INFECTION AND IMMUNITY, May 1995, p. 1745–1753
0019-9567/95/$04.00+0
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Vol. 63, No. 5

Molecular Cloning of a Serine Proteinase Inhibitor from *Brugia malayi*

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Received 22 November 1994/Returned for modification 18 January 1995/Accepted 20 February 1995

The antigens produced by the infective-stage larvae of filarial parasites are potentially important targets for a protective immune response. A major impediment to studies on the biochemistry and molecular biology of antigens from infective larvae is a lack of parasite material. By employing a reverse transcription PCR-based strategy which exploited the presence of a conserved 22-nucleotide spliced leader sequence present at the 5′ end of a proportion of nematode transcripts, spliced leader-containing cDNAs were amplified from the late-vector-stage larvae of the filarial nematode *Brugia malayi*. A major 1.4-kb PCR product was cloned into pBluescript. One of the PCR cDNA clones (BmY8) contained a 1,287-bp insert that encoded the first member of the serine proteinase inhibitor (serpin) superfamily to be described from nematodes. Reverse transcription PCR analysis of RNA isolated from different developmental stages of the parasite showed that transcription of the *B. malayi* serpin (Bmserpin) begins between days 8 and 9 of larval development within the insect vector and continues through to the adult and microfilarial stages. In immunoblot analyses of *B. malayi* somatic extracts, the native protein was estimated to have a molecular weight of 44,000. In immunoblots using excretory-secretory products from infective- and fourth-stage larvae, a single band with an estimated molecular weight of 75,000 was detected. A quantitative analysis of somatic extracts demonstrated that infective-stage larvae contained 10- to 16-fold-more Bmserpin than adults or microfilariae. Bmserpin was immunogenic in gerbils and was recognized strongly by sera from immunized animals. Bmserpin, which has the potential for modifying host defense responses, may play an important role in parasite survival during the early phase of vertebrate-stage development.

The causative agents of lymphatic filariasis, *Wuchereria bancrofti*, *Brugia malayi*, and *Brugia timori*, are estimated to infect over 75 million people in Africa, India, Asia, the South Pacific, and the Caribbean basin. In areas of endemicity, infection with these parasitic nematodes typically results in a chronic infection that can last for decades. For a significant proportion of individuals, infection results in chronic lymphatic obstruction. Although safe and effective drugs that kill certain stages of the parasite are available, the efficacy of chemotherapy programs in many areas is compromised by economic and logistical factors. Evidence for natural immunity in humans (for a review, see reference 28) and for acquired immunity in animal models of human filariasis (42) suggests that a vaccine could be an important component of programs designed to prevent and control infection. One of the possible targets for a protective immune response is the antigens synthesized by the infective, third-stage larval (L3) parasites.

The third-stage larvae of filarial parasites undergo developmentally regulated gene expression during the late vector stage and early vertebrate stage of parasite maturation (2, 19, 41). The evidence suggests that a subset of these developmentally regulated components may be important in a protective immune response (2, 19). A major limitation to the study of the biochemistry and immunobiology of these L3-associated proteins is the restrictions placed on the availability of larval material by the stringent requirement for residence in an insect vector for proper parasite development. In recent years, a standard way to circumvent the problems associated with limited material is to apply the methods of molecular genetics and eventually clone the genes of interest. However, in the case of developing L3s, it has been extremely difficult to isolate the number of larvae required to apply conventional approaches for the cloning of genes that are expressed in a developmentally regulated fashion. Thus, alternative approaches need to be considered.

As part of our ongoing studies to identify and characterize targets of protective immune responses against filarial parasites, procedures have been developed to study genes that are expressed during the late vector stage and the early vertebrate stage of parasite development. Here we report on the use of a reverse transcription polymerase chain reaction (RT-PCR) procedure that exploits the presence of a conserved 22-nucleotide spliced leader (SL) sequence that is *trans*-spliced to the 5′ end of many filarial transcripts (31). SL-containing cDNAs were amplified from small numbers of late-vector-stage *B. malayi* L3s and cloned. One of the dominant genes transcribed at this stage of parasite development was found to encode a serine proteinase inhibitor. Studies on the transcription, protein expression, and immunogenicity of this serine proteinase inhibitor suggest that this protein, which is preferentially expressed during the late vector and early vertebrate stages of development and is found in both somatic extracts and excretory-secretory (ES) products from the parasite, may be an important molecular target for a protective immune response.

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MATERIALS AND METHODS

Parasites. *B. malayi*-infected gerbils (*Meriones unguiculatus*) and live adult male *Dirofilaria immitis* were obtained from the Filariasis Repository Research Service through the auspices of a National Institute of Allergy and Infectious Diseases supply contract (AI 02642), U.S.-Japan Cooperative Medical Science Program. *B. malayi* L2s and L3s were produced by feeding *Aedes aegypti* mosquitoes on infected gerbils with 5,000 to 10,000 microfilariae per ml of blood (20). Microfilariae, L4s (day 15 postinfection), immature adults (day 35 postinfection), and adult male and female worms were obtained by lavaging peritoneal cavities of intraperitoneally infected gerbils. Postvector L3s is the designation given to *B. malayi* larvae that were harvested from the mouthparts of mosquitoes at day 11 of infection, washed, and placed in culture for 9 to 10 h at 37°C.

Isolation of RNA and first-strand synthesis. The procedure for isolation of total RNA from the heads and thoraces of 100 *B. malayi*-infected and 100 control mosquitoes followed a modification of the procedure described by Chomczynski and Sacchi (8). RNAs from *B. malayi* microfilariae, males, and females and from *D. immitis* males were isolated by the procedure of Sambrook et al. (37).

Total RNA was subjected to first-strand cDNA synthesis with Moloney murine leukemia virus reverse transcriptase (37). The cDNA was treated with phenol-chloroform, precipitated with sodium acetate-glycogen (3.0 M sodium acetate [pH 7.0] containing 100 μg of glycogen per ml) and isopropanol (24, 51).

SL RT-PCR. One microliter of the single-stranded cDNA (sscDNA) from infected mosquitoes was mixed with 79.5 μl of water, 10 μl of 10× PCR buffer (100 mM Tris-HCl [pH 8.3], 500 mM KCl, 15 mM $MgCl_2$, 0.01% [wt/vol] gelatin), 4 μl of 20 mM deoxynucleoside triphosphate mixture, 5 μl (each) of a 20 μM solution of primers, and 0.5 μl of 5-U/μl AmpliTaq (Perkin-Elmer Corp., Norwalk, Conn.). Thirty-five cycles of PCR were carried out, with one cycle consisting of 1 min at 94°C, 2 min at 50°C, and 5 min at 72°C, with a 5-min extension period at the end. The primer that extended in the reverse direction contained the SL sequence with a *Not*I restriction site at the 5′ end (NSL; 5′-CATGCGGCCGCGGTTTAATTACCCAAGTTTGAG-3′). The forward primer contained 20 T's and a 5′ extension with one *Xho*I and one *Eco*RI restriction site (XET; 3′-TTTTTTTTTTTTTTTTTTTTCTTAAGAGCTCGC GCG-5′).

