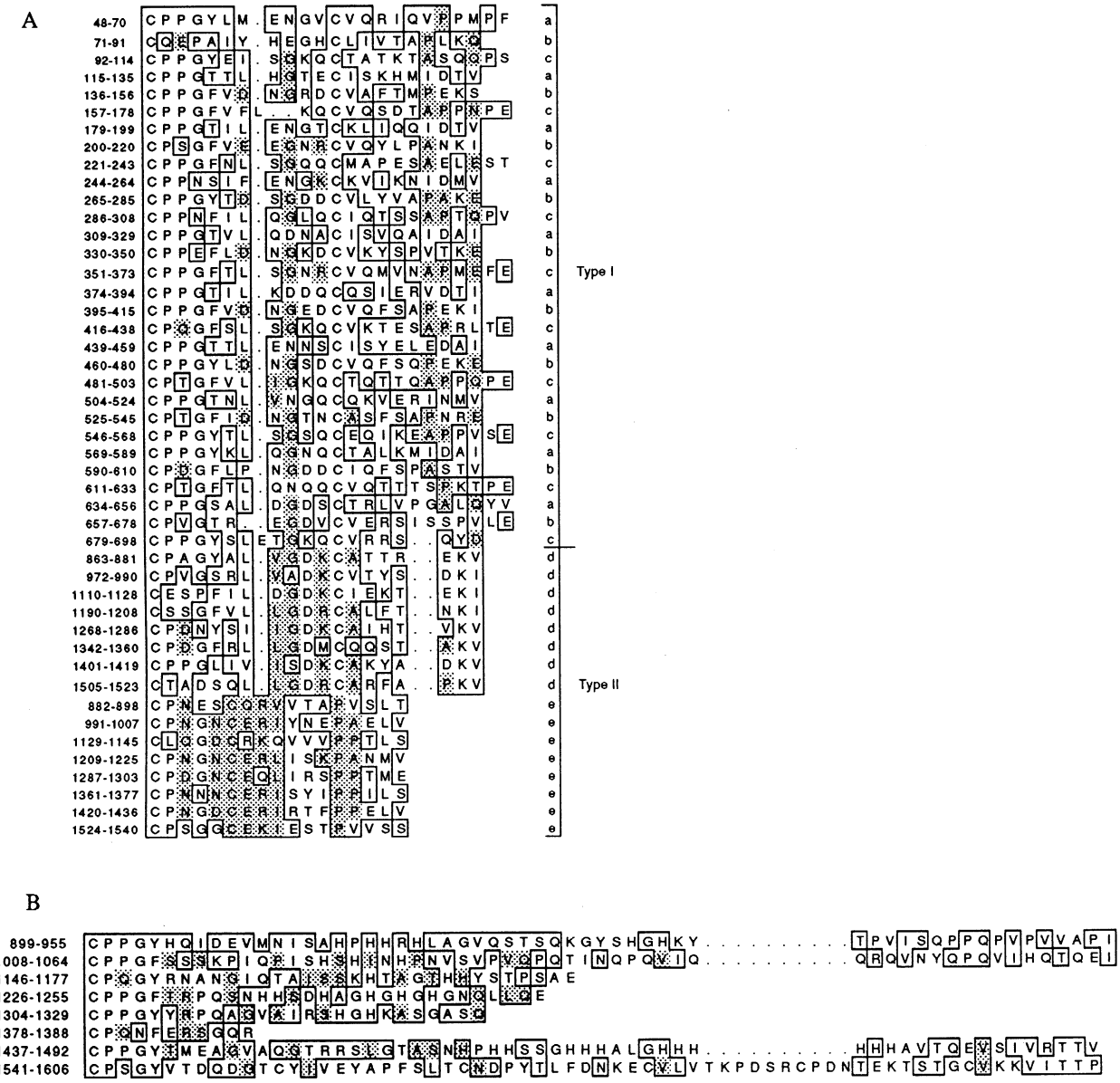

Fig. 4. Alignment of Type I and Type II subrepeats (A) and of the C-terminal inter-repeat histidine-rich sequences (B). Identical or homologous amino acids are boxed. Open and grey boxes indicate amino acid positions with distinct classes of conserved residues. The numbers refer to the position of each amino acid repeat within the sequence of COWP. Gaps have been introduced into the sequences (dots) to optimize the alignment. The lower case letters to the right of Type I (a–c) and Type II (d, e) subrepeats identify their relative position within the corresponding major repeat according to the scheme of Fig. 3.

