

Aedes aegypti: Characterization of a Hemolymph Polypeptide Expressed during Melanotic Encapsulation of Filarial Worms

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BEERNTSEN, B. T., SEVERSON, D. W., AND CHRISTENSEN, B. M. 1994. *Aedes aegypti*: Characterization of a hemolymph polypeptide expressed during melanotic encapsulation of filarial worms. *Experimental Parasitology* 79, 312-321. We report on the initial characterization of an 84-kDa polypeptide that is differentially expressed in *Aedes aegypti* during melanotic encapsulation immune reactions against filarial worms. [³⁵S]Methionine-labeled hemolymph from mosquitoes inoculated with saline, parasites that are melanized, or parasites that are not melanized was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis. Results show that the level of the 84-kDa polypeptide increases considerably in those mosquitoes undergoing encapsulation reactions against parasites but remains down-regulated in those mosquitoes exposed to parasites that are not melanized or are undergoing wound healing responses (saline-inoculated). Experiments involving glycosidase treatment of hemolymph samples indicate that this polypeptide is not heavily glycosylated. Amino acid microsequencing was performed and two internal sequence fragments (15 continuous amino acids and 12 noncontinuous amino acids) were obtained. Analysis of these sequences to known sequences in a protein database did not yield any conclusive information as to the identity of the 84-kDa polypeptide. Therefore, degenerate oligonucleotide primers were designed, based on the sequence of the 15-amino-acid fragment, and used with the polymerase chain reaction (PCR) to amplify from *A. aegypti* genomic DNA the region between the primers. The PCR product was cloned and sequenced to verify that the nucleic acid sequence matched the known protein sequence. Screening of an *A. aegypti* cDNA library with this small PCR-generated clone resulted in the selection of an approximately 540-bp clone. Northern analysis with this larger cDNA clone indicates that it hybridizes to an approximately 2.0-kb message that is differentially expressed in mosquitoes undergoing melanotic encapsulation reactions against filarial worms. Furthermore, sequencing of this approximately 540-bp clone showed that it contained the 15-amino-acid sequence that had been used to design the degenerate PCR primers, indicating that an appropriate clone was selected. However, sequence analysis of this clone at the protein and nucleic acid level did not provide any conclusive answers to the identity or function of the 84-kDa polypeptide. © 1994 Academic Press, Inc.

INDEX DESCRIPTORS AND ABBREVIATIONS: *Dirofilaria immitis*; *Brugia pahangi*; Nematoda; Filarioidea; *Aedes aegypti*; Diptera; Culicidae; Biotechnology Center (BTC); Basic Local Alignment Search Tool (BLAST); Differentially expressed polypeptide; Hemolymph; Immune response; Liverpool (LVP); Low melting point (LMP); Melanotic encapsulation; Phosphate buffered saline (PBS); Polyvinylidene difluoride (PVDF); Polymerase chain reaction (PCR); Postinoculation (PI); Restriction fragment length polymorphisms (RFLP); Relative humidity (RH); Messenger RNA (mRNA); Ribosomal RNA (rRNA); Room temperature (RT); Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

INTRODUCTION

Vector competence is dependent, in part, on the intricate relationship between the

The sequence data reported herein have been submitted to GenBank under Accession No. U11235.

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vector and the parasite that it transmits. This relationship is a delicate balance that can be influenced by inherent vector control mechanisms as well as by parasite-associated products. In some vectors, parasite development to the infective stage is prevented due to a physiological incompat-

ibility (Christensen and Severson 1993), while in other vectors an active immune response is directed against the invading parasite, resulting in parasite destruction (Yamamoto *et al.* 1985; Beerntsen *et al.* 1989).

In mosquitoes, the immune response against the parasites that they transmit involves both cellular and humoral components of the hemolymph, and this response can effectively limit or prevent a species from functioning as a competent vector. Against metazoan and certain protozoan parasites, this response takes the form of a melanotic encapsulation reaction that effectively sequesters and kills parasites in capsules composed of hemocytes and/or protein polyphenolic materials (Christensen and Tracy 1989).

