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Isolation and Expression of a Gene Which Encodes a Wall-Associated Proteinase of *Coccidioides immitis*

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A chymotrypsinlike serine proteinase of *Coccidioides immitis* with an estimated molecular size of 34 kDa has been shown by immunoelectron microscopy to be associated with the walls of the parasitic cells of this human respiratory pathogen. The proteinase has been suggested to play a role in spherule development. We report the isolation of a 1.2-kb cDNA from an expression library of *C. immitis* constructed in the λZAP II phage vector. The cDNA is suggested to encode the 34-kDa protein. We demonstrate identity between segments of the deduced amino acid sequence of the open reading frame of the 1.2-kb cDNA and three distinct sequences obtained from cyanogen bromide cleavage peptides of the purified proteinase. The occurrence of *N*-glycosyl linkage sites in the deduced sequence of 309 amino acids of the open reading frame (ORF) correlates with our identification of such linkage sites in the native glycosylated proteinase. A protein encoded by an 800-bp fragment of the 1.2-kb cDNA, which was produced by transformed *Escherichia coli* XL1-Blue, was recognized by the anti-34-kDa protein antibody in a Western blot (immunoblot). Northern (RNA) hybridization of total poly(A)-containing RNA of *C. immitis* with the labeled 1.2-kb cDNA clone revealed a single band of approximately 1.75 kb. Partial homology was demonstrated between the deduced amino acid sequence of the ORF (927 bp) and reported sequences of α-chymotrypsin and chymotrypsinogens. Expression of the proteinase gene was examined by Northern dot blot analysis of total RNA from different stages of parasitic cell development in *C. immitis*. Maximum levels of specific mRNA were detected during early endospore wall differentiation. The 34-kDa proteinase appears to be concentrated in walls of the parasitic cells at stages of active growth. We suggest that the enzyme may participate in wall plasticization and/or intussusception or in cell wall turnover.

Coccidioides immitis is a respiratory pathogen of humans which is characterized by a unique parasitic life cycle (11). Large multinucleate spherules of up to 60 μm in diameter develop in infected lung tissue from inhaled arthroconidia of the pathogen. The conidia, which are small enough to reach the alveoli of the host (5), are airborne products of the saprobic phase of *C. immitis*. The saprobe inhabits desert soils in southwestern regions of the United States, as well as certain areas of Mexico, Central America, and South America. Arthroconidia undergo conversion from cells with one to three nuclei to large multinucleate spherules within approximately 48 h, as demonstrated both in vitro and in vivo (11, 24, 34). Diametric growth of spherules from cylindrical arthroconidia (3 to 6 by 2 to 4 μm) involves an approximately 200-fold increase in the total surface area of each cell. This diametric growth phase is accompanied by segmentation of the coenocyte, which is initiated by simultaneous invagination of the plasmalemma and intussusception of new wall material at several sites on the inner circumference of the spherule envelope. Completion of this endogenous segmentation wall apparatus triggers morphogenetic events which result in the conversion of the compartmentalized cytoplasm into a multiplicity of uninucleate endospores. Each endospore is encompassed more or less simultaneously by a newly formed thin wall layer. A burst of wall synthetase activity is suggested to occur when this process of endospore differentiation is initiated. As the endospore wall is formed, it separates from the segmentation wall, which undergoes some breakdown while endospore differentiation proceeds within the still-intact spherule envelope. The latter finally ruptures as the endospores begin to enlarge. The newly released endospores (approximately 2 μm in diameter) function in dissemination of the pathogen in vivo and undergo diametric growth and segmentation to differentiate into a second generation of endosporulating spherules. The parasitic cycle is repeated (24).

The focus of this study is on a chymotrypsinlike serine proteinase. Our original approximation of the molecular size of this purified proteinase was 36 kDa (12, 39), but subsequent examinations by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) suggested that 34 kDa is a more accurate estimate. The enzyme has been shown to be localized in the walls of parasitic cells of *C. immitis* on the basis of results of immunoelectron-microscopic studies (5, 9, 41). The 34-kDa-specific immunolabel has been found in the spherule envelope, segmentation wall, and endospore wall. In an effort to better understand the role of this wall-associated proteinase in spherule development, we have made use of recombinant DNA technology. We report the isolation of a cDNA which encodes the 34-kDa proteinase and examine the results of Northern (RNA) blot analysis of expression of the proteinase gene during the parasitic life cycle of *C. immitis*.

MATERIALS AND METHODS

Isolation and purification of the proteinase. The chymotrypsinlike serine proteinase was initially concentrated by cold acetone extraction of a water-soluble conidial wall

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fraction (SCWF) of *C. immitis* (6); the acetone precipitate was resolubilized and subjected to immunoaffinity chromatography with specific, antiproteinase rabbit serum as previously described (12, 39); the affinity-bound fraction was further separated by SDS-PAGE conducted under reducing conditions; and the 34-kDa fraction was isolated and purified by electroelution (12).

CNBr digestion and amino acid sequencing. Attempts to sequence the purified proteinase failed, even when the initial acetone extraction of the SCWF was omitted (7). We concluded that the proteinase is N terminally blocked. CNBr digestion of the enzyme was used to obtain peptide fragments cleaved on the carboxyl side of methioninyl residues (31). Earlier analysis of the amino acid composition of the proteinase had shown that methionine residues were present (39). CNBr digestion was performed by the method of Yuen et al. (42). Three different methods (A to C) were used to isolate distinct CNBr cleavage products for the determination of their N-terminal amino acid sequences. In the first method (A), the digest was separated by SDS-PAGE (15% gel) and components were electrotransferred to an Immobilon membrane (Millipore Corp., Bedford, Mass.) as described by Moos et al. (23). The membrane was stained with Ponceau S (Sigma Chemical Co., St. Louis, Mo.), washed, and dried under a stream of nitrogen gas. The most prominent and well-separated bands were excised, and the bound CNBr digestion fragments were separately sequenced in an Applied Biosystems model 477A gas-phase sequencer by standard methods (18). Since all cleavage peptides isolated by this method showed identical sequences, additional isolation procedures were used. In the second method (B), the CNBr digestion mixture was subjected to sequence analysis in the presence of *o*-phthalaldehyde (OPA; Sigma), which blocks Edman degradation and sequence initiation by reacting with all residues, except for proline (4). The unfractionated CNBr digestion products were initially examined in the amino acid sequencer, which revealed mixed sequence signals but showed proline residues at the fourth cleavage step of Edman degradation. A second sequence analysis of the same digestion mixture was performed by automated addition of the OPA reagent (20 mg of OPA plus 50 μl of β-mercaptoethanol in 10 ml of acetonitrile) to the sample in the sequencer at the end of the third cycle to block further Edman degradation of N-terminal amino acids, except for proline. In the third method (C), the digestion mixture was fractionated by reverse-phase high-pressure liquid chromatography (RP-HPLC) with a Hewlett-Packard model HP 1090 chromatograph equipped with a Beckman Ultrasphere-ODS C_{18} column (10 mm by 25 cm; Beckman Instruments, Palo Alto, Calif.). The lyophilized sample was solubilized in 0.1% (vol/vol) trifluoroacetic acid (Fisher Scientific Co., Fair Lawn, N.J.) in filtered distilled water. Approximately 100-μl aliquots of the sample were injected into the column and eluted with a linear solvent gradient of 0 to 60% (vol/vol) acetonitrile in 0.1% trifluoroacetic acid over a period of 30 min. The flow rate was 1.0 ml/min, and fractions were monitored at 214 and 280 nm simultaneously with the diode array detector of the HP 1090 chromatograph. Major hydrophobic fractions of the cleavage peptide mixture were collected and pooled from several separations, partially dried in a Heto model CT-60-e speed-vacuum concentrator (Heto Lab Equipment, Birkerod, Denmark), and subjected to amino acid sequence analysis.