Cloning PCR cDNAs. The RT-PCR products were separated on a 2% agarose gel. The prominent 1.4-kb band was isolated with a NA45 DEAE-cellulose membrane, and the PCR cDNA products were cut with *Not*I and *Xho*I for directional ligation into the plasmid pBluescript (Stratagene, La Jolla, Calif.). Cloned, plasmid-containing bacteria were arranged in a grid pattern on a Hybond-N membrane (Amersham, Arlington Heights, Ill.) and grouped on the basis of cross hybridization of their insert cDNAs. The enhanced chemiluminescence (Amersham) procedure was used as the detection system.

Sequencing BmY8. pBluescript carrying BmY8 was prepared by using the plasmid Maxi preparation Qiagen kit (Qiagen, Inc., Chatsworth, Calif.). The PCR cDNA insert was sequenced by the dideoxy method (38) using Sequenase version 2.0 (United States Biochemical, Cleveland, Ohio). Both strands of the BmY8 insert were completely sequenced.

Southern blot. About 5 μg of *B. malayi*, *Brugia pahangi*, *Onchocerca volvulus*, *D. immitis*, *Panagrellus redivivus*, *A. aegypti*, and human DNAs were digested with 10 U of *Eco*RI. The digested DNAs were separated on a 0.8% agarose gel and transferred to a Hybond-N membrane. The enhanced chemiluminescence hybridization technique was carried out as outlined above with BmY8 insert cDNA as the probe. The final wash conditions were 0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–6 M urea at 42°C.

BmY8 genomic DNA. PCR was used to obtain a fragment of *B. malayi* genomic DNA that encoded the central portion of BmY8. The primers 8.5.1 and 8.3.1 were used in 35 cycles of amplification, with 1 cycle consisting of 1 min at 94°C, 2 min at 50°C, and 5 min at 72°C. The 1,130-bp PCR product was isolated and cloned into pBluescript for sequencing as outlined above.

BmY8 transcription. RNAs from *B. malayi*-infected mosquitoes at days 2, 4, 6, 8, 9, and 10 after infection, postvector L3s, L4s, immature adults, mature males, mature females, and microfilariae were isolated for RT-PCR analysis of BmY8 transcription. sscDNA was produced as outlined above. The second strand was synthesized with the SL primer and *Taq* DNA polymerase under the conditions of 1 min at 94°C, 3 min at 50°C, and 15 min at 72°C. The double-stranded SL cDNAs were used as templates in a PCR employing the SL primer paired with an internal BmY8 primer. The results of PCR of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase were used to gauge the quantity and quality of the cDNA templates. PCR was carried out with a hot start for 20, 30, or 40 cycles of amplification, with 1 cycle consisting of 1 min at 94°C, 1 min at 50°C, and 1 min at 72°C, with a 5-minute extension period at the end. The reaction products were separated on 2% agarose gel, stained with ethidium bromide, and transferred to Hybond-N membranes for Southern blot hybridization.

Subcloning and expression. The intact BmY8 cDNA insert was directionally cloned into the *Stu*I and *Xba*I sites of pIH902 (New England Biolabs, Beverly, Mass.), which produced a fusion protein with the maltose-binding protein (MBP). To produce a construct that would result in the expression of a truncated form of the fusion protein (tBmserpin-MBP), the BmY8 cDNA was excised from BmY8/pIH902 with *Sac*I and *Hin*dIII. A 3′ coding fragment of 129 bp was removed from BmY8 cDNA by *Pvu*II digestion, and the truncated BmY8 cDNA was directionally cloned into the *Sac*I and *Stu*I sites of pIH902.

The bacteria containing plasmid with the full-length or truncated BmY8 insert were grown at 37°C for 18 h. The cultures were then diluted 1/50 and incubated for an additional 6 h at 20°C. Isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.3 mM, and the cultures were incubated for 18 h at 20°C. After centrifugation, the bacterial pellets were dissolved in lysis buffer [10 mM Na_2HPO_4, 30 mM NaCl, 0.25% Tween 20, 10 mM β-mercaptoethanol, 10 mM EDTA, 10 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA); pH 7.0] and stored at −70°C.

Bmserpin synthetic peptides. Peptides, one of 23 amino acids (Bmserp23) and the other of 21 amino acids (Bmserp21) were synthesized with an ABI peptide synthesizer model 438 (Applied Biosystems, Foster City, Calif.).

Antibodies. Bmserp23 and Bmserp21 were coupled individually to bovine serum albumin (BSA; Sigma) by using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (Sigma) (15). Individual rabbits were immunized either with 600 μg of Bmserp23-BSA or 500 μg of Bmserp21-BSA in Freund's complete adjuvant. Subsequent immunizations were performed at 3-week intervals, using Freund's incomplete adjuvant mixed with 300 μg of Bmserp23-BSA or 250 μg of Bmserp21-BSA. The sera used in this study were taken 10 days after the fourth boosting dose of peptide-protein conjugate.

Gerbil sera were collected from infected animals within days after the microfilariae were first detected in the blood (early chronic sera, $n = 4$) or from animals that had harbored a patent infection for about 6 months (late chronic serum, $n = 5$). Naive gerbils ($n = 4$; Tumblebrook Farm, West Brookfield, Mass.) were infected subcutaneously with 85 L3s, and sera were obtained 35 days postinfection (L3-L4 exposed, $n = 4$). Immunized sera were obtained from gerbils exposed to 1,100 ^{60}Co-irradiated L3s. The irradiated larvae were introduced subcutaneously in doses of 100 to 200 parasites over a 3-month period. The immunized animals ($n = 5$) were challenged with a total of 85 viable L3s over a 7-day period, and sera were collected 50 days after challenge.

Parasite antigens. The larvae and adult *B. malayi* were washed to remove host tissues and proteins and homogenized on ice in phosphate-buffered saline (PBS). For the antigens used in the competitive enzyme-linked immunosorbent assay (ELISA), sodium dodecyl sulfate (SDS) was added to the parasite extraction buffer to a final concentration of 0.5%. The bicinchoninic acid assay (Pierce, Rockford, Ill.) was used to estimate the protein concentration of parasite extracts.

ES products were obtained from postvector L3 and L4 parasites. *B. malayi* L3s were harvested from infected mosquitoes at day 11 postinfection, washed, and injected into the peritoneal cavity of a gerbil. The larvae were lavaged from the gerbil after 6 h to obtain "vertebrate-stage" L3s. L4s were obtained by lavaging peritoneal cavities of intraperitoneally infected gerbils at 15 days postinfection. The larvae were placed in the wells of a 24-well plate (Costar, Cambridge, Mass.) containing 1.5 ml of Dulbecco modified Eagle medium without a phenol red indicator (GIBCO BRL Life Technologies, Grand Island, N.Y.), 100 U of penicillin per ml, 100 μg of streptomycin per ml, 0.25 μg of amphotericin B per ml, and 1% glucose. Approximately 150 L3s were placed in each well and cultured at 37°C with a 5% CO_2 atmosphere for 72 h. About 1 ml of medium was removed every 8 to 12 h and replaced with an equal amount of fresh medium. A mixture of proteinase inhibitors (1 mM EDTA, 1 mM EGTA, 2 mM phenylmethylsulfonyl fluoride [PMSF], 0.2 mM *N*α-*p*-tosyl-L-lysine chloromethyl ketone [TLCK], 1 mM tolylsulfonyl phenylalanyl chloromethyl ketone [TPCK]; Sigma, St. Louis, Mo.) was added to the harvested media. The ES samples were pooled, concentrated, and stored at −70°C.