This melanotic encapsulation response functions as the control mechanism in select strains of *Anopheles gambiae* by destroying the ookinete stage of several species of *Plasmodium* (Collins *et al.* 1986) and the microfilariae of *Brugia malayi* in the mosquito, *Armigeres subalbatus* (Yamamoto *et al.* 1985; Beerntsen *et al.* 1989). It also has been suggested that this melanization reaction might decrease the parasite burden in natural vectors, resulting in an increased survival for both the parasite and the mosquito (Christensen 1981).

Although a thorough understanding of the biochemical and cellular aspects of melanotic encapsulation is not yet available (Christensen and Tracy 1989; Christensen and Severson 1993), it is known that hemocytes are necessary for effective melanization of parasites (Chen and Laurence 1985, 1987; Forton *et al.* 1985; Christensen and Forton 1986). Following immune activation, hemocyte populations increase in number (Christensen *et al.* 1989), and specific alterations occur in hemocyte surface molecules (Nappi and Christensen 1986; Li and Christensen 1990; Spray and Christensen 1991). Furthermore, immune activation results in increases in the activity of hemocyte phenol oxidase, an enzyme nec-

essary for the production of melanotic materials (Li *et al.* 1992), and the differential expression of an 84-kDa polypeptide that seems to be released into the plasma (Beerntsen and Christensen 1990).

Although the role and importance of hemolymph plasma proteins in the immune responses of insects against bacteria have been well documented (Dunn 1990), analysis of hemolymph proteins in mosquitoes undergoing melanotic encapsulation reactions against filarial worms is limited to our report of an 84-kDa polypeptide that is differentially expressed in the hemolymph of *Aedes aegypti* that are undergoing wound healing and/or melanotic encapsulation responses against filarial worms (Beerntsen and Christensen 1990). This polypeptide is not a heat shock (stress) protein and seems to be produced by hemocytes and not by the fat body, a common source of immune proteins in insects (Dunn 1990). Herein, we describe additional experiments designed to better identify this 84-kDa polypeptide and characterize its role in melanotic encapsulation responses.

MATERIALS AND METHODS

Mosquito maintenance. *Aedes aegypti* (LVP) were obtained originally from the University of London in 1977 and were reared as previously described (Christensen and Sutherland 1984). Mosquitoes from all experiments were 2–6 days old and were maintained in an environmental chamber at $26.5 \pm 0.3^\circ\text{C}$, $80 \pm 5\%$ RH, and with a 16-hr light and an 8-hr dark cycle, including a 90-min crepuscular period at the beginning and end of each light cycle.

In vivo labeling of mosquitoes with [^{35}S]methionine. Mosquitoes were exposed to the label contained in a hanging drop of sucrose and separated using a Geiger counter following previously described methods (Beerntsen and Christensen 1990). Labeled mosquitoes used in inoculation experiments were allowed to recover from CO_2 and cold anesthesia for 1 hr prior to inoculation.

Isolation and inoculation of microfilariae. *Brugia pahangi* and *Dirofilaria immitis* microfilariae were isolated from blood of infected jirds, *Meriones unguiculatus*, and beagles, respectively, following previously described protocols (Christensen *et al.* 1984). Midgut-derived microfilariae were obtained by allowing micro-

filariae to penetrate a mosquito midgut *in vitro* prior to inoculation into [³⁵S]methionine-labeled mosquitoes (Lafond *et al.* 1985). Inoculation of microfilariae followed established procedures (Christensen 1981). Controls included uninoculated and saline-inoculated [³⁵S]methionine-labeled mosquitoes.

Hemolymph collection and protein determination. Hemolymph was collected by perfusion of the mosquito body cavity with *Aedes* saline (Hayes 1953) as previously described (Beerntsen and Christensen 1990). Cells were removed from the hemolymph by centrifugation and the plasma was then used for analysis. Protein concentration was determined using a commercially available microassay kit (Pierce, Rockford, IL).

Electrophoresis and fluorography. Sample preparation and SDS-PAGE followed previously published protocols (Beerntsen and Christensen 1990).