Carbohydrate composition of the proteinase. The monosaccharide content of the purified proteinase was examined by gas chromatography (GC)-mass spectroscopy (MS) with authenticated, spectroscopic-grade sugar standards as previously described (8). Two methods of deglycosylation of the isolated 34-kDa fraction were used to examine the carbohydrate composition of the proteinase: trifluoromethanesulfonic acid (TFMS) treatment as reported by Edge et al. (15) and sodium periodate treatment as previously described (7). The method of TFMS treatment which was used cleaves *O*-glycosyl linkages, while *N*-glycosyl linkages remain largely intact (15). The TFMS-treated sample was examined by GC-MS for the presence of residual sugar. The TFMS-treated and untreated proteinase samples were also examined by SDS-PAGE under reducing conditions to determine differences in molecular size. Gel bands were detected by the silver staining technique (22). Deglycosylation of the purified proteinase was also performed by incubation of the sample with 0.2 M $NaIO_4$ (7). The periodate-treated sample was subsequently examined by GC-MS and SDS-PAGE as described above.

Construction of a cDNA expression library from the parasitic phase of *C. immitis*. The cDNA expression library was prepared from poly(A)$^+$ RNA isolated from spherules of *C. immitis* (strains 634 and 735). The spherules were grown in liquid Converse medium at 39°C for 72 h as previously described (10). Isolation of poly(A)$^+$ RNA and preparation of double-stranded cDNA were performed as described in an earlier report (19). Internal restriction sites in the cDNA were protected by methylation, and the double-stranded cDNA was phenol-chloroform extracted and precipitated with ethanol. An *Eco*RI adapter, with the sequence d(5′-AATTCGGCACGAG-3′), was added by ligation, and the cDNA was digested with *Xho*I. A Sepharose CL-4B column (15 by 3 mm; Pharmacia Fine Chemicals, Piscataway, N.J.) was used to isolate cDNA fragments larger than 500 bp. The isolated cDNA was ligated into the λZAP II phage vector (Stratagene, La Jolla, Calif.). The cDNA was packaged with Gigapack (Stratagene), and *Escherichia coli* PLK-F (Stratagene) was used as the host for the recombinant phage. Approximately 10^6 recombinants were obtained.

Antibody screening of the cDNA expression library. Rabbit antiserum specific for the 34-kDa protein, as determined by immunoblot analysis (41), was used to screen the cDNA expression library. The rabbit antiserum was diluted 1:100 in Tris-buffered saline (20 mM Tris-HCl [pH 8.0] plus 150 mM NaCl) containing 5% skim milk (Carnation Co., Los Angeles, Calif.). The antiserum was preabsorbed with an *E. coli* lysate (5 Prime-3 Prime Inc., West Chester, Pa.) at a final concentration of 0.35 mg/ml of diluted serum. Approximately 1×10^4 to 5×10^4 phage were plated on LB agar (Difco Laboratories, Detroit, Mich.) in 150-mm-diameter petri plates which had previously been inoculated with *E. coli* XL1-Blue (Stratagene) and incubated at 37°C. The plaque-forming cultures were subsequently incubated at 42°C. The fusion proteins were bound to a nitrocellulose membrane (Schleicher & Schuell, Keene, N.H.) and screened with the proteinase-specific antiserum. Adsorbed rabbit immunoglobulin was visualized with goat anti-rabbit immunoglobulin G conjugated with peroxidase as previously described (19). Two positive clones were isolated, replated, and rescreened with the antiproteinase antibody until 100% positive plaques were obtained.

DNA sequencing. The two recombinant λZAP II phage containing 600- and 800-bp cDNA inserts were isolated and treated separately by the in vivo excision protocol of Short et al. (30) to subclone them into the pBluescript SK (+/−) plasmid (Stratagene). The nucleotide sequences of the

TABLE 1. Amino acid sequences determined for isolated CNBr cleavage fractions of the 34-kDa proteinase

Method used for the isolation of CNBr fractions[a]	Amino acid sequence[b]
A (Membrane immobilization and excision)	Met-Arg-Ser-Ser-Leu-Ile-Thr-Val-Tyr-Gly-Ile-Thr-Lys-Pro-Gly-Thr-Asp-Lys-Gln-Tyr-Ile-Ser-Val-Ile-Ser-Pro-Thr-Tyr-Asn-Leu-Ile
B (OPA blockage)	Met-(**Ser-Ala-Ser**)-Pro-Leu-Ala-Ala-Thr-Thr-Ile-Thr-Asn-Val-Gln-Thr-Arg-Ser-Met-Thr-Ile-Phe-Leu-Tyr-Tyr-Met
C (RP-HPLC)	Met-Lys-**Val**-Asp-Thr-**Leu-Leu**-Thr-Gly-**Val-Val-Val**-Glu-Glu-Lys

[a] Details of the isolation methods are given in Materials and Methods.

[b] The underlined amino acid residues were used to design the synthetic oligonucleotide probe (35-mer) used in the RACE protocol (see Materials and Methods). Sequences of amino acid residues in boldface type within parentheses were deduced from mixed sequence signals of unfractionated CNBr cleavage products prior to the addition of OPA to the reaction mixture. A high percentage of hydrophobic amino acids (43%; residues in boldface type but not within parentheses) contributed to the affinity of this CNBr fragment for the C_{18} column.

cDNA inserts were determined by the dideoxy chain termination method as previously reported (19).

RACE. We were unable to obtain a full-length cDNA copy of the mRNA transcript of the proteinase gene by antibody screening of the expression library. The protocol of rapid amplification of cDNA ends (RACE [16]) made use of the polymerase chain reaction (PCR) to amplify a copy of the region between the sequenced part of the transcript and its 5′ end. A gene-specific primer (18-mer) was designed on the basis of part of the nucleotide sequence determined for the 800-bp cDNA clone (see Fig. 4), which was isolated as described above. The probe was synthesized in an Applied Biosystems model 381A oligonucleotide synthesizer. The sequence of the gene-specific primer was as follows: d(5′-TGTGGCAGATTCAGGTAG-3′). The λZAP II (M13) reverse primer (Stratagene) was used as the upstream oligonucleotide probe for hybridization with total cDNA from the expression library. The conditions used for PCR were the same as those previously reported (19). The PCR products were electrophoresed in 1.0% agarose prepared in 0.04 M Tris-acetate buffer (pH 8.0) containing 0.001 M EDTA. The separated products were transferred to nitrocellulose filters by the method of Southern (33) and hybridized to a synthesized 3′-end-labeled oligonucleotide probe. The latter was designed on the basis of the amino acid sequence of a CNBr cleavage fragment of the purified proteinase (Table 1) and was used to identify the missing terminal cDNA fragment of the proteinase gene in the PCR products. The synthetic oligonucleotide probe contained 35 bp. Inosine was used to minimize degeneracy and maximize base-pairing promiscuity (28). The nucleotide sequence of the 35-mer was as follows: d(5′-TATTGTTTATCIGTICCIGGTTTIGTIATIC CATA-3′). Prior to Southern hybridization, the oligonucleotide probe was labeled with biotin-21-dUTP by the 3′-end-labeling method as described by the manufacturer (Clontech Laboratories Inc., Palo Alto, Calif.). The nitrocellulose filters were washed, blocked, and incubated with streptavidin-alkaline phosphatase conjugate, and the bound product was visualized by exposure to substrate as previously reported (19). The selected PCR product (540 bp) was purified by agarose gel electrophoresis and digested with *Eco*RI. A 420-bp cDNA fragment was subcloned into the pBluescript II KS (+/−) plasmid (Stratagene) as previously reported (19) and sequenced as described above.