compasses the first 698 amino acids and consists of a putative leader peptide followed by 10 tandemly arrayed Type I repeats of approximately 65 amino acids each and a carboxyl-terminal domain (position 851–1622) that encompasses 772 amino acids and contains 8 Type II repeats of 53 amino acids alternated with histidine-rich sequences of variable length. The 2 putative domains are separated by a region containing 2 elements: (i) an histidine-rich stretch similar to those found in the C-terminal domain (position 738–750) and (ii) a degenerated Type I repeat (positions 763–837). Computer analysis showed in both Type I and Type II repeats the presence of lower order amino acid repetitive units indicated as subrepeats *a–e* (Fig. 3). The alignment of the sequences of all Type I and Type II subrepeats indicated that these amino acid units are closely related (Fig. 4A). The motif CPPG is well conserved at the beginning of all subrepeats, which display invariable amino acid positions and a number of conservative substitutions. Each subrepeat shares between 80 and 30% similarity with each of the others. These data strongly suggest that the 2 major domains of COWP, although differing in the overall architecture, may have originated from a single primordial sequence. We also aligned, from the common CPPGY/F motif, the amino acid sequences of the inter-repeat regions that separate Type II repeats and found that they share on average 30% similarity. These amino acid regions, so far con-

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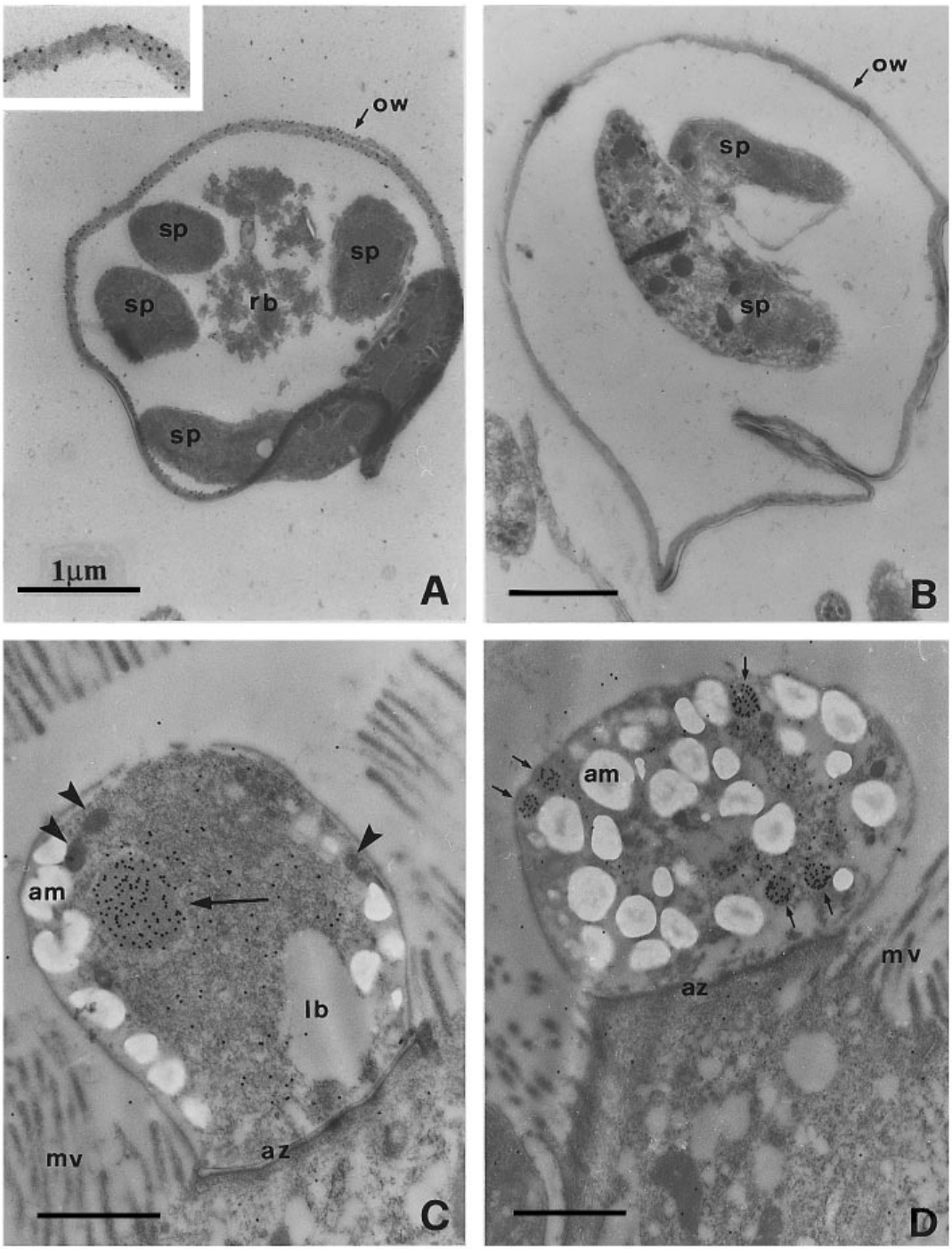

