Schistosomes utilize proteases (termed hemoglobinases) for degradation of host globin. cDNA clones encoding *Schistosoma mansoni* protease were isolated by immunologically screening an expression cDNA library with antisera raised against purified hemoglobinase. Confirmation of the identities of the clones was obtained immunologically and biochemically. The bacterially produced fusion protein encoded by one clone, \( \alpha \text{Hb}2 \), degraded hemoglobin *in vitro*. The sequence of this clone suggested that this *S. mansoni* protease is synthesized in a precursor form *in vivo*. Gene titrations indicated that *S. mansoni* contains multiple genes corresponding to this cDNA. The expression of these genes may be regulated during the organism's life cycle since adult, female worms contained the highest abundancies of homologous mRNA and protein compared to other stages.

The multicellular, human parasite *Schistosoma mansoni* utilizes proteases throughout its life cycle. For example, egg release from the host animal and micradaclinal penetration of the intermediate snail host may be facilitated by proteolytic activities (1, 2). Infective cercariae and the first parasitic stage, schistosomula, also contain proteases thought to be involved in penetration of human skin (3-5). In spite of their possible biologic significance, relatively little is known of the specificities and biochemistry of these enzymes. Proteases described in adult schistosomes, however, play a significant role in parasite nutrition. Adult worms parasitize the hepatic portal system and have been shown to consume considerable volumes of host erythrocytes. When reticulocytes containing radioactive hemoglobin were injected into infected mice, adult schistosomes incorporated the label throughout their tissues (6). Furthermore, when hemoglobin, red cells, or red cell ghosts containing residual hemoglobin were added to worms in culture, schistosomes were able to mature (7-9). The nutrients contained in host globin may also be essential for egg production as addition of red cells to worms in culture enabled oviproduction.

A schistosome hemolytic activity capable of lysing host red cells has been described (10). Released globin is degraded to peptides containing phenylalanine at their amino termini by proteases (11), first described in 1959 by Timms and Bueding.

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**EXPERIMENTAL PROCEDURES**

**Materials**—Human globin, 4-chloro-1-naphtol, phosphate-buffered saline (PBS)\(^*\) and Sephadex G-200 were obtained from Sigma. Protein A-horseradish peroxidase conjugate and DEAE-Bio-Gel A (100-200 mesh) were purchased from Bio-Rad. Goat anti-rabbit IgG was from Cappell Cooper Organon, and agaro-1,4-phenylalanine was from P. L. Pharmac or ICN Biochemicals. Rabbit anti-\( \beta \)-galactosidase antisera was obtained as described (18). Freund's complete and incomplete adjuvant were obtained from DIFCO. An ELISA kit, [\( ^{35} \text{S} \)]dATP and [\( ^{32} \text{P} \)]dCTP were from Du Pont-New England Nuclear. Restriction enzymes and the Klenow fragment of *E. coli* DNA polymerase were purchased from Boehringer Mannheim. Deoxyribonucleoside triphosphates and M13 single-strand sequencing primers were obtained from P. L. Pharmac and New England Biolabs.

*S. mansoni* worms of a Puerto Rican strain were obtained by perfusion of the portal veins of 8-week infected mice (19). Cercariae were shed from infected snails (Biomphalaria glabrata). Schistosomula were prepared by mechanical transformation of cercariae followed by incubation in culture for 3 h (20) and eggs were obtained from intestines of 6-week infected mice (21).

**Hemoglobinase Purification and Preparation of Antiserum**—The enzyme was purified according to the method of Sauer and Senft (11). One hundred mg of lyophilized adult worms were homogenized in 10 ml of 0.2 M sodium acetate, pH 4.0, and subjected to gel filtration on Sephadex G-200, followed by affinity chromatography on agaro-1,4-phenylalanine. Hemoglobinase activity of different fractions was determined using human globin as substrate. Aliquots of 0.2 ml from each fraction were incubated with 0.4 ml of globin (10 mg/ml) in 0.2 M sodium acetate, pH 4.0, at 37°C. The reaction was stopped after 1 h by addition of an equal volume of 10% trichloroacetic acid and centrifugation at 1000 \( \times \) g to remove precipitated protein. Enzyme activity was determined by measuring the absorbance of the supernatant at 280 nm. Protein concentrations were determined by Lowry assays using bovine serum albumin as the standard (22). Units of activity were as described (11).