Polyclonal antibody production. To obtain large enough quantities of the 84-kDa polypeptide for injection into a rabbit, hemolymph from 75 *D. immitis*-inoculated mosquitoes was collected at 24–30 hr PI. The sample was subjected to lyophilization to remove fluid, sample buffer was added, and the mixture frozen at –70°C. Three such samples were separated by SDS-PAGE and stained with Coomassie blue, and the bands containing the 84-kDa polypeptide were excised from the gel. The bands were prepared for injection following previously described methods (Dunbar 1987). Because the quantity of antigen (18 µg) was limited, it was mixed with complete Freund's adjuvant and injected directly into the rabbit's surgically exposed popliteal lymph nodes, which were better visualized by the presence of previously injected Evan's blue dye (Sigel *et al.* 1983). An initial booster shot, containing two 84-kDa polypeptide bands (12 µg) in incomplete Freund's adjuvant and PBS, was injected subcutaneously at 4 weeks after the primary injection. After a second booster shot (12 µg injected intradermally), anti-84-kDa polyclonal antibodies were detected by Western blot analysis using immune-activated hemolymph, collected at 24–30 hr PI, as the antigen.

Glycoprotein analysis. Immune-activated hemolymph samples (5 µg) were denatured in 1% SDS and incubated at 100°C for 2 min, and an equal volume of incubation buffer (50 mM Na₂HPO₄, pH 7.2, 50 mM EDTA, 1% β-mercaptoethanol, and 5% octylglucoside) was added. The prep was placed at 100°C for 2 min, and then 0.4 units of *N*-glycosidase F (Boehringer Mannheim, Indianapolis, IN), an enzyme that cleaves *N*-linked high mannose, hybrid, and complex oligosaccharides from glycoproteins, was added and the prep incubated at 37°C for 18 hr. A control hemolymph prep lacked enzyme. *N*-Glycosidase F also was added to alkaline phosphatase, a glycosylated protein that served as a positive control. Coomassie blue staining

of the SDS-PAGE gel containing this positive control was performed to observe the expected mobility shift in the protein.

The 84-kDa polypeptide was identified in glycosidase-treated and control hemolymph samples using anti-84-kDa polyclonal antibodies. The samples were separated by SDS-PAGE and blotted onto a PVDF membrane using a transphor electrophoresis unit (Hoefer, San Francisco, CA). Following blockage with PBS-1% instant nonfat dried milk (blocking buffer) at RT for 2 hr, the membrane was incubated with anti-84-kDa polyclonal antibodies (1:1000 dilution) in blocking buffer at RT for 1 hr. The membrane was washed four times with PBS/0.05% Tween (Sigma, St. Louis, MO) (5 min/wash) and then incubated in goat anti-rabbit IgG conjugated to alkaline phosphatase (1:1000 dilution) for 1 hr at RT. The membrane was washed four times with PBS/0.05% Tween (5 min/wash) and incubated in developing buffer (50 mM Tris, 3 mM MgCl₂, pH 9.8) for 5 min at RT. To detect antibody binding, the membrane was incubated in developing buffer containing nitro blue tetrazolium (0.01%) and 5-bromo-4-chloro-3-indolyl phosphate (0.05 mg/ml).

Amino acid microsequencing. Microsequence data were obtained by following the procedures outlined by Matsudaira (1987). Briefly, hemolymph from *D. immitis*-inoculated mosquitoes was collected and concentrated, separated by SDS-PAGE, and then electroblotted to a PVDF membrane using a CAPS transfer buffer (10 mM 3-[cyclohexylamino]-1-propanesulfonic acid, 10% MeOH, pH 11.0, 0.4 A, 3.5 hr). The membrane was stained with Coomassie blue to visualize the polypeptide and the band cut out and directly sequenced by the UW-Madison BTC. Because the N-terminus of the sequence was blocked, the BTC subjected the sample to a cyanogen bromide cleavage to obtain internal sequence information.