Fig. 5. Immunogold electron microscopy of ultrathin cryosections of purified oocysts (A and B) and of LR White-embedded *Cryptosporidium*-infected ileum (C and D). (A) Reactivity of serum M10/01 with a sporulated oocyst containing longitudinal and transversal sections of the sporozoites. Note the presence of numerous gold granules on the oocyst wall. Insert: higher magnification of a portion of the oocyst wall. (B) Section of a sporulated oocyst stained with control serum T8. (C) Early macrogamete stained with serum M10/01 characterized by the presence of a heavily labelled large granule (arrow) and by cytoplasmic staining; note the lack of labelling in the peripheral granules (arrowheads). (D) Reactivity of serum M10/01 with a mature macrogamete containing 5 intensely stained wall-forming bodies (arrows). am, Amylopectin; az, attachment zone; lb, lipid body; ow, oocyst wall; mv, microvilli; rb, residual body; sp, sporozoite.

sidered unrelated and scattered throughout the C-terminal region of the COWP molecule, may represent degenerated versions of a common ancestral sequence that was probably duplicated as an integral part of Type II repeats.

Ultrastructural localization of COWP

We have attempted to localize COWP by immunogold electron microscopy on ultrathin cryosections of highly purified *C. parvum* oocysts. For this

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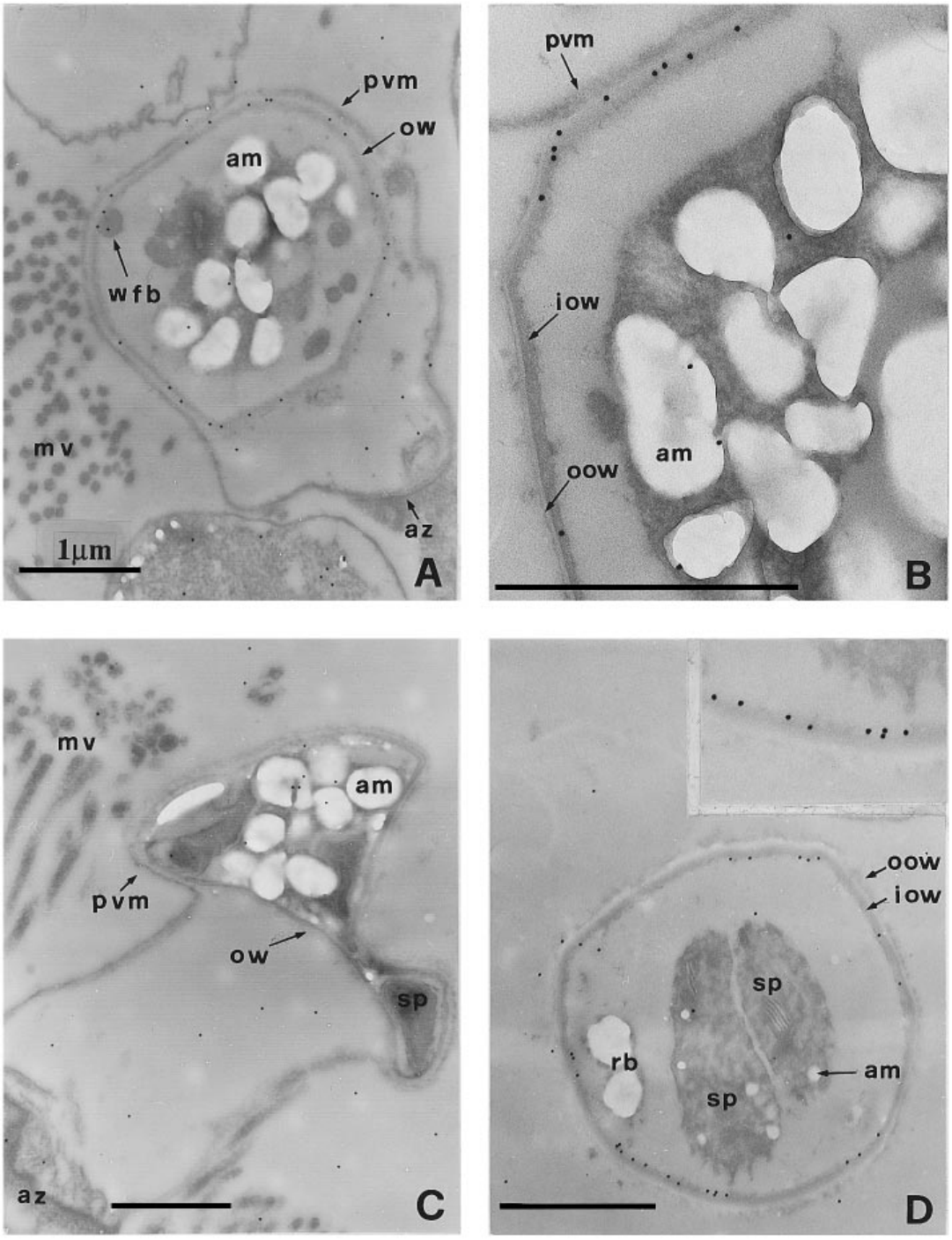