Polyclonal antisera against purified hemoglobinase were raised in New Zealand white rabbits by subcutaneous injection of 30 \( \mu \)g of

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1. M. Phillips, personal communication.
purified enzyme in complete Freund's adjuvant. Initial immunization was followed by boosts of 30 μg of enzyme in incomplete adjuvant at 14 days and antigen alone at 28 days. Antisera against β-galactosidase fusion protein were also prepared in rabbits. The animals were first immunized with gel slices containing fusion protein. The resulting antisera had low titers. The animals were subsequently immunized with gel slices prepared on DEAE Bio-Gel (below). Immunization was in complete Freund's adjuvant followed by boosts in incomplete adjuvant and with antigen alone; 1 week later animals were bled.

Western blotting experiments were performed using protein from various stages of the S. mansoni life cycle. Organisms were homogenized in 0.2 M sodium acetate, pH 4.0, and aliquots were added to sodium dodecyl sulfate (SDS) gel sample buffer. Proteins were electrophoresed on SDS gels (23) and electrophoretically transferred to nitrocellulose (24). Five percent non-fat, dry milk was used to block nonspecific binding sites (25). The nitrocellulose was reacted with anti-hemoglobinase or antifusion protein antisera. The bound immunoglobulin was detected using Protein A-horseradish peroxidase conjugate and 4-chloro-1-naphthol as substrate.

**Antibody Screening of cDNA Library and DNA Sequencing—Polyclonal anti-hemoglobinase antisera diluted 1:25 were preabsorbed with lysates of E. coli and then used to screen a cDNA expression library made from adult S. mansoni mRNA in λgt11 ampicillin (18). Thermally induced lysogens in E. coli strain BTA282 (26) were lysed by freeze-thaw, and proteins were electrophoresed and visualized by Coomassie Blue staining and Western blot analyses. Two clones, λHb1 and λHb2, were selected for characterization. Phage DNA was prepared from lysogens, digested with EcoRI, cloned into plasmid pBR322, and mapped. Appropriate restriction fragments were cloned into M13 mp18 and M13 mp19 and subjected to dyeodeoxy sequencing (27). DNA sequences were analyzed with Beckman Microgenie, version 4, and dastP (28) software. Data banks included the Dayhoff protein sequence bank and banks in the Microgenie program.

**Purification and Characterization of Bacterially Produced Protein—The β-galactosidase fusion protein encoded by cDNA clone λHb2 was insoluble. It was made soluble in 7 M guanidine hydrochloride, 50 mM Tris-HCl, pH 7.5, 2 mM dithiothreitol, and was dialyzed extensively against 20 mM Tris-HCl, pH 8.3, 0.1 mM EDTA, 0.1 mM dithiothreitol, 7.0 M urea (DEAE buffer). Fusion protein was purified on a DEAE-Bio-Gel column as described (29). Fractions were concentrated on an Amicon B15 Minicon apparatus and examined for the presence of fusion protein by electrophoresis on a 10% polyacrylamide gel containing SDS. Fractions containing purified fusion protein were pooled. Another insoluble fusion protein from an unrelated schistosome, cloned from Xgtll amp3-infected BTA282. Following dialysis against PBS, the dialysates were subjected to chromatography on 1 ml agarose-1.1-phenylalanine column as described above.

To examine shared antigenic determinants between the native schistosome hemoglobinase and fusion protein encoded by cDNA clone λHb2, the procedure of Engvall and Perlman (30) was used to bind the native enzyme to the wells of polyclonal chloroform microtiter plates. One percent bovine serum albumin in PBS was used to block nonspecific protein-binding sites. A 1:5000 dilution of rabbit anti-hemoglobinase antisera was preincubated for 2 h with increasing concentrations of purified fusion protein (0–10 μg/ml) and was then added to each well. The bound immunoglobulin was detected using goat anti-rabbit IgG conjugated to alkaline phosphatase and p-nitrophenyl phosphate as substrate. Color development was complete in 30 min. The absorbance was read on a Dynatech Laboratories microELISA analyzer at 405 nm. Anti-hemoglobinase antisera preincubated with β-galactosidase or an unrelated schistosome fusion protein served as controls.

**Gene Titration—To estimate the gene copy number of sequences homologous to the λHb2 cDNA, schistosome DNA was isolated as described (31). DNA was digested with PstI, and purified by phenol/chloroform extractions and ethanol precipitation. DNA was suspended in 10 mM Tris-HCl, pH 8.0, 1 mM EDTA (TE). Plasmid pBR322 containing the EcoRI insert from λHb2 was diluted to 0.1 ng/μl in TE containing 10 μg/ml sonicated salmon sperm DNA and was subjected to PstI digestion and purification as described above. Two-fold serial dilutions of both the plasmid DNA and schistosomulum DNA were made. The total DNA concentration in each aliquot was adjusted to 5 μg/100 μl using sonicated salmon sperm DNA. One hundred μl of 2 M NaCl, 0.2 M NaOH was added to each aliquot, and the DNA was heated at 65 °C for 3 min. Twenty-five μl each of 0.5 M Tris-HCl, pH 7.6, and 0.8 M HCl were added. One hundred and twenty-five μl of each sample were immobilized on nitrocellulose using a Schleicher & Schuell Minifold apparatus. The nitrocellulose was baked at 75 °C for 2 h and was hybridized with the λHb2 cDNA insert as described (18).