Western blot (immunoblot) analysis of the fusion protein. The isolated 800-bp cDNA phage insert, which had been subcloned into the pBluescript SK (+/−) plasmid, was expressed in *E. coli* XL1-Blue. The bacteria were grown in LB-ampicillin liquid medium plus 1% glucose at 37°C until the optical density at 600 nm reached 0.5 (1). Isopropyl-β-D-thiogalactopyranoside (5 Prime-3 Prime) was added to the culture medium at a final concentration of 10 mM, and the cells were incubated for an additional 2 h at 37°C. The cells were pelleted, resuspended in SDS-PAGE sample buffer (38), boiled for 2 min, and recentrifuged. The supernatant was separated on a 12% polyacrylamide gel, and the polypeptide components were electrotransferred to nitrocellulose as reported previously (41). The nitrocellulose-bound components were reacted with the rabbit anti-34-kDa protein serum preabsorbed with an *E. coli* lysate as described above, and the bound immunoglobulin was visualized by reaction with goat anti-rabbit immunoglobulin G conjugated with peroxidase (19). As a negative control, *E. coli* containing the pBluescript SK (+/−) vector without a cDNA insert was examined by Western blot analysis with the anti-34-kDa protein serum under the same conditions. The SCWF was used in the Western blot analysis as a positive control.

Northern hybridization. Poly(A)-containing RNAs were isolated (29) from 24-, 48-, 96-, 120-, and 168-h spherule cultures of *C. immitis* grown in Converse medium as described above. Each culture was inoculated with 2×10^7 arthroconidia in 50 ml of medium. The cells of the parasitic-phase cultures remained well synchronized through at least the end of the first generation (approximately 96 h) of arthroconidium-spherule-endospore transformation (11). Dot hybridization analysis of total RNA preparations from each developmental stage was performed by the method of White and Bancroft (37). The nucleotide probe used was the 1.2-kb cDNA produced by ligation of the 800- and 420-bp cDNA fragments described above. The overlapping fragments were ligated at the *Acc*I restriction site (see Fig. 5B). The probe was labeled with biotin-21-dUTP by the mixed-primer labeling method as described previously (19). Dot hybridization was performed on Biotrans nylon membranes (ICN Biomedicals Inc., Costa Mesa, Calif.) with a Bio-Dot microfiltration apparatus (Bio-Rad Laboratories, Richmond, Calif.). Prehybridization of the membrane was performed as previously described (19). For analysis, 3 μg of total RNA from cells at each sampling time was solubilized in 100 μl of reaction buffer (37) and hybridized directly with 200 ng of the cDNA probe per ml or, after dilution of the total RNA (1:2 to 1:8), with reaction buffer as described previously (37). The negative control was a total RNA preparation derived from Alaska pea (a gift from S. Roux, Department of Botany, University of Texas).

Northern hybridization was also performed with total poly(A)-containing RNAs from 48-h spherules which had been electrophoresed in a 1.0% formaldehyde-agarose gel (27) and processed as reported earlier (19). The hybridization probe was the same biotin-21-dUTP-labeled 1.2-kb cDNA as that described above. The same negative control RNA preparation as that described above was also used.

Immunofluorescence and lectin affinity microscopy. Spher-

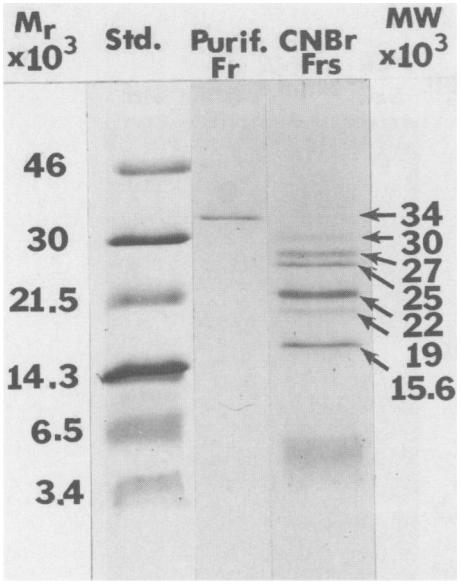

FIG. 1. SDS-PAGE separation of the purified 34-kDa proteinase (Purif. Fr) and cleavage products of a CNBr-treated sample (CNBr Frs). The molecular sizes of components of the standard mixture (Std.) and the samples are indicated.

ules grown in cultures for various periods as outlined above were chemically fixed, embedded in low-viscosity resin which had been cured at 37°C, and thick sectioned (approximately 1 μm) in preparation for fluorescence microscopy as previously described (10). Thick sections were pretreated as reported previously (6) and incubated with the rabbit anti-34-kDa protein serum (41) and then with goat anti-rabbit immunoglobulin G conjugated with fluorescein isothiocyanate (FITC; Miles Laboratories, Elkhart, Ind.). Controls included sections reacted with secondary antibody-FITC alone or preimmune primary antibody followed by secondary antibody-FITC. Thick sections of spherules in various stages of development were reacted separately with wheat germ agglutinin (WGA) conjugated with FITC (Sigma). Thick sections were pretreated as reported previously (6) and reacted with WGA-FITC (50 μg/ml) in phosphate-buffered saline (0.1 M; pH 7.6) for 1 h at room temperature (21). The sections were washed, mounted in glycerin, and examined by fluorescence microscopy as described previously (11). Sections reacted with FITC alone served as controls.

Nucleotide sequence accession numbers. The GenBank and EMBL nucleotide sequence accession numbers for the cDNA reported in this paper are M81863 and X63114, respectively.

RESULTS

Amino acid sequences of CNBr digests. SDS-PAGE separation of the CNBr cleavage products is shown in Fig. 1. Fractions with molecular sizes of 15.6 to 30 kDa were well defined. Fractions with molecular sizes of less than 6.5 kDa were not separated in the 15% gel. The faintly stained, 34-kDa band is suggested to represent residual, intact proteinase which was not digested during incubation with CNBr. After electrotransfer to the Immobilon membrane, selected Ponceau S-stained bands were isolated and subjected to sequence analysis. The amino acid sequences of the 15.6-, 22-, and 27-kDa polypeptides were identical (Table 1), suggesting the presence of a common N-terminal methionine (CNBr cleavage site) for each isolated fraction. As expected, the 34-kDa fraction isolated from the reaction mixture by electrotransfer to the Immobilon membrane was N-terminally blocked. The amino acid sequences of the other, less abundant CNBr cleavage products shown in Fig. 1 were not determined. The amino acid sequences of the CNBr cleavage products isolated by the OPA reaction during sequence analysis and by RP-HPLC are also reported in Table 1. Distinct sequences were obtained, indicating that the three isolation methods used were effective for obtaining different cleavage peptides of the native proteinase.