Western blots. SDS-polyacrylamide gel electrophoresis (PAGE) (22) was performed on a 10% acrylamide gel. After separation, the proteins were electrotransferred onto a nitrocellulose membrane and the membranes were processed for immunostaining (47).

ELISA and competitive ELISA. The ELISAs for the detection of anti-Bmserp23 and Bmserp21 activities in gerbil and rabbit sera were carried out by the procedures outlined by Tamashiro et al. (48).

For the competitive ELISA, microtiter plates were sensitized with 50 ng of the Bmserp23 peptide per well. After the wells were washed with PBS containing 0.05% Tween 20, the wells were incubated with 2% normal goat serum in PBS containing 0.05% Tween 20 (blocking solution) at 37°C for 30 min. Anti-Bmserp23 was diluted 1:2,000 in blocking solution which contained known amounts of either Bmserp23 peptide or *B. malayi* extract. The mixtures were placed in the wells of a microtiter plate, and serial twofold dilutions were carried out with a diluent that contained anti-Bmserp23 at a 1:2,000 dilution. The concentrations of Bmserp23 peptide, *B. malayi* microfilaria, L3, and adult male and adult female extracts in the first-row wells were 0.01, 40, 5, 75, and 120 μg/100 μl, respectively. Plates were incubated at 37°C for 2 h and washed, and a horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (Cappel) was used to detect the bound antibodies. The concentrations of Bmserp23 epitopes contained in the parasite somatic antigen preparations were estimated from a standard curve and expressed in units per milligram of extract.

Nucleotide sequence accession number. The nucleotide sequence reported in this article has been submitted to the GenBank data base and has the accession number U04206.

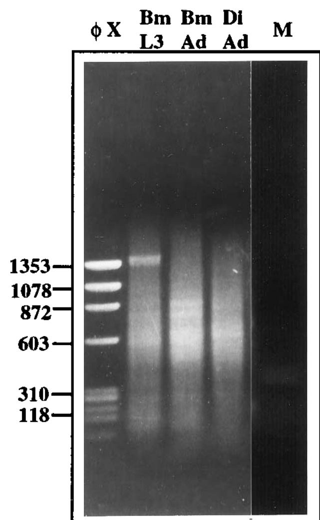

FIG. 1. Agarose gel analysis of SL-containing PCR cDNA products. The SL-containing cDNAs from *B. malayi* L3s (Bm L3) and adult males (Bm Ad), *D. immitis* adult males (Di Ad), and mosquitoes (M) were used as templates in a PCR employing primers NSL and XET. After 35 cycles of amplification, 10 μl of a 100-μl reaction mixture was separated on a 2.0% agarose gel and stained with ethidium bromide. φX, *Hae*III-digested φX174 replicative-form phage DNA markers. The sizes of selected marker components in base pairs are indicated at the left margin of the figure.

RESULTS

PCR cDNA products of *B. malayi* L3 cDNA. When an amount of sscDNA equivalent to the material from a single mosquito containing 5 to 10 *B. malayi* larvae was used as the template for 35 cycles of amplification, the PCR products consisted of a smear of PCR cDNAs ranging from just over 2,000 bp to about 150 bp in which there was a predominant band of approximately 1.4 kb (Fig. 1). Under these conditions, PCR amplification using one mosquito equivalent of sscDNA as the template gave only a trace product of approximately 350 bp. A PCR was also carried out under the same conditions with sscDNAs isolated from male *B. malayi* and male *D. immitis*, a filarial parasite of dogs, as the templates. The products from the male parasites also separated as smears between 2,000 and 150 bp, but there was no indication of the 1.4-kb band observed when larval cDNAs were used as the template (Fig. 1).

The 1.4-kb L3 PCR band was excised from a gel for cloning. By utilizing the *Not*I and *Xho*I sites at the 5′ and 3′ ends of the PCR products, respectively, the PCR cDNAs were directionally cloned into pBluescript. Of the more than 200 bacterial colonies produced, 75 clones, containing plasmids with inserts of 1.2 to 1.5 kb, were grouped by cross hybridization. Further analyses demonstrated that of the 75 SL PCR cDNA clones, 12% (9 of 75) contained the gene encoding glyceraldehyde-3-phosphate dehydrogenase, 11% (8 of 75) contained an insert designated BmY8 (see below), 7% (5 of 75) contained a gene coding for a cuticular collagen, and 4% (3 of 75) contained a gene with similarity at the amino acid level to *Bordetella pertussis* cyclolysin (data not shown). Therefore, although certain genes were represented in higher frequency than others, the 1.4-kb band appeared to be made up of a relatively diverse population of transcripts.

Sequence of BmY8. BmY8 contained 1,287 bp which included the SL sequence and a polyadenylated tail of 19 A's (Fig. 2). The initiation codon (ATG) was separated from the SL sequence by a single adenosine residue. The presumed stop codon (TAG) was 1,170 bases from the initiation codon and 54