**Northern Blot Hybridization—**The expression of genes corresponding to λHb2 during the S. mansoni life cycle was examined by Northern blot analyses. RNA was isolated, electrophoresed through agarose gels containing formaldehyde, transferred to nitrocellulose, and hybridized with nick-translated insert from λHb2 (18).

**RESULTS**

**Purification of S. mansoni Hemoglobinase and Isolation of cDNA Clones—**Hemoglobinase was purified by gel filtration and affinity chromatography of schistosome proteins solubilized in 0.2 M sodium acetate, pH 4.0. In six separate preparations, an average of 200 μg of hemoglobinase was isolated from 100 mg of lyophilized adult worms. The specific activity of the enzyme preparation was approximately 10 units/mg of protein and on silver-stained SDS gels the preparation appeared as a single major protein species of Mr = 31,000 (Fig. 1). Minor contaminants included a protein of Mr = 27,000. Polyclonal rabbit antisera were raised against the enzyme preparation. These antisera recognized only protein of Mr = 31,000 in Western blots of the starting homogenate and final enzyme preparation (Fig. 1).

To isolate cDNA clones encoding schistosome hemoglobinase, approximately 106 recombinants from an amplified portion of an expression cDNA library in λgt11 ampicillin were immunologically screened with anti-hemoglobinase antisera. Six recombinants were identified which remained immunoreactive during subsequent plaque purification, and two clones, λHb1 and λHb2, were chosen for further study based upon ease of handling. The λHb2 clone encoded a β-galactosidase fusion protein of Mr = 150,000 recognized by anti-hemoglobinase antisera (Fig. 2); anti-β-galactosidase antisera also recognized this protein. The other clone (λHb1) did not produce a fusion protein but produced a protein of Mr = 52,000 recognized by anti-hemoglobinase antisera (Fig. 2).

The fusion protein produced by λHb2 was partially purified

![Fig. 1. Immunologic reactivity of anti-hemoglobinase antisera with worm homogenate and purified hemoglobinase. Approximately 100 μg of protein in homogenates of adult S. mansoni worms (lanes 1 and 3) and 7 μg of enzyme released from agarose-1-phenylalanine (lanes 2 and 4) were subjected to electrophoresis on a 12.5% SDS gel. Lanes 1 and 2 were silver stained, and lanes 2 and 4 were transferred to nitrocellulose and reacted with anti-hemoglobinase antisera, followed by protein A coupled to horseradish peroxidase. Numbers refer to estimated molecular weight of the purified enzyme. The band near the top of lane 2 was an artifact also seen in lanes without loaded protein.](image-url)
Antibody binding was visualized with protein A coupled to horseradish peroxidase and stained with Coomassie Blue.

The anti-hemoglobinase antisera were preabsorbed to electrophoresed proteins in lysogens containing XHb1 (lane 1) and XHb2 (lane 2) were subjected to electrophoresis on a 10% SDS gel and transferred to nitrocellulose. The anti-hemoglobinase antisera were preabsorbed to remove E. coli antibodies and was added to the nitrocellulose. Antibody binding was visualized with protein A coupled to horseradish peroxidase. B, proteins in lysogens of XHb2 (lane 1) and in pooled fractions eluted from a DEAE Bio-Gel column (lane 2) were electrophoresed and stained with Coomassie Blue.

The anti-hemoglobinase antisera were added to the wells and antibody binding determined using goat anti-rabbit IgG coupled to alkaline phosphatase. One-hundred nanograms of native enzyme eliminated binding to the enzyme immobilized on the microtiter plates. Furthermore, control absorption experiments using up to 1 µg of β-galactosidase or an unrelated fusion protein had no significant effect on the antisera binding to hemoglobinase. Further confirmation of the identity of the polypeptide encoded by λHb2 was obtained using rabbit antisera raised against the partially purified fusion protein. In Western blotting experiments these antisera recognized protein of $M_r = 31,000$ in native enzyme preparations and homogenates of S. mansoni worms (Fig. 4).