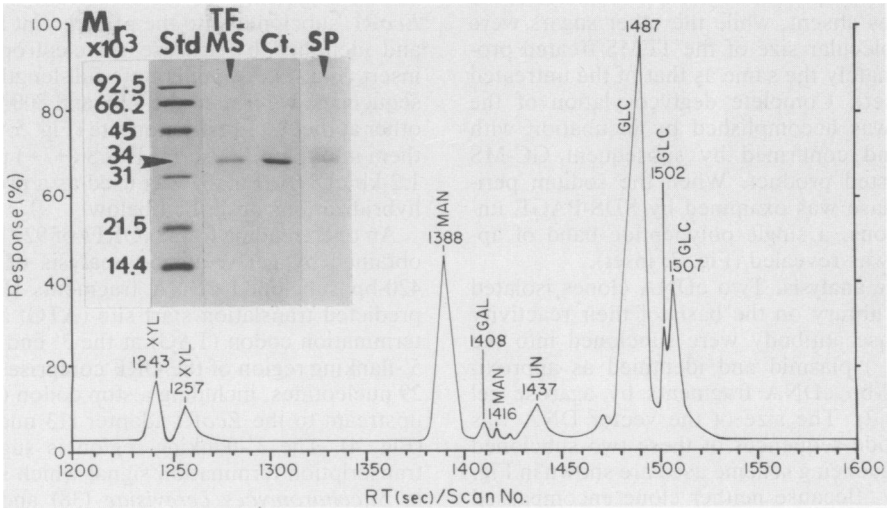

FIG. 2. GC of trimethylsilylated methylglycosides of monosaccharides present in the purified 34-kDa proteinase. The inset shows SDS-PAGE separation of the 34-kDa fraction (control [Ct.]) after TFMS or sodium periodate (SP) treatment. The monosaccharide components of the carbohydrate fraction were as follows: XYL, xylose; MAN, mannose; GAL, galactose; GLC, glucose. An unidentified sugar (UN) was labeled at a retention time (RT) of 1,437 s. Std, standard.

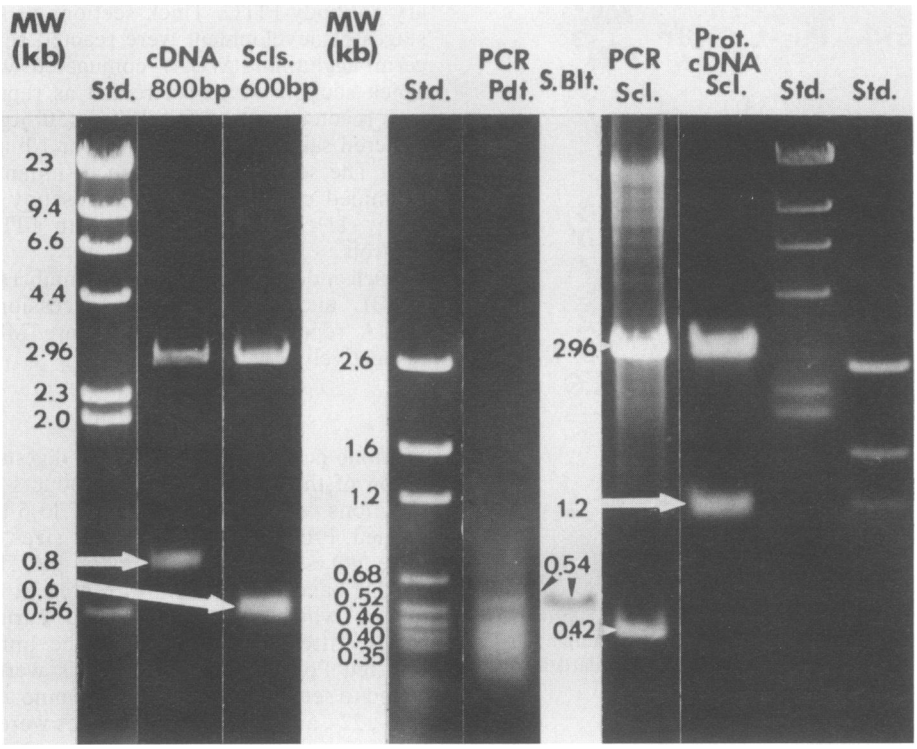

FIG. 3. (Left panel) Agarose gel electrophoresis separation of subcloned (Scls.) 800- and 600-bp cDNA fragments and the standard mixture (Std.). (Center panel) Separation of the PCR products (Pdt.) which were used in the Southern blot (S.Blt.) with the biotin-21-dUTP-labeled 35-mer to identify and isolate a 540-bp cDNA fragment. The 540-bp cDNA was digested with *Eco*RI and identified as a 420-bp cDNA (in the right panel) after it was subcloned into the pBluescript II KS (+/−) plasmid (2.96 kb). (Right panel) Ligation and identification of the 420- and 800-bp cDNAs as a 1.2-kb cDNA, which represented the subcloned 34-kDa proteinase gene (Prot. cDNA Scl.).

Analysis of the carbohydrate composition of the proteinase. The chromatogram of neutral sugar components of the purified proteinase in Fig. 2 reveals the presence of xylose, mannose, galactose, and glucose in a ratio of 11.6:22.1:1.4:64.9, respectively. A minor, unidentified sugar (UN) was also detected. After TFMS treatment and examination of the sugar composition of the product by GC-MS analysis, we found that xylose was absent, while the other sugars were still present. The molecular size of the TFMS-treated proteinase was approximately the same as that of the untreated fraction (Fig. 2, inset). Complete deglycosylation of the purified proteinase was accomplished by incubation with sodium periodate and confirmed by subsequent GC-MS analysis of the digested product. When the sodium periodate-treated proteinase was examined by SDS-PAGE under reducing conditions, a single polypeptide band of approximately 34 kDa was revealed (Fig. 2, inset).

Nucleotide sequence analysis. Two cDNA clones isolated from the expression library on the basis of their reactivity with the antiproteinase antibody were subcloned into the pBluescript SK (+/−) plasmid and identified as approximately 800- and 600-bp cDNA fragments by agarose gel electrophoresis (Fig. 3). The size of the vector DNA was 2.96 kb. The nucleotide sequences of these two subcloned fragments and the sequencing scheme used are shown in Fig. 4 and 5, respectively. Because neither clone encompassed the entire gene, the RACE protocol was used to generate the missing cDNA fragment. The result of agarose gel separation of the PCR product is shown in Fig. 3. A 35-mer oligonucleotide probe designed on the basis of the amino acid sequence of the membrane-immobilized CNBr cleavage fragment reported in Table 1 (method A) was used to identify the proteinase gene-specific PCR product by Southern blot analysis. Southern hybridization of the 3′-end-labeled oligonucleotide probe with the electrophoresed PCR product revealed a single band of approximately 540 bp (Fig. 3). This cDNA fragment was isolated from the gel, digested with *Eco*RI, subcloned into the pBluescript II KS (+/−) plasmid, and identified by agarose gel electrophoresis as a 420-bp insert (Fig. 3). To generate a full-length clone of the partial sequences, we ligated the 420- and 800-bp fragments to each other at the *Acc*I restriction site (Fig. 5A to C) and subcloned them into the pBluescript II KS (+/−) plasmid. The isolated 1.2-kb cDNA (Fig. 3) was used as a probe for the Northern hybridizations described below.

An open reading frame (ORF) of 927 nucleotide bases was obtained by gel sequence analysis of the 600-, 800-, and 420-bp subcloned cDNA fragments (Fig. 4 and 5A). The predicted translation start site (ATG) at the 5′ end and the termination codon (TAG) at the 3′ end were identified. The 5′-flanking region of the ORF comprises a short sequence of 29 nucleotides, including a stop codon (Fig. 4), and is linked upstream to the *Eco*RI adapter (13 nucleotides) and vector (Fig. 4). The 3′-flanking region is suggested to contain a transcription termination signal which is comparable to that in *Saccharomyces cerevisiae* (38) and is identified as 5′-TAG...TAGT...TTT-3′.