Primer synthesis and DNA amplification. Degenerate oligonucleotide primers, containing 5' *Eco*RI restriction sites at the amino terminus (5' CCCGAATTCATGCYNACNGGNATHTA 3') and at the carboxy terminus (5' CCCGAATTCARYTGNCNGTDA-TRTC 3') of the 15-amino-acid base sequence fragment, were synthesized and then used in PCR (Saiki *et al.* 1988). The PCR reaction mixture (total volume of 100 µl) contained reaction buffer provided by the *Taq* DNA polymerase supplier (Promega, Madison, WI), 200 µM each dNTP, 0.12 µM each of the two sets of primers, 2.0 units of *Taq* DNA polymerase, and 1.5 µg of female LVP genomic DNA. The reaction mixture was subjected to an initial 5 cycles of PCR with 1 min at 93°C, 1 min at 42°C, and 1 min at 71.5°C before undergoing 25 cycles with 1 min at 93°C, 1 min at 62°C, and 2 min at 71.5°C. The resulting PCR products (7.5 µl) were then reamplified (2×) using 25 cycles with 1 min at 93°C and 4 min at 71.5°C. A portion of the PCR

products was separated on a 4% NuSieve (FMC Bio-products, Rockland, ME) agarose gel and stained with ethidium bromide to determine that the appropriate-size product (62 bp) had been amplified.

Cloning and sequencing of the PCR products. The PCR products were ethanol precipitated and then *EcoRI*-digested prior to electrophoresis on a 2% LMP agarose gel. The *EcoRI*-digested fragment was visualized by ethidium bromide staining and excised from the gel. Ligations of this fragment to the *EcoRI*-digested and dephosphorylated plasmid vector, pGEM3Zf⁻ (Promega, Madison, WI), were performed following the rapid cloning protocol of Sambrook *et al.* (1989). This plasmid then was used to transform JM109 cells made competent by the CaCl₂ method (Maniatis *et al.* 1982). Colonies were screened using PCR as previously described (Severson *et al.* 1993). These PCR products were separated on 0.6% agarose gels to ensure that the appropriate-size insert was present. A large scale plasmid prep was performed using lysis by alkali followed by purification of the plasmid DNA by precipitation with polyethylene glycol (Sambrook *et al.* 1989). The insert was sequenced using a commercially available kit (Sequenase) following the supplier's recommendations (United States Biochemical, Cleveland, OH). The sequence data obtained indicated that the correct sequence was amplified. The insert, designated B6, then was amplified using PCR with SP6 and T7 primers following a previously described protocol (Severson *et al.* 1993) and LMP purified (Sambrook *et al.* 1989) prior to use in probe production.

Probe preparation. ³²P-labeled DNA probes were produced by specific priming (B6 insert) with the SP6 and T7 primers or random priming (Feinberg and Vogelstein 1983, 1984) to a specific activity of about 1.0–1.6 × 10⁹ cpm/μg. Free dNTPs were separated by selective precipitation of the insert in 2 M ammonium acetate or by column chromatography through Biogel P60 (Bio-Rad, Richmond, CA) equilibrated in elution buffer (0.5% NaDodSO₄, 1 mM EDTA, 5 μg/ml Torula yeast RNA, 20 mM Tris, pH 7.4).

DNA and RNA isolation. DNA from individual adult mosquitoes was isolated using previously described methods (Severson *et al.* 1993). Total RNA was prepared from uninoculated and parasite-inoculated (i.e., immune-activated) adult female *A. aegypti* mosquitoes using a protocol described elsewhere (Chomczynski and Sacchi 1987).

Southern and Northern analysis. *EcoRI*-digested restriction fragments of genomic DNA from individual mosquitoes were size-fractionated on 0.9% agarose gels. DNA was photonicked by exposure at about 7 cm to a uv germicidal lamp for 4 min, denatured (150 mM NaOH, 3 mM EDTA), neutralized (150 mM NaPO₄, pH 7.8), and transferred to GeneScreen Plus (NEN, Boston, MA) by capillary action in 25 mM Na₂P₂O₇. For Northern blots, 2 μg total RNA from uninoculated