We subsequently compared the biochemical characteristics of the protein encoded by λHb2 with hemoglobinase. The fusion protein was subjected to chromatography on agarose-

Cloning of S. mansoni Hemoglobinase

**Fig. 2. Identification of λgtll amp3 clones-encoding hemoglobinase and purification of λHb2 fusion protein.** A, proteins produced by lysogens containing λHb1 (lane 1) and λHb2 (lane 2) were subjected to electrophoresis on 10% SDS gels and transferred to nitrocellulose. The anti-hemoglobinase antisera were preabsorbed to remove E. coli antibodies and was added to the nitrocellulose. Antibody binding was visualized with protein A coupled to horseradish peroxidase. B, proteins in lysogens of λHb2 (lane 1) and in pooled fractions eluted from a DEAE Bio-Gel column (lane 2) were electrophoresed and stained with Coomassie Blue.

**Fig. 3. Absorption of antibodies against native hemoglobinase by λHb2 fusion protein.** One-hundred nanograms of native hemoglobinase were bound to polyvinyl chloride microtiter wells in 100 µl of PBS. Anti-hemoglobinase antiserum in a 1:5000 dilution were electrophoresed on a 10% SDS gel, transferred to nitrocellulose, and reacted with anti-λHb2 fusion protein antiserum followed by Protein A-horse radish peroxidase conjugate.

**Fig. 4. Anti-fusion protein antisera recognized the native hemoglobinase.** Approximately 80 µg of protein in homogenates of adult S. mansoni worms (lane 1) and 25 µg of native enzyme (lane 2) were electrophoresed on a 10% SDS gel, transferred to nitrocellulose, and reacted with anti-λHb2 fusion protein antiserum followed by Protein A-horse radish peroxidase conjugate.

**Fig. 5. The λHb2 fusion protein bound to agarose-L-phenylalanine and exhibited enzymatic activity.** Partially purified λHb2 fusion protein (A) and native enzyme purified by chromatography on Sephadex G-200 (B) were loaded onto a 1-ml agarose-L-phenylalanine column equilibrated in 0.2 M sodium acetate, pH 4.0. Protein was eluted with 0.2 M acetic acid. Total protein was monitored by absorbance at 280 nm (open circles). Globin was incubated with aliquots of each fraction. Following precipitation with trichloroacetic acid, the absorbance of the supernatant at 280 nm was determined (closed circles).

L-phenylalanine. The bacterially synthesized fusion protein bound to, and was released from, the affinity column in a similar fashion as was S. mansoni hemoglobinase (Fig. 5). Aliquots of column fractions eluted with 0.2 M acetic acid were capable of digesting hemoglobin. Neither β-galactosidase nor the unrelated fusion protein bound to the affinity column. Furthermore, no proteolytic degradation of globin was observed using aliquots of fractions containing material washed through the column with 0.2 M sodium acetate, pH 4.0, or released with 0.2 M acetic acid.

**DNA Sequence of Clones λHb1 and λHb2—** The nucleotide-
Cloning of S. mansoni Hemoglobinase

**Fig. 6. Sequence of \( \lambda Hb2 \) insert.** A partial restriction map, sequencing strategy, nucleotide sequence, and translation of the frame in frame with \( \beta \)-galactosidase are shown. A sequence conforming to a consensus sequence near the start of translation of eucaryotic mRNAs (32, 33) is underlined. Boxes indicate polyadenylation addition signals.

sequencing strategy, partial restriction map, and nucleotide sequence of the \( \lambda Hb2 \) insert are shown in Fig. 6. A long open reading frame which was in frame with \( \beta \)-galactosidase is also indicated. The predicted protein starting with the indicated methionine is 353 residues long and would encode a 41,000 protein. A short sequence conforming to a consensus sequence near the start of translation of eucaryotic mRNAs is also shown (32, 33). There were two polyadenylation signals centered 16 and 43 base pairs upstream of the poly A tract. The G-C content of the \( \lambda Hb2 \) insert was 34.5%. The \( \lambda Hb1 \) cDNA sequence was also determined. It was identical to residues 613-950 in the sequence of the \( \lambda Hb2 \) insert. Computer searches comparing nucleotide and amino acid sequences encoded by \( \lambda Hb2 \) against the Dayhoff and Microgenie data banks yielded no significant homologies.

**Hemoglobinase Gene Copy Number and Expression during the S. mansoni Life Cycle**—To estimate the gene copy number corresponding to \( \lambda Hb2 \), we performed Southern blot analyses. Eight, six-base pair recognition restriction enzymes each produced 10-12 fragments of schistosome DNA which hybridized with the \( \lambda Hb2 \) insert. A subset of these bands hybridized with the insert from \( \lambda Hb1 \). To quantify the gene copy number, we performed gene titration experiments (Fig. 7). Comparison of the intensity of hybridization of known amounts of genomic DNA and plasmid DNA containing the \( \lambda Hb2 \) insert indicated that hemoglobinase genes were present in multiple copies in the S. mansoni genome.