A selected restriction enzyme map of the ORF is shown in Fig. 5C. As previously indicated, the *Acc*I restriction site is in the region of the overlap of the 800- and 420-bp cDNA

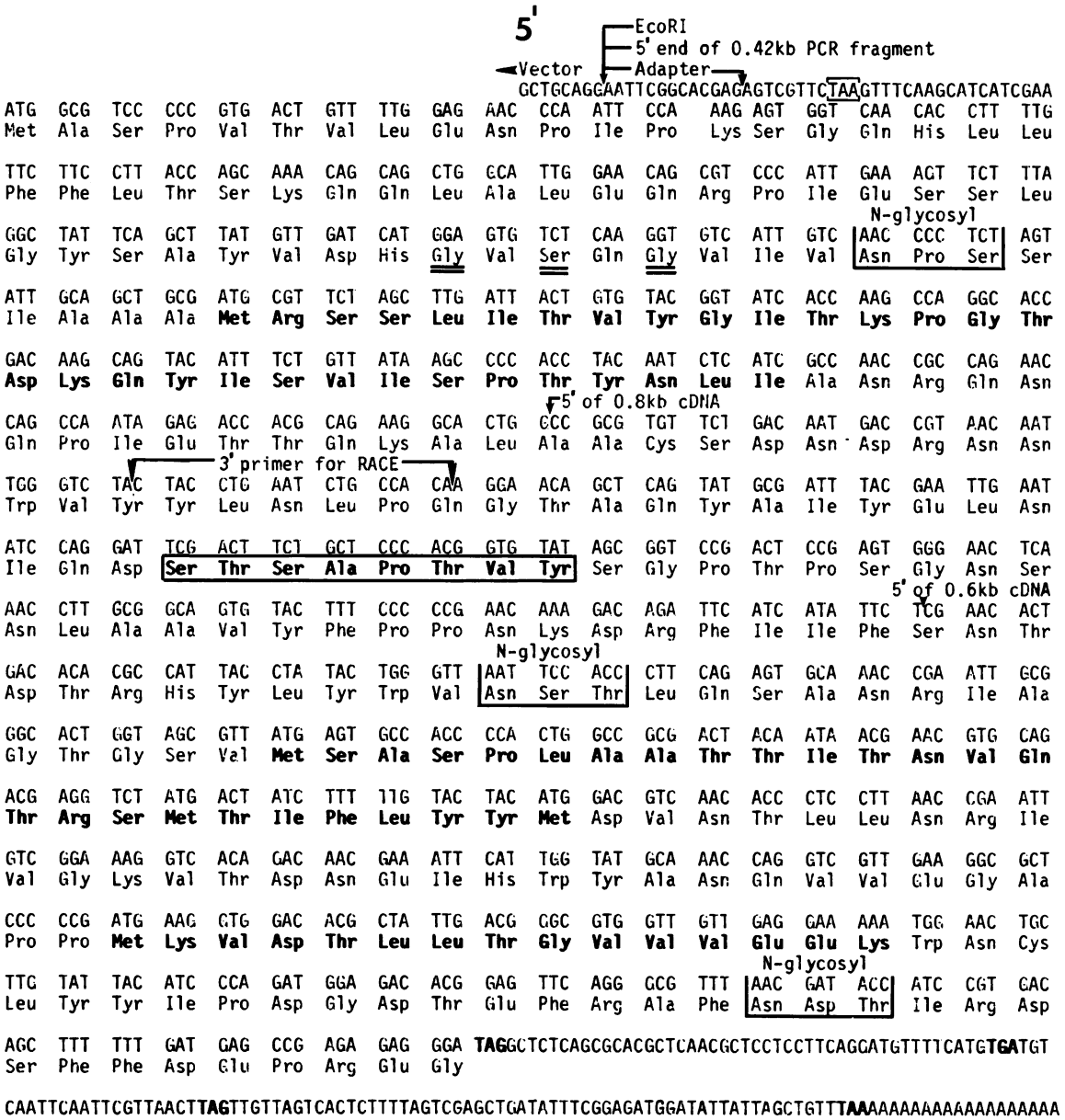

FIG. 4. Nucleotide sequence of the 1.2-kb cDNA and deduced amino acid sequence of the ORF (927 bp, encoding a 309-amino-acid protein). The 5'-untranslated region upstream from the ORF contains a stop codon (TAA in brackets). The 5' end of the 420-bp PCR-derived cDNA is shown upstream from this region. The ORF contains three potential sites of *N*-glycosyl linkages (brackets). Amino acid sequences which are identical to sequences determined for isolated CNBr cleavage peptides are in boldface type. Amino acid sequences showing partial homology to sequences of serine hydrolases (doubly underlined), α-chymotrypsin, and chymotrypsinogens (boldface type enclosed in a box) are also shown. Termination codons in the 3'-untranslated region are shown in boldface type.

fragments. *Dpn*I and *Nla*III restriction sites are located adjacent to the conserved sequence of serine hydrolases (Fig. 4) described below. *Hin*fI and *Sec*I restriction sites lie adjacent to an apparently conserved sequence of α-chymotrypsin also discussed below (Table 2). No restriction sites for many of the commonly used restriction enzymes, including *Eco*RI, *Pst*I, *Bam*HI, *Hin*dIII, *Sac*I, *Xho*I, *Kpn*I, and *Sal*I, are present within the ORF.

Analysis of the deduced amino acid sequence. The ORF of 927 bp can encode a 309-amino-acid polypeptide. The calculated molecular size of 34.3 kDa and the isoelectric point of 5.1, which are based on this deduced sequence, are close to the gel estimates of the molecular size (34 kDa) and isoelectric point (4.5) determined for the purified proteinase of *C. immitis* (39). Three regions of the deduced sequence indicated by boldface type in Fig. 4 demonstrate total identity with the amino acid sequences of the CNBr fragments reported in Table 1. Close similarities exist between the

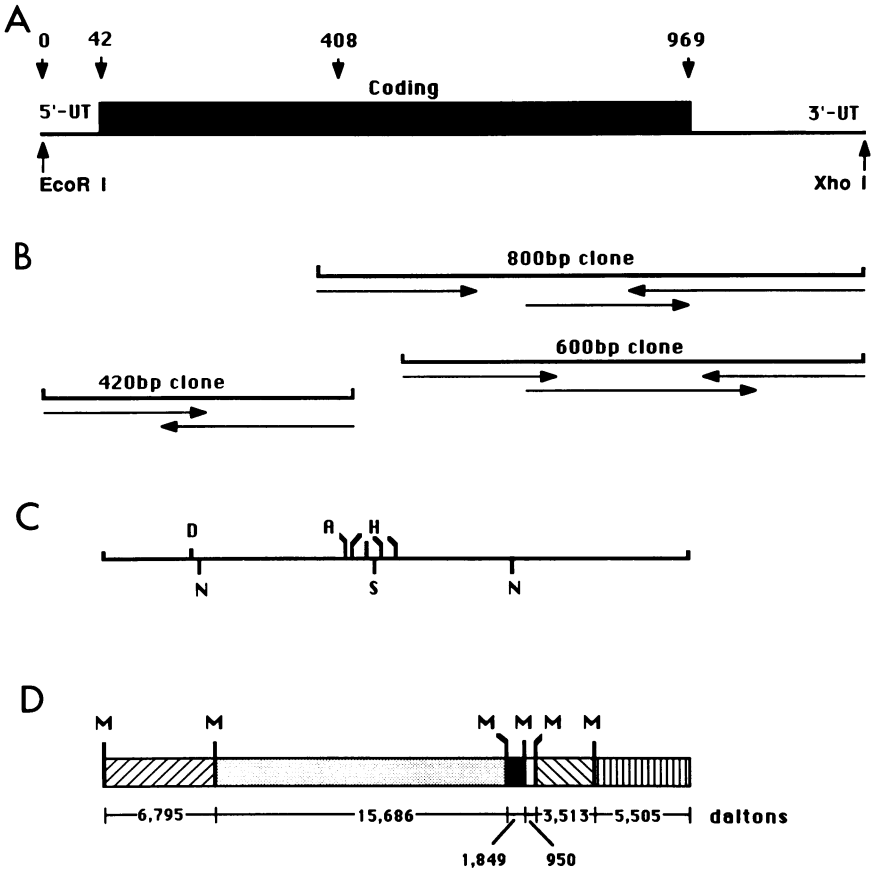

FIG. 5. (A) cDNA clone (1.2 kb) which includes the fragment encoding the 34-kDa proteinase. *Eco*RI and *Xho*I restriction sites and untranslated (UT) 5′ and 3′ regions are also shown. (B) Strategy for sequencing the 1.2-kb cDNA clone. The arrows indicate the direction and extent of sequence determination. (C) Restriction enzyme map of the 1.2-kb cDNA clone (A, *Acc*I; D, *Dpn*I; H, *Hin*fI; N, *Nla*III; S, *Sec*I). (D) Molecular sizes of potential CNBr cleavage peptides (showing Met [M] cleavage sites) calculated on the basis of the deduced amino acid sequence.