Fig. 6. Reactivity of serum M10/01 (A, B and D) and control serum T8 (C) with LR White sections of *Cryptosporidium parvum*-infected mouse ileum. (A) Sporulating oocyst showing the localization of the gold granules on the oocyst wall. Note the presence of a labelled wall-forming body fused to the oocyst wall. (B) Detail of a sporulating oocyst. The labelling is localized only on the inner layer of the oocyst wall. (C) Sporulating oocyst labelled with control serum T8 showing no specific staining of parasite structures. (D) Sporulated, double-walled oocyst found in the intestinal lumen showing the distribution of the gold particles on the inner layer of the oocyst wall. Insert: higher magnification of a portion of the oocyst wall. am, Amylopectin; az, attachment zone; iow, inner wall; mv, microvilli; oow, outer wall; ow, oocyst wall; pvm, parasitophorus vacuole membrane; rb, residual body; sp, sporozoite; wfb, wall-forming body.

purpose we used the mouse serum M10/01 raised against the recombinant polypeptide 6xHis-cpRL3. Although the morphological resolution obtained using ultrathin cryosections did not allow discrimination between the inner and outer layer of the oocyst wall, the labelling pattern clearly indicated that COWP is an abundant constituent of this parasite structure (Fig. 5A). The specificity of the labelling pattern observed using serum M10/01 was demonstrated by the lack of reactivity of the control

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serum T8 (Fig. 5B). The presence of few gold particles over sporozoite structures (Fig. 5A) was interpreted as non-specific binding of Protein A/gold conjugates as this labelling was also observed with the control serum T8 (Fig. 5B).

We have also employed immunogold electron microscopy to study the expression and the localization of COWP during the parasite life-cycle within infected enterocytes. The antiserum M10/01 was incubated on ultrathin sections of LR White-embedded mouse ileum experimentally infected with *C. parvum*. While the asexual forms of the parasite, trophozoites and schizonts at various maturation stages, did not show any significant reactivity (data not shown), the macrogametes were consistently labelled by the anti-COWP serum. Macrogametes at a relatively early developmental stage, as judged by the limited number of peripheral amylopectin granules, were heavily stained within a single large electron-dense vesicle, while the cytoplasm showed a diffuse labelling (Fig. 5C). On macrogametes at a later maturation stage, serum M10/01 strongly reacted with a class of smaller, frequently subpellicular, cytoplasmic granules that closely resembled Type-I wall-forming bodies (Fig. 5D). We have also immunolocalized COWP during the development of *C. parvum* oocysts inside the parasitophorous vacuole. As indicated by the distribution of gold granules in experimental (Fig. 6A and B) and control (Fig. 6C) sections, serum M10/01 specifically labelled the wall of sporulating oocysts. The high morphological definition obtained using the LR White-embedded ileum sections allowed us to identify the 2 layers of the oocyst wall and to localize COWP only in the inner layer (Fig. 6B). The same ultrastructural localization was also observed in a sporulated oocyst shed in the intestinal lumen (Fig. 6D).