We subsequently characterized hemoglobinase gene expression during the S. mansoni life cycle using the \( \lambda Hb2 \) cDNA insert as a probe on Northern blots. Fig. 7 shows that the insert hybridized to a mRNA class of approximately 1,500 bases in adult and immature worms. The intensity of hybridization was strongest in females compared to male and immature worms. In cercariae, a smaller mRNA class of approximately 1,400 bases was observed while no mRNA was observed in eggs. The presence of protein reactive with the anti-hemoglobinase antisera in the different stages of the parasite life cycle was also examined (Fig. 7). A \( M_f = 31,000 \) protein class was observed in eggs, cercariae, 3-h-old schistosomula and adult worms. This protein was present in higher abundance in females compared to other stages. Higher molecular weight proteins were also visualized in cercariae and schistosomula.
DISCUSSION

We report isolation and characterization of cDNAs encoding *S. mansoni* protease. Our strategy involved purification of the \( M_r = 31,000 \) native hemoglobinase and immunologic screening of an expression cDNA library with antisera directed against the purified protein. The \( \alpha \text{Hb2} \) fusion protein shared antigenic determinants with hemoglobinase. Up to 50% of the antibodies raised against the native enzyme could be absorbed with the fusion protein but not with \( \beta \)-galactosidase or an unrelated fusion protein encoded by a schistosome cDNA. The lack of complete absorption may reflect features of bacterial production of the fusion protein in that carbohydrates may be lacking or that the native enzyme preparation contained multiple proteases. Further immunologic confirmation of the identity of \( \alpha \text{Hb2} \) was offered by the reaction of bacterial production of the fusion protein in that carbohydrate or an unrelated fusion protein encoded by a schistosome cDNA. The lack of complete absorption may reflect features of bacterial production of the fusion protein in that carbohydrate may be lacking or that the native enzyme preparation contained multiple proteases. Further immunologic confirmation of the identity of \( \alpha \text{Hb2} \) was offered by the reaction of the anti-fusion protein antisera with hemoglobinase. Biochemical properties exhibited by the \( \alpha \text{Hb2} \) fusion protein were also similar to those shown by the native enzyme. Both proteins bound to agarose-\( L \)-phenylalanine and exhibited the ability to degrade hemoglobin following release. The enzymatic activity of the fusion protein probably did not result from a contaminating bacterial protease since an unrelated fusion protein purified under similar conditions was incapable of hemoglobin degradation. The finding of hemoglobin degradation is one of a few cases in which a \( \beta \)-galactosidase fusion protein exhibited activity (34-37). Taken together, these results indicate that \( \alpha \text{Hb2} \) encodes \( S. \text{mansoni} \) hemoglobinase.

The cDNA sequence of \( \alpha \text{Hb2} \) predicted one long open reading frame which coincided with the lac gene reading frame. Starting with the first methionine in this frame, the protein would be \( M_r = 41,000 \), somewhat larger than the native enzyme (\( M_r = 31,000 \)). Two reports have described \( M_r = 41,000 \), somewhat larger than the native enzyme (\( M_r = 31,000 \)). Two reports have described \( M_r = 27,000 \) species (16, 17).

Members of the hemoglobinase gene family may be differentially expressed during the *S. mansoni* life cycle. Hemoglobinase protein and mRNA levels were in highest abundance in female worms compared to male worms, immature worms, and cercariae. This was in agreement with previous findings indicating 8-fold higher hemoglobinase activity in female worms compared to males (17). Since schistosomula in culture begin to consume red blood cells after four days (38, 39), protein reactive with the antisera in cercariae and 3-h-old schistosomula may represent homologous enzymes used for different functions. It is of further interest that mRNA hybridizing with the \( \alpha \text{Hb2} \) cDNA was not observed in eggs while protein reactive with the anti-hemoglobinase antisera was. The egg mRNA might have been below the limits of detection. Alternatively, the egg protein may have been of maternal origin.

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Note Added in Proof—A cDNA extending 84 base pairs upstream of the sequence in Fig. 6 has been recently isolated. In the same reading frame it predicts 30 of 32 amino acids determined for the N terminus of *S. mansoni* hemoglobinase (Shawar, S. M., Cook, R. G., and Dresden, M. H. (1987) *Fed. Proc.* **46**, 2148).

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