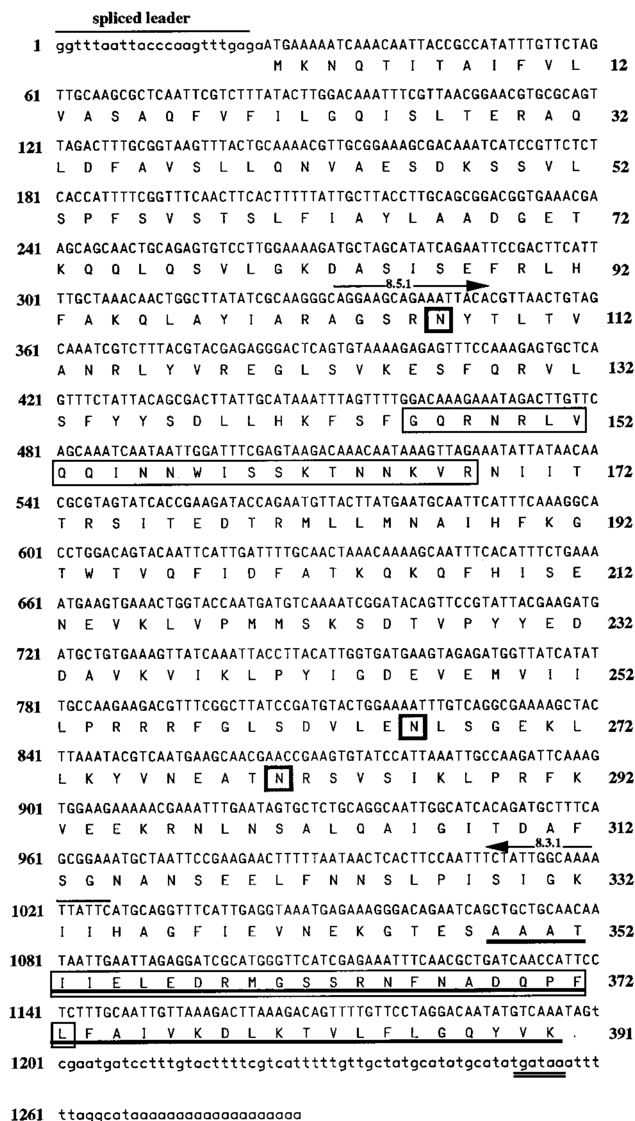

FIG. 2. Nucleotide and deduced amino acid sequences of BmY8. The nucleotides that make up the noncoding regions are in lowercase letters, and the nucleotides of the presumed coding region are in uppercase letters. The SL sequence is overscored and labeled. The positions and sequences of Bmserp23 and Bmserp21 are boxed. The positions of the internal PCR primers 8.5.1 and 8.3.1 are indicated with an arrow over the sequence. The positions of potential N-linked glycosylation sites at Asn-108, Asn-267, and Asn-281 are boxed. The amino acid sequence that was deleted from the truncated Bmserpin fusion protein is indicated by the bold underline. The potential polyadenylation signal is designated by a double underline. The nucleotides and amino acids are numbered along the left and right margins, respectively.

bases upstream from a possible polyadenylation signal sequence (GATAAA). The putative polyadenylation signal was 11 bp upstream from the poly(A) tail.

Southern blot analyses. To determine the genomic distribution and species specificity of BmY8, Southern blot analyses were carried out on *B. malayi* and selected nematode DNAs, respectively (Fig. 3A). The major bands observed in the five restriction enzyme digests of *B. malayi* DNA after hybridization with the BmY8 insert cDNA were consistent with the patterns predicted by the distribution of the restriction sites known to occur in the cDNA and genomic sequences (data not shown). The intensities of the major hybridizing bands were

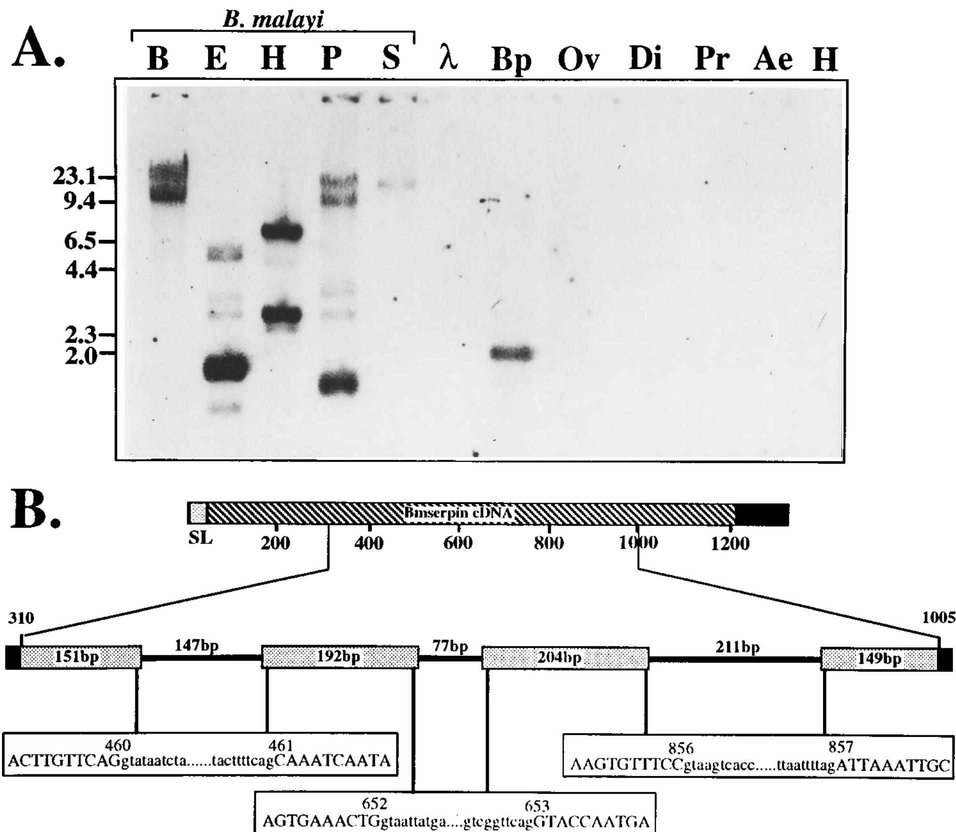

FIG. 3. Southern blot analysis and genomic organization of BmY8. (A) Lanes B, E, H, P, and S contained *B. malayi* DNA digested with *Bam*HI, *Eco*RI, *Hin*dIII, *Pst*I, and *Sal*I, respectively. Lanes Bp, Ov, Di, Pr, Ae, and H contained *Eco*RI-digested *B. pahangi*, *O. volvulus*, *D. immitis*, *P. redivivus*, *A. aegypti*, and human DNAs, respectively. Lane λ contained *Hin*dIII-digested λ phage DNA. The standard marker locations in kilobases are indicated at the left margin of the blot. The DNAs were separated on a 0.8% agarose gel. The hybridization was performed by the enhanced chemiluminescence method with the BmY8 insert as the probe. The film was processed after a 3-h exposure. (B) The top portion represents a graphic map of the BmY8 insert cDNA. The SL, coding and noncoding regions are indicated by the stippled, hatched and solid areas, respectively. The bottom portion of the figure outlines features of the genomic organization of BmY8 between nucleotides 310 and 1005. Stippled boxes indicate exons, and the horizontal lines denote introns. The number of nucleotides in each exon and intron is shown in the boxes or over the lines, respectively. The sequences of donor and acceptor splice junctions are shown in open boxes under the corresponding positions. The numbers above each junction indicate nucleotide positions as they occur in the intact BmY8 cDNA.

similar to the levels obtained when probes from known single-copy genes were used on the same blot under similar conditions (data not shown). This result suggests that BmY8 may occur as a single copy in the *B. malayi* genome. The lower-intensity bands, which were a constant feature of *B. malayi* DNA digested with *Eco*RI, *Hin*dIII, and *Pst*I, were not predicted by the known restriction enzyme. These bands may indicate that other genes with sequences similar to BmY8 exist in the genome.

Of the related filarial nematode species, *B. pahangi*, *O. volvulus*, and *D. immitis*, only the genomic DNA isolated from *B. pahangi* contained sequences that hybridized with the BmY8 probe under the conditions used (Fig. 3A). In addition, the probe did not hybridize to DNA from the free-living nematode *P. redivivus*, from the mosquito *A. aegypti*, or from humans.