DISCUSSION

The isolation from a *C. parvum* genomic library of the phage insert cpFS1 enabled us to clone the entire nucleotide sequence of the COWP gene. By combining the existing information (Ranucci *et al*. 1993) with the sequence data obtained from the insert cpFS1 we have shown that the COWP gene consists of an open reading frame of 4866 bp encoding a polypeptide of 1622 amino acids. We propose the ATG codon at position 501 as the putative translation start site of the gene. This possibility is supported by the observation that the encoded methionine is followed by a typical signal peptide and preceded by a nucleotide sequence of low coding potential. The putative initiation codon is immediately preceded by a pyrimidine-rich stretch containing an adenine at position −3. This structural feature is consistent with the notion that the proposed codon is the translation start site as a purine (usually an A) at position −3 is the most conserved nucleotide found in eukaryotic mRNAs (Kozak, 1989). Available information did not allow us to rule out the presence of introns within the sequence of the COWP gene. However, several facts argue against this possibility: (i) the amino acid sequence deduced from the uninterrupted 4866 bp reading frame has a complex repetitive architecture that would be dramatically altered by the presence of an intron sequence; (ii) protozoan genes usually contain few introns and their presence has not been reported in any of the *Cryptosporidium* genes so far characterized (Kim *et al*. 1992; Khramtsov *et al*. 1995, 1996; Steele *et al*. 1995); (iii) accurate analysis of the coding region of COWP failed to detect suitable pairs of donor/acceptor splicing sites. The cloning of the cDNA or RT-PCR products would permit assessment of the presence of introns within the coding sequence of COWP. However, this experimental approach is very problematic, as *Cryptosporidium* sexual stage-specific mRNA can be obtained only from an infected gut mucosa.

The deduced amino acid sequence of COWP predicts a precursor polypeptide of 175·6 kDa and a mature polypeptide, after the removal of the putative leader signal, of 174 kDa. This value is slightly different from the apparent molecular weight of the parasite protein (190 kDa) as observed in immunoblot experiments (Lally *et al*. 1992; Ranucci *et al*. 1993). The discrepancy between the calculated and the apparent molecular weight of COWP may reflect either artefacts in the electrophoretic mobility of the native protein, possibly due to the high content of cysteine (7%) and proline (11%) residues, or the existence of post-translation modifications. The first possibility is supported by previous observations indicating that the recombinant polypeptide 6xHis-cpRL3 produced in *E. coli* migrated in SDS–PAGE as a 105 kDa band, despite a predicted molecular weight of 85·5 kDa (Ranucci *et al*. 1993).

Although the process of oocyst wall formation has been extensively studied and accurately described at the ultrastructural level (Chobotar & Scholtyseck, 1982), COWP is the first fully characterized oocyst wall component among cyst-forming coccidia. The cloning of the COWP gene and the production of specific antisera offered a unique opportunity to follow the synthesis of an oocyst wall protein during the sexual development of *C. parvum*. We demonstrated, by immunoelectron microscopy, that in developing macrogametes COWP is detected in 2 distinct types of cytoplasmic inclusions showing similar electrondensity. The presence of the gold staining either in a single large vesicle or in Type-I wall-forming bodies (Current & Reese, 1986), in early and late macrogametes respectively, suggests a functional relationship between the 2 classes of granules. This is, to our knowledge, the first evidence suggesting that Type-I wall-forming bodies derive

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from a larger vesicular precursor. The labelling pattern observed at the oocyst stage allows establishment of a direct relationship between *Cryptosporidium* Type-I wall-forming bodies and the inner layer of the oocyst wall. This evidence is in contrast with ultrastructural observations relating Type-I wall-forming bodies of other coccidia to the formation of the outer oocyst wall layer (Chobotar & Scholtyseck, 1982) and may be peculiar to the *Cryptosporidium* genus.

The relevance of COWP with respect to oocyst physical properties and survival in the environment is difficult to assess. It is reasonable that as an abundant and cysteine-rich protein COWP plays a structural role, possibly conferring rigidity to the oocyst wall through a net of intermolecular disulfide bridges. A distinctive feature of the C-terminal domain of COWP is the presence of several histidine-rich amino acid stretches. Although the function of these sequences is not understood, it is worth mentioning that some of the cysteine-rich chorion proteins of *Drosophila* (Spradling *et al*. 1987), that assemble and cross-link to form the egg-shell and the p48 protein of the egg-shell of *Schistosoma mansoni* (Chen, Rekosh & LoVerde, 1992) contain a series of histidine-rich sequences very similar to those found in COWP.

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