molecular sizes of the CNBr fragments determined by SDS-PAGE (Fig. 1) and the calculated sizes of the fragments between adjacent methionines (Fig. 5D). The SDS-PAGE estimates of molecular sizes appear to be slightly higher, a result which may have been due to glycosylation of the native proteinase. The Gly-Val-Ser-Gln-Gly sequence (residues 49 to 53; Fig. 4) shows partial homology with the conserved sequence (Gly-X-Ser-X-Gly) of all reported serine hydrolases (20). Comparison between the deduced amino acid sequence of the *C. immitis* proteinase and previously reported sequences of α-chymotrypsin and chymotrypsinogens revealed a conserved region of 75% homology (Table 2).

Western blot analysis of the fusion protein. The aim of the preliminary experiments in this part of the study was to show that the fusion protein expressed by pBluescript SK (+/−) plasmid-transformed *E. coli* XL1-Blue cells was recognized by an antiserum raised against the native *C. immitis* proteinase. The antibody showed a high affinity for a band of approximately 28 kDa in Western blots of the reduced and boiled lysate preparation of bacteria transformed with the 800-bp fragment-containing plasmid (Fig. 6). The adjacent

TABLE 2. Partial homology between the deduced amino acid sequence of the *C. immitis* proteinase and the reported sequences of α-chymotrypsin and chymotrypsinogens

Enzyme	Source	Sequence (residues 144 to 151 in Fig. 4)[a]	Reference or source
Chymotrypsinlike serine proteinase	*C. immitis*	**Ser-Thr-Ser**-Ala-**Pro**-Thr-**Val-Tyr**	Fig. 4
α-Chymotrypsin	Human	**Ser-Thr-Ser-Thr-Pro-Gly-Val-Tyr**	36
Chymotrypsinogen 2	Dog	**Ser-Thr-Ser-Thr-Pro-Gly-Val-Tyr**	26
Chymotrypsinogen A	Bovine	**Ser-Thr-Ser-Thr-Pro-Gly-Val-Tyr**	3
Chymotrypsinogen B	Bovine	**Ser-Thr-Ser-Thr-Pro-Ala-Val-Tyr**	32
	Rat	**Ser-Thr-Ser-Thr-Pro-Ala-Val-Tyr**	2

[a] Boldfacing indicates identical residues.

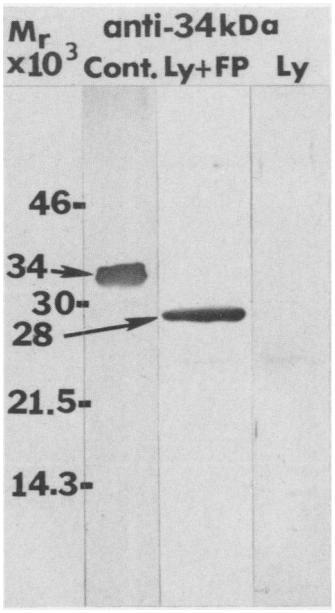

FIG. 6. Western blot of SDS-PAGE separation of the SCWF (Cont.), a lysate of *E. coli* transformed with the 800-bp cDNA-containing plasmid (Ly+FP), and a lysate of *E. coli* transformed with the control plasmid (Ly), each reacted with the rabbit anti-34-kDa protein serum–peroxidase conjugate.

control lane shows the results of an immunoblot of the SDS-PAGE-separated lysate of the same *E. coli* strain transformed with the pBluescript SK (+/−) vector lacking a cDNA insert. A faint band (approximately 25 kDa) was visible in both the test lysate (Ly+FP) and the control lysate (Ly) and appeared to be a minor *E. coli* product recognized by the rabbit antiserum. The second control lane (Cont.) shows the result of reaction of the antiproteinase antibody with the SCWF. A single 34-kDa band was recognized. A lysate of *E. coli* XL1-Blue transformed with the 1.2-kb cDNA which had been subcloned into the pBluescript SK (+/−) vector was also examined in a Western blot with the anti-34-kDa protein serum. No polypeptide was recognized by the antiserum.

Northern hybridization. Converse medium inoculated with arthroconidia of strains 735 and 634 produced spherule cultures which showed synchronous development at least through the endosporulation stage of the first generation of the parasitic life cycle (Fig. 7). The stages of development of strain 735 shown in Fig. 7A to D include round cell formation from arthroconidia at 12 h postinoculation of the culture (A), the development of diametrically expanded and segmented spherules at 24 h (B), early differentiation of endospores within spherules at 48 h (C), and endospore release at 96 h (D). When the endospores were permitted to develop into the second generation, endospore differentiation within these newly formed spherules occurred at approximately 168 h postinoculation. Cells isolated from cultures at five different time periods (24, 48, 96, 120, and 168 h) were used as sources of poly(A)-containing RNA. The latter was used in Northern dot blots to estimate the temporal expression of the *C. immitis* 34-kDa proteinase gene during development of the parasitic cells (Fig. 8). The maximum detected level of hybridization between the biotin-21-dUTP-labeled 1.2-kb cDNA probe and specific mRNA corresponded to the early stage of endospore differentiation in both the first and the second generations of the parasitic life cycle (Fig. 8). Lower but still detectable levels of hybridized probe and mRNA