Genomic sequence. A PCR-based strategy was employed to isolate a 1,130-bp genomic fragment of BmY8 which contained three introns and two complete exons and two partial exons (Fig. 3B). The introns contained 147, 77, and 211 bases. The splice acceptor and splice donor sites conformed to the consensus splice junctions for most nematodes (14) and other eukaryotic organisms (25) (Fig. 3B).

Developmental regulation of BmY8 transcription. RT-PCR was carried out on RNA isolated from *B. malayi* at different stages during the life cycle (Fig. 4). A 1,027-bp fragment of BmY8 was amplified with the SL primer and the BmY8 internal primer 8.3.1 (Fig. 2). Parallel PCRs were carried out with the SL primer and an internal primer specific for the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase to control for the quality and quantity of the template cDNAs. The identities of the BmY8 and glyceraldehyde-3-phosphate dehydrogenase PCR products were confirmed by Southern blot hybridization with nested sequences as the probes (data not shown). It is important to note that the use of the SL primer in these reactions allows for the selective amplification of cDNA and essentially eliminates any problems caused by the presence of genomic DNA contamination in the cDNA template.

Even after 40 rounds of amplification, no bands were detected in ethidium-stained gels or Southern blots of the PCR products derived from larvae at days 2 and 4 postinfection (Fig. 4). This apparent lack of transcription extended through day 8 postinfection (data not shown), indicating that either BmY8 is not transcribed during the early phase of vector-stage development or that the BmY8 message is unstable during early larval development. Transcription was first detected at day 9 postinfection and transcription continued through day 10 of vector-stage development and into the early phase of verte-

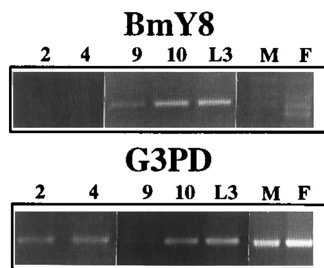

FIG. 4. Transcription of BmY8 at different developmental stages of *B. malayi*. RNAs isolated from infected mosquitoes at day 2 (lane 2), day 4 (lane 4), day 9 (lane 9), and day 10 (lane 10) postinfection, postvector L3 (lane L3), mature male (lane M), and mature female (lane F) worms were processed to obtain double-stranded SL cDNA. The SL cDNAs were used as templates to amplify a 1,027-bp fragment from BmY8 or a 1,082-bp fragment of *B. malayi* glyceraldehyde-3-phosphate dehydrogenase (G3PD) as outlined in Materials and Methods. After 30 (BmY8) or 20 (G3PD) rounds of PCR amplification, the products were separated on a 1.2% agarose gel and stained with ethidium bromide. The amounts of template used per reaction were equivalent to the cDNAs from 1 to 5 larvae from infected mosquitoes, 10 larvae from the postvector L3s, 1 adult male, and 0.4 adult female.

brate-stage development (Fig. 4). BmY8 transcription could also be detected in L4s and immature adults harvested on day 35 of infection (data not shown). Transcription of BmY8 was reduced but nominally detectable in mature male and female parasites (Fig. 4) and in microfilariae (data not shown). An analysis of the relative rate of accumulation of PCR products from the different stages tested after 20, 30, or 40 rounds of amplification suggested that the late-vector-stage and postvector L3s had levels of transcription that were 5- to 10-fold higher than that observed in the adult parasites.

Predicted amino acid sequence of BmY8. The BmY8 open reading frame encoded 391 amino acids (Fig. 2). Following an initial stretch of hydrophilic residues, amino acids 6 through 25 formed a strong hydrophobic region that may function as a signal peptide for transport to the endoplasmic reticulum.

A search of the protein databases using the BLAST network service at the National Center for Biotechnology Information showed that the protein encoded by BmY8 had similarity to a number of proteins, all of which were members of the serine proteinase inhibitor, or serpin, superfamily. Included in the list of serpins were silkworm (*Bombyx mori*) antitrypsin (46) and antichymotrypsin II (39), tobacco hornworm (*Manduca sexta*) alaserpin (21), human α_1-antitrypsin (26), and the chicken gene Y, an ovalbumin-related protein (16). In light of this similarity, the protein encoded by BmY8 was designated Bmserpin.

An alignment of the amino acid sequence of the serpins from silkworm, hornworm, human and chicken to the Bmserpin sequence is presented in Fig. 5. Although the highest similarity between the protein sequence of Bmserpin and the serpins from the other species was only 30% (for *M. sexta* alaserpin and the human leukocyte elastase inhibitor), the Bmserpin sequence aligned exactly to a number of amino acids that are highly conserved within the serpin superfamily. Bmserpin matched at 13 positions that are conserved in all 16 of the serpin sequences used for comparison (Fig. 5). Included in this group is the highly conserved serpin motif E-X-G-X-E (positions 343 to 347) that corresponds to the "hinged" region that is located immediately upstream of the serpin reactive center. Moreover, Bmserpin agreed with the consensus sequence for over 80% of the 16 serpin sequences at 22 additional amino acid sites. Bmserpin did not agree with the serpin superfamily consensus sequence at four positions (Ala to Leu at position 151, Val to Ile at position 159, Ile to Val at position 167, and Pro to stop at position 392).

Anti-Bmserpin antibodies. After evaluating the protein sequence for predicted hydrophilic stretches (Kyte-Doolittle) and for the regions that may be functional domains of the protein, two regions of the Bmserpin sequence were selected for the production of synthetic polypeptides (Fig. 2). One region was a 23-amino-acid stretch located near the center of the protein extending from Gly-145 through Arg-168 (Bmserp23). The second area was 21 amino acids positioned near the carboxy terminus of Bmserpin from Ile-353 through Leu-373 (Bmserp21) that included a portion of the putative reactive site of Bmserpin (Fig. 2; see Discussion). After synthesis, Bmserp23 and Bmserp21 were conjugated separately to BSA and the two conjugated preparations were used to immunize rabbits for the production of peptide-specific antibodies.

The antibodies were partially characterized with the MBP fusion proteins. Bacterial extracts from cultures of induced and noninduced full-length (Bmserpin-MBP) or truncated (tBmserpin-MBP) fusion proteins were separated by SDS-PAGE and then blotted onto a nitrocellulose membrane. tBmserpin-MBP was missing the carboxy-terminal 42 amino acids, which include the epitopes specifically recognized by anti-Bmserp21 (Fig. 2). The anti-Bmserp23 antibodies recognized Bmserpin-MBP and tBmserpin-MBP in extracts from induced, but not from noninduced, cultures (Fig. 6). Anti-Bmserp21 recognized the full-length fusion protein in extracts from induced cultures (Fig. 6). As expected, anti-Bmserp21 did not recognize the truncated fusion preparation from either induced or noninduced cultures. In the induced and noninduced fusion protein preparations, the antibodies also demonstrated low-level nonspecific recognition of a limited number of bacterial proteins. Neither antiserum recognized MBP (data not shown).