and parasite-inoculated mosquitoes (collected at 12 and 24 hr PI) was separated in formaldehyde agarose gels as previously described (Sambrook *et al.* 1989). A mosquito ribosomal protein cDNA, designated pL8 (Durbin *et al.* 1988), was used as a positive control to ensure that equal amounts of RNA were present. Following transfer, the filters were air-dried and baked *in vacuo* at 80°C for 3–5 hr. Prehybridization/hybridization and membrane washes were conducted at 65°C in glass bottles in a rotating oven (National Labnet, Woodbridge, NJ). Conditions for prehybridization and hybridization have been described previously (Severson *et al.* 1993). Filters were washed twice in 2× SSC with 0.1% NaDodSO₄ at 65°C for 15 min and twice in 1× SSC (B6 probe) or 0.2× SSC (D6.12) with 0.1% NaDodSO₄ at 65°C for 15 min. Kodak XAR-5 film was exposed to the filters for 3–6 days at –70°C with an intensifying screen.

Library screening. Plaque lifts from an *A. aegypti* (Bahama strain) cDNA library (James *et al.* 1989) were screened with ³²P-labeled B6 insert and positive clones were selected. Clones were plaque-purified and phage DNA isolated with Magic Lambda Preps (Promega). An approximately 540-bp *EcoRI*-digested fragment subsequently was subcloned into the *EcoRI* site of pGEM3Zf⁻ (Promega). This clone, designated D6.12, was sequenced and then used to probe an *EcoRI*-digested genomic *A. aegypti* blot and a Northern blot of RNA from parasite-inoculated (immune-activated) and uninoculated mosquitoes.

Sequence analysis of the D6.12 clone. The translated protein sequence, encoding the open reading frame of the D6.12 clone, was analyzed against sequences in the Swiss-Prot or Pir protein databases using the BLAST program (Altschul *et al.* 1990) or the FastA program of the GCG sequence analysis software package (Genetics Computer Group, Madison, WI). The nucleic acid sequence was analyzed against sequences in the GenBank/EMBL database using the FastA program for nucleic acids. A motifs program (Genetics Computer Group) that searches for sequence motifs also was used in the analysis.

RESULTS

SDS-PAGE analysis of the expression of the 84-kDa polypeptide. [³⁵S]Methionine-labeled mosquitoes were inoculated with microfilariae that are not normally melanized to assess whether the presence of a parasite, in the absence of melanotic encapsulation reactions, results in increased levels of the 84-kDa polypeptide. SDS-PAGE analysis of hemolymph collected from labeled mosquitoes inoculated with blood-

isolated *B. pahangi* (melanized) and midgut-derived *B. pahangi* (not melanized) indicates that expression of the 84-kDa polypeptide in mosquitoes inoculated with midgut-derived microfilariae is similar to that observed in a wound healing control (i.e., saline-inoculated) and is not as elevated as that seen when blood-isolated microfilariae are inoculated (Fig. 1). Therefore, differential expression of the 84-kDa polypeptide seems to be limited to situations in which melanotic materials are required for either parasite destruction or wound healing responses.

Effect of glycosidase treatment on the 84-kDa polypeptide. Because carbohydrate staining techniques, e.g., periodic-shiff staining, were not sensitive enough to detect proteins in dilute hemolymph samples, enzymatic deglycosylation with *N*-glycosidase F, followed by Western blotting with

anti-84-kDa polyclonal antibodies, was used to determine whether the 84-kDa polypeptide contained carbohydrate residues. Results show that there is no or only a very slight mobility shift in the 84-kDa polypeptide between glycosidase-treated and untreated hemolymph samples (Fig. 2), indicating that this polypeptide contains few, if any, N-linked sugar residues. The appropriate mobility shift was observed in the alkaline phosphatase positive control, indicating that the assay was working properly (data not shown).