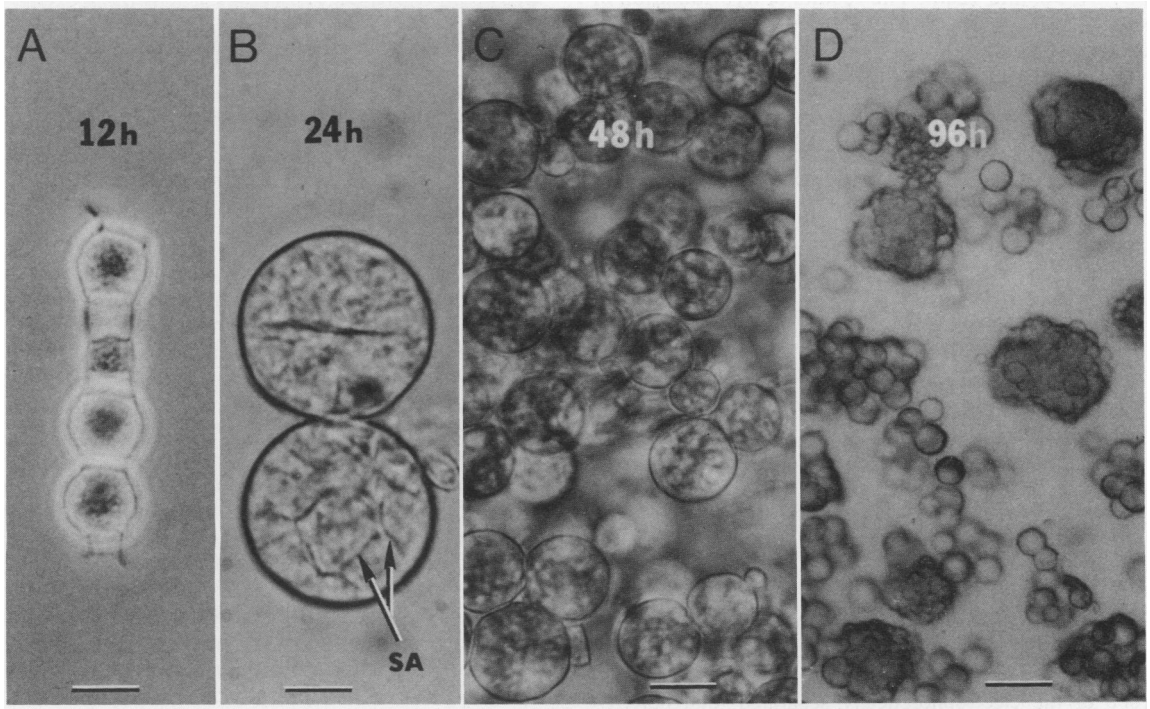

FIG. 7. Light micrographs of cell suspensions of *C. immitis* from liquid cultures inoculated with 30-day-old arthroconidia after 12 h (A), 24 h (B), 48 h (C), and 96 h (D) of incubation. SA, endogenous segmentation wall apparatus. Bars in A to D represent 3.0, 10, 40, and 10 μm, respectively.

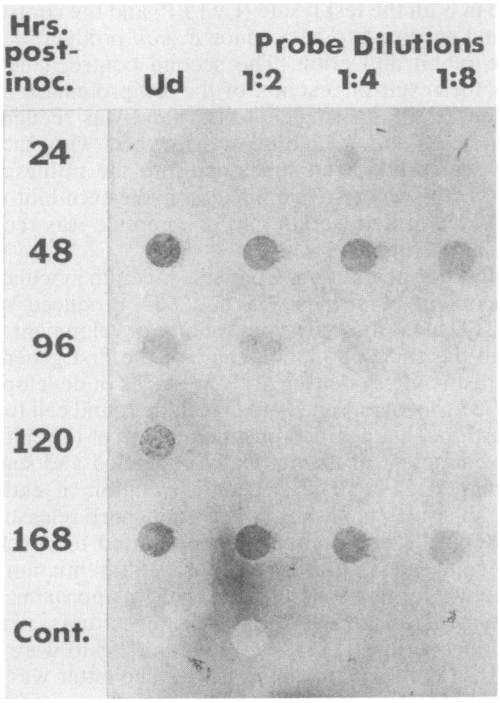

FIG. 8. Northern dot blot hybridization with the labeled 1.2-kb cDNA probe of poly(A)-containing RNA isolated from spherule cultures at 24, 48, 96, 120, and 168 h postinoculation (postinoc.) (compare with Fig. 7). The RNA was used undiluted (Ud) or diluted 1:2 to 1:8 with reaction buffer. The negative control (Cont.) was a total RNA preparation from Alaska pea.

were recorded for all developmental stages examined, represented by 24, 96, and 120 h. The results of hybridization of the labeled cDNA probe with control RNA were consistently negative (Fig. 8).

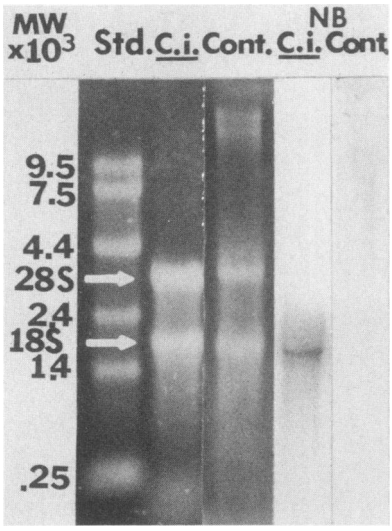

FIG. 9. Northern hybridization (NB) with the labeled 1.2-kb cDNA probe of the electrophoretically separated total poly(A)-containing RNA of *C. immitis* (C.i.) and total RNA of Alaska pea (Cont.). The molecular sizes of the standard mixture (Std.) are indicated.

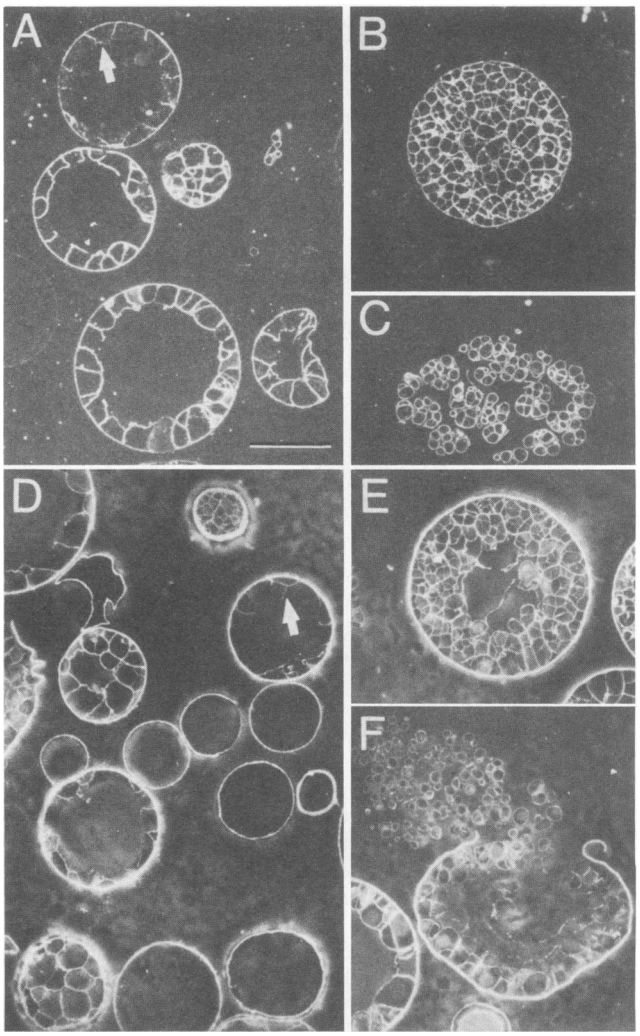

FIG. 10. Thick sections of parasitic cells of *C. immitis* reacted with either the rabbit anti-34-kDa protein antibody and then with goat anti-rabbit immunoglobulin G conjugated with FITC (A to C) or an WGA-FITC conjugate (D to F). Arrows in panels A and D indicate comparable early stages of segmentation wall formation. Bar, 10 μm (same magnification for all panels).

The results of Northern hybridization of the electrophoresed, total poly(A)-containing RNA from 48-h spherules with the labeled 1.2-kb cDNA are shown in Fig. 9. A single, 1.75-kb band was identified on the basis of comparison with components of the standard mixture. No hybridization between the cDNA probe and electrophoresed total RNA of the control preparation was detected.

Colocalization of proteinase and chitin. The expression of the proteinase gene correlated temporally with the differentiation of the segmentation wall apparatus and endospore initials. We had earlier demonstrated immunolocalization of the proteinase in walls of parasitic cells which were fixed in the apparent stages of active growth of the segmentation wall and early differentiation of endospores (5, 9, 41). In this study, we compared the distribution of the FITC-labeled anti-34-kDa protein antibody in spherules and endospores (Fig. 10A to C) with that of the FITC-labeled lectin WGA (Fig. 10D to F). The latter binds to newly deposited *N*-acetylglucosamine polymers as well as crystalized chitin in cell

walls and, therefore, identifies walls which are in stages of active growth as well as fungal walls which have thickened and ceased to grow. Thick sections of the same developmental stages were examined with the immunolabel and lectin by fluorescence microscopy. The earliest stages of segmentation wall formation in young spherules showed both antiproteinase antibody and WGA labels (arrows in Fig. 10A and D, respectively). The segmentation wall in later stages of development also reacted with both labels. However, the lectin showed affinity for the thickened spherule envelope (Fig. 10E), while little immunolabel was visible in this wall layer, which encompasses the mature, segmented spherule (Fig. 10B). The young endospore wall was clearly labeled with the anti-34-kDa protein antibody–FITC conjugate (Fig. 10C). Comparatively less but clearly visible WGA-FITC label was also present in the wall of the newly released endospores (Fig. 10F).