Native protein. Anti-Bmserp23 recognized a protein with an apparent molecular weight of 44,000 (Fig. 7). This result was in good agreement with the predicted molecular weight of the protein encoded by the BmY8 open reading frame (44,100). In addition to the band at 44 kDa, there was strong recognition of a band of >200 kDa and three to five minor bands with apparent molecular weights between 200,000 and 100,000. Anti-Bmserp23 produced a nearly identical recognition pattern in protein extracts from immature adult parasites and from an extract from mixed male and female parasites (Fig. 7). The recognition of the 44,000- and high-molecular-weight complex of proteins by anti-Bmserp23 was abolished by preincubation of the antibody with Bmserp23 peptide (data not shown). Unexpectedly, the antibodies directed against the carboxy-terminal portion of Bmserpin (anti-Bmserp21) did not recognize any proteins in the extracts from L3s or immature adults (data not shown).

Western blot analyses were also performed with ES products obtained during in vitro culture of *B. malayi* L3 parasites. In contrast to the results from parasite extracts, anti-Bmserp23 recognized a single component with an apparent molecular weight of 75,000 in L3 ES products (Fig. 7). Anti-Bmserp21 failed to bind to any component in the L3 ES (data not shown). Preincubation of anti-Bmserp23 with Bmserp23 peptide effectively eliminated recognition of the 75-kDa ES protein (data not shown). Immunoreactivity at the 75-kDa band was also detected in the ES products from *B. malayi* L4 (data not shown).

Quantitative analysis. A competitive ELISA was developed to detect the concentration of the Bmserp23 antigenic determinants in parasite extracts. The extract from *B. malayi* L3s had the highest level of Bmserp23 epitopes at 6,600 U/mg of L3 extract. The microfilarial extract contained only 400 U of

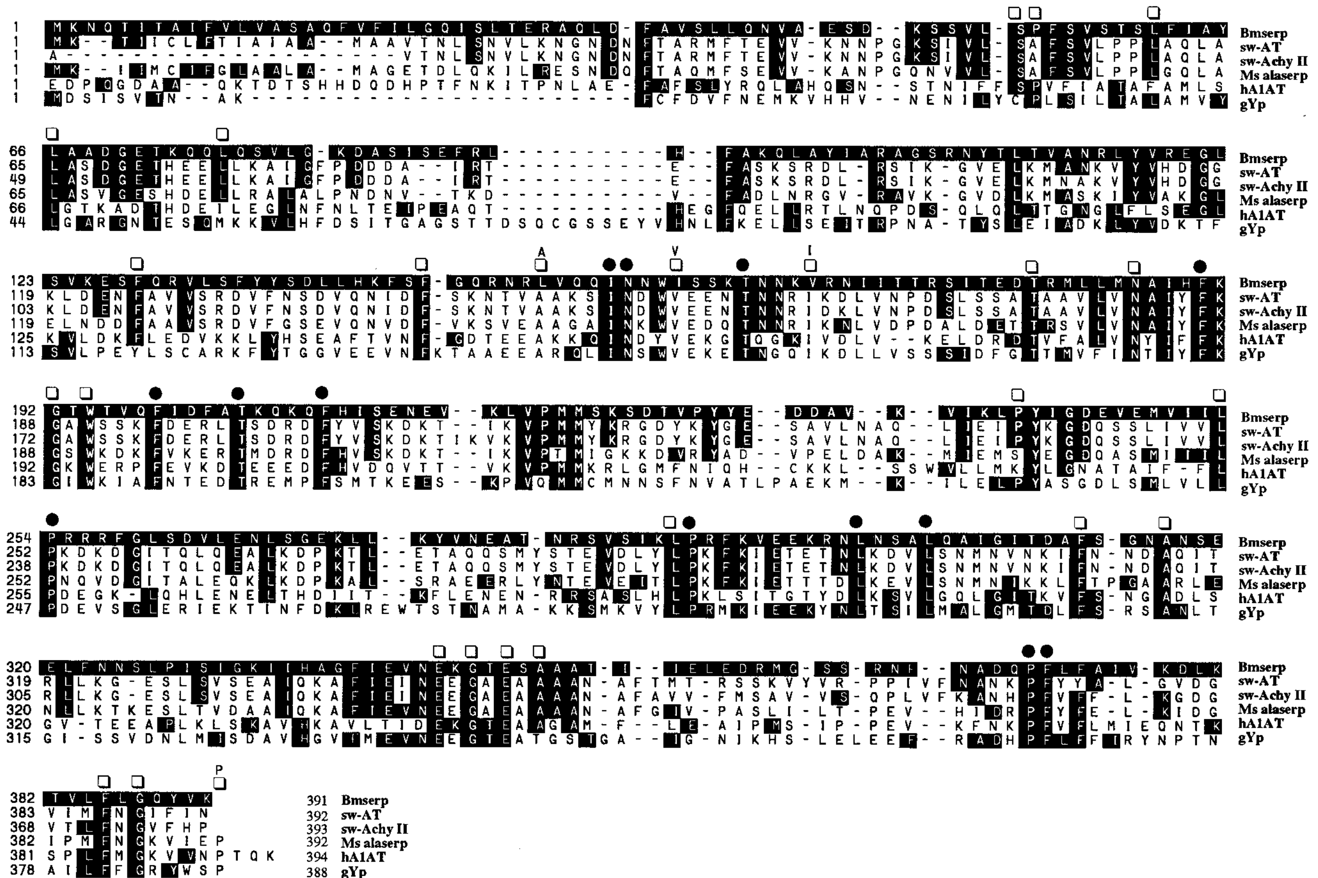

FIG. 5. Alignment of the deduced amino acid sequence of the BmY8 open reading frame (Bmserp) with five members of the serpin superfamily. Solid boxes indicate amino acids that are identical to those of Bmserpin at a given position. The numbers along the margin and at the end of the last line designate the positions of amino acid residues. In addition to the 5 serpins shown, the sequences of 11 additional serpins (total of 16) were surveyed for conserved sites. A solid circle indicates that 100% of the 16 serpins surveyed have the same amino acid residue at that position. An open square indicates that >80% of 16 serpins have the same amino acid residue at that position. The consensus amino acid is shown above the square if the consensus residue differs from the Bmserpin residue. The 16 serpins used were Bmserpin, silkworm antitrypsin (sw-AT) (46), silkworm antichymotrypsin II (sw-Achy II) (39), *M. sexta* alaserpin (Ms alaserp) (21), human α_1-antitrypsin (hA1AT) (26), chicken gene Y protein (gYp) (16), α_1-antichymotrypsin (6), α_2-antiplasmin (17), antithrombin III (7), cowpox virus hemorrhage-specific protein (34), human glia-derived nexin (43), ovalbumin (29), plasminogen activator inhibitor (32), protein C inhibitor (45), rat angiotensinogen (49), and silkworm antichymotrypsin (30).

Bmserp23 epitopes per mg of extract. The extracts from both male and female worms contained 660 U/mg of worm extract. Thus, L3 extracts contained 10 and 16 times the number of Bmserp23 epitopes compared with those of adult worm and microfilarial extracts, respectively.