Cloning of a partial amino acid sequence product. To identify this polypeptide and its potential role in melanotic encapsulation reactions, protein sequence data were obtained by blotting the 84-kDa polypeptide to a membrane, staining it with Coomassie blue, and then directly sequencing it. Because the amino terminus was blocked, a cyanogen bromide cleavage of the 84-kDa

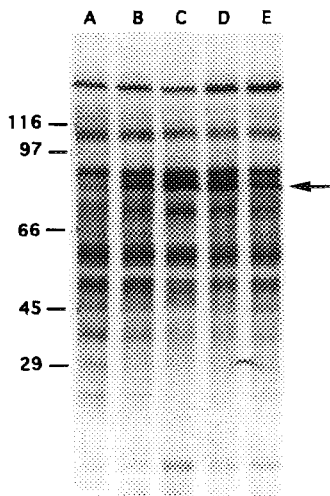


FIG. 1. Fluorogram of [35 S]methionine-labeled hemolymph polypeptides separated by SDS-PAGE (10% gel). Samples include cell-free hemolymph from uninoculated (A), saline-inoculated (B), blood-isolated *Dirofilaria immitis* microfilariae-inoculated (C), blood-isolated *Brugia pahangi* microfilariae-inoculated (D), and midgut-derived *Brugia pahangi* microfilariae-inoculated (E) *Aedes aegypti* at 48 hr postinoculation. The arrow indicates the position of the 84-kDa polypeptide. Molecular weight standards (kDa) are to the left of the figure.

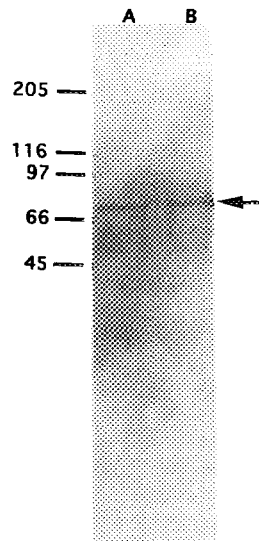


FIG. 2. Western blot of glycosidase-treated (A) and non-glycosidase-treated (B) cell-free hemolymph samples of *Aedes aegypti*. Samples were loaded onto a SDS gel and analyzed by Western blot using rabbit anti-84-kDa polyclonal anti-sera as the first antibody and alkaline phosphatase-conjugated rabbit anti-IgG as the second antibody. Molecular weight standards (kDa) are to the left of the figure.

polypeptide was performed, resulting in two internal amino acid sequence fragments. The first fragment consisted of 15 amino acids with the following sequence: amino terminus-M-P-T-G-I-Y-V-R-K-D-I-T-G-Q-L-carboxy terminus. The second sequence consisted of 12 noncontinuous identified residues: amino terminus-M-X-X-P-D-A-A-I-Q-V-Q-X-X-A-G-X-D-carboxy terminus. Degenerate primers, made from the amino and carboxy termini of the 15-amino-acid fragment, were used, in conjunction with PCR, to amplify from mosquito genomic DNA the region between the primers. This procedure resulted in the amplification of a product that when cloned, sequenced, and translated was identical to the 15-amino-acid fragment.

Southern analysis. This PCR-amplified clone, designated B6, then was used to probe a Southern blot of *Eco*RI-digested genomic DNA from individual female and male mosquitoes to ensure that it indeed hybridized to mosquito DNA. It hybridized to three restriction fragments ranging in size from 1.0 to 2.5 kb (data not shown). Screening of an *A. aegypti* cDNA library with this clone resulted in the selection of an approximately 540-bp clone, designated D6.12, that was subsequently subcloned into a plasmid vector. This larger clone then was used to probe the same *Eco*RI-digested genomic mosquito blot of individual mosquitoes. It also recognized the same fragments (a_1 , a_2 , and a_3) as the smaller clone (Fig. 3), indicating that an appropriate clone had been isolated. This blot also shows that there are three different RFLP alleles (a_1 , a_2 , and a_3) present and that for any individual mosquito there are two RFLP alleles with several combinations possible (e.g., a_1a_1 , a_1a_2 , a_1a_3 , and a_2a_3). This information is valuable because it suggests that the D6.12 clone is probably a single-copy gene.