DISCUSSION

Evidence has been presented in this paper that we have isolated and cloned the gene which encodes the 34-kDa proteinase of *C. immitis*. The supporting data are derived from analysis of the nucleotide sequence of the cDNA, characteristics of the deduced protein encoded by the cDNA, and features of the native proteinase. We have demonstrated identity between parts of the deduced amino acid sequence and three sequenced CNBr cleavage peptides of the purified enzyme. The molecular size and isoelectric point of the protein calculated on the basis of the gene sequence are comparable to those determined for the isolated proteinase (39). However, since this is a secreted proteinase, we predict that cleavage of a signal peptide (35) occurs as the protein is released to the cell wall. No meaningful data are so far available on the size of the prepeptide on the basis of examination of the hydrophobic profile and secondary structural analyses of the deduced sequence (data not shown). Results of GC-MS analysis of the carbohydrate content of the proteinase before and after incubation with the selected deglycosylation reagent TFMS suggest that sugar residues are bound to the protein by *N*-glycosyl linkages. Three potential *N*-glycosyl linkage sites were identified in the deduced amino acid sequence of the gene. No change in the molecular size of the proteinase after deglycosylation with sodium periodate was noted in SDS-PAGE, compared with the results for the untreated enzyme. Although the percentage of carbohydrate per unit weight of the glycoprotein was not determined, we suggest that the sugar content of the enzyme is low.

The 800-bp cDNA fragment, which was subcloned into the pBluescript SK (+/−) plasmid and used to transform *E. coli* XL1-Blue, expressed a 28-kDa polypeptide that was recognized by the anti-34-kDa protein serum in Western blots. We were unable to obtain expression of the 1.2-kb cDNA clone in this same vector, possibly because the untranslated 5′-flanking region of 29 nucleotides upstream from the terminal methionine contains a stop codon. The nucleotide sequence of the 5′-untranslated region of the 1.2-kb cDNA in Fig. 4 was in frame with the adapter-plus-vector sequence. The failure to express the 1.2-kb clone in *E. coli* may also have been due to the absence of a suitable ribosome binding site. Northern hybridization between the biotin-21-dUTP-labeled 1.2-kb cDNA probe and the electrophoresed total poly(A)-containing RNA from 48-h spherules revealed a single 1.75-kb band. An mRNA of this size would encode a protein of 34 kDa if we assume the presence of a poly(A) tail of about 100 nucleotides and combined 5′- and 3′-untranslated regions of approximately 700 nucleotides. The length of a poly(A) segment in mammalian cells is consistently between 100 and 250 nucleotides but is shorter in fungal cells (13). The length of the poly(A) tail in *C. immitis* is unknown. The 5′-untranslated region of the mRNA can be variable in length in eukaryotic cells, but typically 10 to several hundred nucleotides occur between the methylated cap structure at the 5′ end of the transcript and the first AUG initiation codon (13). The 5′-untranslated region may include several stop codons. We have determined that the 3′ end of the mRNA contains an untranslated region of 133 nucleotides between the translation stop codon and the poly(A) addition site. Within the 3′-untranslated region, a transcription termination signal which has been reported to occur in *S. cerevisiae* was identified (38). However, a polyadenylation signal typical of higher eukaryotic cells (e.g., 5′-AATAAA-3′ [14]) was not recognized.

Our final line of evidence that we have cloned the 34-kDa proteinase gene is derived from a comparative analysis of the deduced amino acid sequence of *C. immitis* and sequences of other serine proteinases. Köller (20) has pointed out that all serine hydrolases have a conserved common sequence, Gly-X-Ser-X-Gly. This sequence is present near the N terminus of the ORF (residues 49 to 53). A conserved sequence of 8 amino acids (Ser-Thr-Ser-X′-Pro-X″-Val-Tyr) has also been recognized for α-chymotrypsin from humans, chymotrypsinogens from dog, bovine, and rat sources, and the *C. immitis* proteinase. One of the mismatched residues of *C. immitis* (residue 149; X″) aligns with amino acids which differ even between the chymotrypsin and chymotrypsinogens studied, suggesting a higher percent homology between the sequences than is indicated by direct alignment.

Application of the labeled 1.2-kb gene as a probe in Northern dot blots of poly(A)-containing RNA of the parasitic cells showed that the peak expression of the gene correlated with early endospore differentiation. Immunoelectron microscopy revealed that the anti-34-kDa protein antibody was concentrated in the thin wall layer of young endospores. The myriad of endospores which forms within each mature spherule would account for the burst of synthesis of the 34-kDa protein, which is subsequently incorporated into the developing endospore wall. Both immunoelectron microscopy (5, 9, 41) and immunofluorescence showed that the 34-kDa protein-specific immunolabel was localized in the envelope of endospores and young spherules which were still in their diametric growth phase. The immunolabel was also shown to concentrate in the segmentation wall from the earliest stage of development of the segmentation wall apparatus. These phases of parasitic cell development during which the antiproteinase antibody reacted with the wall are suggested to correspond to periods of intususception of chitin microfibils into newly synthesized wall layers, as supported by the results of WGA-FITC labeling studies in this report. The apparent high affinity of the 34-kDa protein for walls of actively growing cells of *C. immitis* suggests that the enzyme may participate in wall plasticization and/or intususception of new wall material during diametric growth of the parasitic cells, the formation of the segmentation wall apparatus, and the development of endospores. The active proteinase has been isolated from the walls of intact and viable endosporulating spherules by extraction with octyl-β-D-thioglucoside, a mild nonionic detergent (41). Hector and Pappagianis (17) have suggested that the segmentation wall contains a mannan-protein complex which is synthesized "prior to the fracturing of the cleavage planes

into endospore walls." This protein component may represent the immunolabeled 34-kDa fraction or the substrate for the proteinase. We showed that the isolated wall of endosporulating spherules, pretreated with phenylmethylsulfonyl fluoride to inhibit endogenous serine proteinase activity, was partially digested by the purified proteinase (41).

A wall-bound autolysin may be protected from proteolysis, in contrast to an autolysin released from growing cells, which is rapidly inactivated by proteases (14). Another possible function of the wall-bound 34-kDa proteinase is that it may be involved in cell wall turnover, a process of in situ excision from preexisting wall material of wall proteins, which are released at the cell surface as muropeptides (14). The latter may or may not be reutilized by the cell. Cell wall-bound mannoproteins of *S. cerevisiae* have been shown to be susceptible to turnover, but only shortly after incorporation into the insoluble wall (14, 25). The mechanism of in situ regulation of the activity of the wall-bound proteinase has been suggested to involve a 5-kDa protein which has been isolated from the wall of segmented spherules and shown to be a competitive inhibitor of the enzyme (40). The inhibitor and the 34-kDa proteinase react in a 1:1 stoichiometry and colocalize in the spherule envelope, segmentation wall apparatus, and endospore wall, as determined by the double-label technique of immunoelectron microscopy (5). Attempts to isolate and sequence the inhibitor gene and to examine its expression are underway. Ultimately, we hope to develop a transformation system for *C. immitis* which, when combined with the construction of 34-kDa proteinase mutations derived from gene disruptions, will permit a rigorous assessment of the role of the proteinase in the morphogenesis of this respiratory pathogen.

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