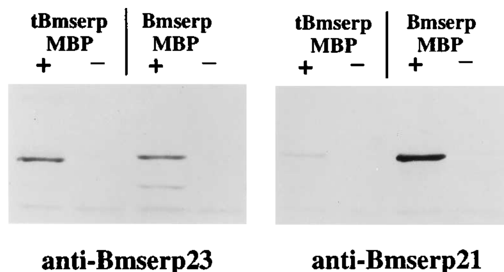

FIG. 6. Western blots of Bmserpin-MBP fusion proteins immunostained with anti-Bmserp23 and anti-Bmserp21 antibodies. Bacterial lysates containing the full-length (Bmserp MBP) or truncated (tBmserp MBP) fusion proteins were separated by SDS-PAGE on a 10% acrylamide gel and then blotted onto nitrocellulose membranes. The separated material included lysates from both induced (+) and noninduced (−) bacteria. The membranes were immunostained with either rabbit anti-Bmserp23 or anti-Bmserp21.

Immunogenicity in gerbils. The immunogenicity of Bmserpin was tested in the gerbil system with pooled sera from *B. malayi*-infected animals (2 to 3 serum samples per pool). The sera were tested for the presence of antibodies that reacted to the Bmserp23 and Bmserp21 peptides by ELISA. The sera from L3-immunized and L3-L4-exposed animals had the highest mean titers of both anti-Bmserp23 (1:6,400) and anti-Bmserp21 (1:6,400) antibodies. The titers of anti-Bmserp23 and anti-Bmserp21 reactivities of the sera from early and late chronic infections were 4- to 16-fold lower than the titers from animals exposed to only the early infective forms of the parasite. The mean titers of anti-Bmserp23 activities of the sera from early and late chronic infections were 1:1,600. The mean titers of anti-Bmserp21 activities of the sera from early and late chronic infection were 1:800 and 1:400, respectively.

DISCUSSION

SL library. A major impediment to studies on the biochemistry and molecular biology of certain parasitic organisms is a lack of parasite material. The issue of limited parasite material is particularly critical for the early infective forms of filarial nematode parasites. We describe here a SL RT-PCR proce-

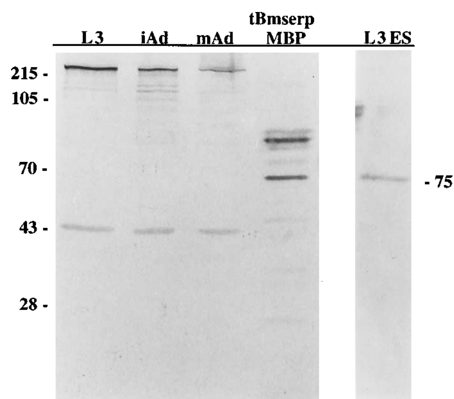

FIG. 7. Western blots of *B. malayi* somatic extracts and ES products immunostained with anti-Bmserp23. (Left) Western blot of *B. malayi* extracts separated by SDS-PAGE on a 10% acrylamide gel and then blotted onto a nitrocellulose membrane. Lane L3 contained the extract from 350 *B. malayi* L3s. Lane iAd contained the extract from 16 immature (day 36) adults. Lane mAd contained the extract from five mature male and female *B. malayi*. Lane tBmserp MBP contained an extract from bacteria expressing the truncated Bmserpin-MBP fusion protein. The positions of the molecular weight markers (in thousands) are shown at the right margin of the blot. The prestained high-molecular-weight markers (Bethesda Research Laboratories) were myosin, H chain (215,000), phosphorylase *b* (105,000), BSA (70,000), ovalbumin (43,000), and carbonic anhydrase (28,000). (Right) A Western blot of *B. malayi* ES products immunostained with anti-Bmserp23 serum. The ES products were obtained as outlined in Materials and Methods. The concentrated L3 ES products were separated by SDS-PAGE on a 10% polyacrylamide gel and blotted onto a nitrocellulose membrane. The blot of L3 ES products was immunostained with anti-Bmserp23. The apparent molecular weight (in thousands) of the immunoreactive band is indicated at the right.

dure that can be used in situations in which only a limited amount of *B. malayi* starting material is available to produce an amount of cDNA sufficient to generate stage- and substage-specific cDNA libraries to investigate developmentally regulated gene expression.

Although SL RT-PCR was applied to *B. malayi* in this study, this approach can be used to study gene transcription in a number of parasitic and nonparasitic nematode species. The SL sequence is a highly conserved element that is present in all of the nematode species in which it has been studied (31). Therefore, the SL RT-PCR strategy can be applied to all of the filarial species, nonfilarial parasitic nematodes of animals and plants, and important soil nematode species. In addition, since *trans*-spliced sequences are not confined to nematodes, this general strategy may be useful for studies of a number of organisms. All members of the family *Trypanosomatidae trans*-splice a leader sequence to the 5′ end of each transcript (4). More recently, SL sequences have been described for *Schistosoma mansoni* (35), the protist *Euglena gracilis* (50), and the protozoan parasite *Herpetomonas samuelpessoai* (1).

Bmserpin. Although the predicted amino acid sequence of the *B. malayi* protein has a similarity of only 22 to 30% to the sequences of serpins from other species, the Bmserpin sequence was identical at 35 of 39 highly conserved amino acid positions. Presumably, this high degree of conservation at specific amino acid positions is necessary for the serpins to adopt the tertiary conformation that is critical for serpin function (18, 44).

The serpin superfamily of proteins has been constructed on the basis of sequence similarities at the protein level. Over 40 proteins, derived from sources as diverse as humans, viruses, and plants, are members of this superfamily (12). The majority of serpins are single-chain glycoproteins with molecular weights between 40,000 and 100,000 that contain a single reactive site located in the carboxy terminus. The serpins function as substrates by forming stable 1:1 complexes with their target serine proteinase. Although the exact nature of the complex has not been established, it has been determined that the serpin is cleaved by and then covalently linked to the proteinase (11). The target serine proteinase cleaves a specific peptide bond in the serpin's reactive center, the carboxy end of the serpin is released, and a covalently linked serpin-proteinase complex is formed by an acyl-ester linkage between the carboxyl group of the amino acid residue at the reactive center of the serpin (designated P_1) and an hydroxyl group of the active site of the proteinase (12).

In addition to interactions that lead to the production of covalently linked complexes, under certain conditions, serpins become substrates for their target serine proteinase (12, 33). In these reactions, the serpin is cleaved at the reactive site, and the carboxy end of the serpin is released, but no complex is formed with the proteinase (33). After serpins are cleaved as a substrate, they lose their ability to inhibit proteinase activity and have no known additional physiological function (5). The ability to catalytically inactivate serpins in vivo is thought to be an important regulatory mechanism during inflammation (5).

The reactive site of Bmserpin has not been determined. However, on the basis of information collected from other serpins, a tentative reactive site for Bmserpin can be assigned. A majority of the known serpin reactive sites occur 10 amino acids downstream from a highly conserved alanine residue. This conserved alanine appears to occur at position 349 in Bmserpin. An alignment of the sequences flanking the reactive center of selected serpins is presented in Fig. 8. According to the convention of Schechter and Berger (40), the amino acids on the N- and C-terminal sides of the cleavage site are termed P_1 and P_1', respectively. By using the criterion of the 10th amino acid downstream from Ala-349, the reactive site of Bmserpin would be Arg-259 and the P_1-P_1' would be Arg-Met. This P_1-P_1' pair is also found in α_2-antiplasmin (Fig. 8), a serpin that functions in regulating blood coagulation (23). The low level of identity between Bmserpin and the *S. haemato-*

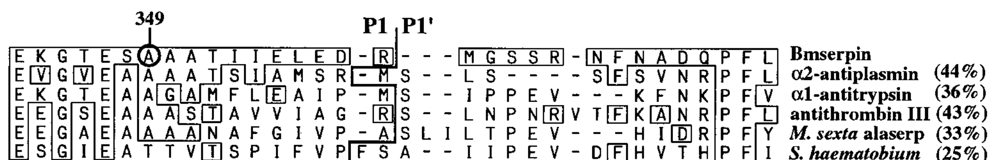

FIG. 8. Alignment of the deduced amino acid sequence of the putative reactive center of Bmserpin with the reactive centers of the serpins human α_2-antiplasmin (17), human α_1-antitrypsin (26), human antithrombin III (7), *M. sexta* alaserpin (21), and *Schistosoma haematobium* serpin (3). Boxed amino acids are those identical to the amino acid at that site in Bmserpin. The conserved alanine residue at position 349 of Bmserpin is circled. The vertical line marks the documented sites of proteinase cleavage for the human and *M. sexta* serpins and the proposed cleavage site for Bmserpin and the *S. haematobium* serpin. The P_1 residues of the reactive center are to the left, and the P_1' residues are to the right side of the thick line. The percentages at the end of each sequence show the levels of sequence similarity to Bmserpin over the reactive center.

bium serpin over their presumed reactive region (25%) and at the estimated reactive sites (Fig. 8) suggests that these two serpins have distinct proteinase targets.

Using antibodies that recognized epitopes formed by amino acids 145 through 168 to immunostain Western blots of proteins extracted from several stages of parasite development, a major band with an apparent molecular mass of 44 kDa (Fig. 7). The 44-kDa band was close to the mass predicted for the protein produced by the BmY8 open reading frame (44.1 kDa). However, despite the similarity in mass, experimental evidence suggests that the 44-kDa band detected by anti-Bmserp23 may represent the cleaved or inactivated form of Bmserpin and not the intact molecule. The results of immunostaining in which antibodies directed against epitopes near the reactive site (anti-Bmserp21) failed to recognize either the high-molecular-mass or 44-kDa molecules on Western blots suggest that the Bmserpin in these extracts was cleaved so that the epitopes were no longer present. It may be that Bmserpin is either complexed or cleaved as soon as it is synthesized by the parasite so that there is little or no unprocessed or active Bmserpin present in the extracts. It is also possible that the methods used to obtain the parasite proteins resulted in the production of cleaved Bmserpin during the extraction process. The parasite proteins used in the Western blots were extracted and held at low temperatures, conditions that have been shown to promote the production of cleaved or inactive serpin in other systems (33).

If the 44,000-molecular-weight band does represent the cleaved Bmserpin, it should have contained 31 fewer amino acids than the intact protein. This loss of the carboxy end of Bmserpin should have reduced the molecular weight to about 40,000. It is possible that Bmserpin, which has three potential sites for the attachment of N-linked sugars (Asn-108, Asn-267, and Asn-281; Fig. 2), is glycosylated like most members of the serpin superfamily and that these carbohydrate moieties may account, at least in part, for the observed molecular weight. The results of preliminary experiments in which the native protein was treated with *N*-glycanase suggest that Bmserpin contains enough N-linked glycans to account for the additional 4 kDa in mass (data not shown).

In addition to the 44-kDa molecule, a number of bands with high molecular mass were detected in the extracts from all of the stages tested. It is likely that these immunoreactive high-molecular-mass bands represent complexes of Bmserpin covalently linked to target serine proteinases. Previous studies have shown that serpin-proteinase complexes do not dissociate during SDS-PAGE (33). The presence of multiple complexes in the whole-parasite extracts suggests that Bmserpin may have multiple serine proteinase targets and that Bmserpin could be playing a role in the regulation of biological processes such as morphogenesis or molting.

Anti-Bmserp 23 recognized a 75-kDa protein in the ES products from *B. malayi* L3s (Fig. 7) which is significantly larger than expected for the Bmserpin monomer. Although the nature of this band is unclear, the apparent increase in molecular mass and the lack of recognition by anti-Bmserp21 suggest that the 75-kDa band may represent a complex between Bmserpin and a target molecule.

An analysis of the immunostained Western blots of protein extracts from different stages of parasite development suggested that the concentration of Bmserpin was significantly elevated in extracts from infective-stage larvae. The intensities of the bands from L3s, immature adults, and mature adults were approximately equal (Fig. 7) despite the fact that the L3 lane contained only about 1/10 the amount of parasite mass. The assertion that Bmserpin was expressed at elevated levels by L3s was supported by the results of the RT-PCR analysis of Bmserpin transcription (Fig. 4) and the results of competitive ELISA which showed that L3s contained 10 to 16 times more of the epitopes associated with the Bmserp23 peptide than adults or microfilariae, respectively.

The synthesis of Bmserpin at elevated levels just prior to and during the early stages of parasite development in the vertebrate host and its release as part of the ES products suggest that it may have an important function in parasite survival. In a number of systems, serpins have been identified as factors that modulate or inhibit host immune responses (27, 36). It has been reported that *B. malayi* larvae can inhibit the initiation of thrombosis through a suppression of the intrinsic coagulation pathway by inhibiting the function of Hageman factor, a serine proteinase (10). This inhibitory activity was found in *B. malayi* somatic extracts and in the supernatants from cultured microfilariae. Bmserpin, which is also associated with both somatic and secreted products, may be an important part of this inhibitory activity. The ability to inhibit coagulation during the early stages of parasitism may be important for the survival of *B. malayi* and other helminth parasites in the human host. Somatic extracts and the ES products of *S. mansoni* also contain molecules that inhibit Hageman factor (9). Interestingly, serpins isolated through biochemical purification (13) and by gene cloning (3) have recently been reported from *S. mansoni* and *S. haematobium*, respectively. If Bmserpin does prove to be an important component of the *B. malayi* survival strategy, then it would be a strong candidate for one of the antigens used in a protective vaccine.

ACKNOWLEDGMENTS

This work was supported by research grants from the U.S. Public Health Service (AI29411), The Edna McConnell Clark Foundation (EMCF 02090), and the MacArthur Foundation Consortium on the Biology of Parasitic Diseases, The Johns Hopkins Program.

Parasites were supplied under the auspices of an NIAID supply contract (AI#02642), U.S.-Japan Cooperative Medical Science Program. We thank Nithya Raghavan for helpful suggestions.

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