Northern analysis. Northern analysis of total RNA from uninoculated and parasite-inoculated mosquitoes indicates that this

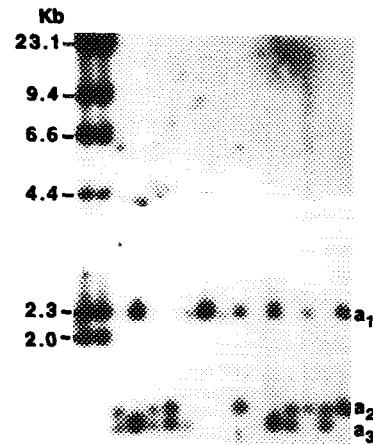


FIG. 3. Southern blot analysis of *Eco*RI-digested *Aedes aegypti* genomic DNA from individual male (odd lanes 3–15) and female (even lanes 4–16) mosquitoes hybridized with the 32 P-labeled cDNA clone D6.12. *Hind*III-digested phage DNA markers (kb) are in the left two lanes. Hybridization was performed at 65°C and the filter washed as described under Materials and Methods.

larger clone hybridizes to an approximately 2.0-kb message (Fig. 4), a size that would be expected from a nonglycosylated 84-kDa protein. Although equal amounts of total RNA were loaded onto each lane as evi-

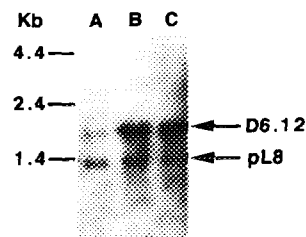


FIG. 4. Northern blot analysis of RNA from immune-activated (parasite-inoculated) and non-immune-activated (uninoculated) mosquitoes hybridized with 32 P-labeled D6.12 and pL8, a mosquito ribosomal protein cDNA that illustrates equivalent amounts of RNA. Two micrograms of total RNA from non-immune-activated (A), 12-hr immune-activated (B), or 24-hr immune-activated (C) *Aedes aegypti* was subjected to electrophoresis and then blotted onto a nylon membrane. The top arrow indicates the position of the 2.0-kb message. RNA size markers are to the left of the figure. Hybridization was performed at 65°C and the filter washed as described under Materials and Methods.

denced by the pattern from the ribosomal protein cDNA probe pL8 (particularly evident in lanes A and C), the clone recognizes an increased message in those mosquitoes undergoing melanotic encapsulation reactions as compared with nonimmune-activated controls. These Northern analysis data correlate well with the known differential expression of the 84-kDa polypeptide in the immune-activated mosquitoes. In uninoculated mosquitoes (non-immune-activated) there is low-level production of the 84-kDa polypeptide present, but upon immune activation with inoculated parasites (blood-isolated), there is increased expression of this polypeptide beginning at approximately 24 hr PI.

Sequence analysis of D6.12. The D6.12 clone was sequenced and translated to determine whether any open reading frames were present (Fig. 5). Only one frame was open, and it contained the same 15-amino-acid sequence as that obtained from amino acid microsequencing. A stop codon was

found near the 3' end of the sequence, as was a short poly(A)⁺ tail.

Sequence analysis of this clone with the BLAST and FastA programs for proteins or FastA program for nucleic acids was inconclusive, as was analysis with the Motifs program. No significant identity to any known motif, nucleic acid, or protein sequence was observed.

DISCUSSION

Until recently, extensive research into the cellular, biochemical, and molecular mechanisms responsible for melanotic encapsulation responses of mosquitoes against filarial worms has been hampered by small sample sizes that made detailed analysis difficult. Now, however, with the advent of sophisticated molecular and biochemical techniques, it is possible to investigate these mechanisms despite a limited amount of material. Therefore, the present study was undertaken to better characterize and attempt to elucidate the role of an

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1 TCG GAC CCA CGT GCC GTA TCT GCA GCC CTG TTC TTC AAC GAC AAG CGC CAG CAG AAG GCC
1 S D P R A V S A A L F F N D K R Q Q K A

61 TGC CCG AAC AAG CTC GCC TCA TCA CAG GGT GCC CCG ATC CGG ATT GTG TCC GCC CAG GAC
21 C P N K L A S S Q G A P I R I V S A Q D

121 CTG ACC AGG CTG GGA GCG AAC AAG CGG CTG ATC TGC CCG AAC CTG GAG CTG CAG GCG ATT
41 L T R L G A N K R L I C P N L E L Q A I

181 GGA AAC TAT CAG GCG TGC AAC GTG GAC TAC ACC ATG CCG ACG GGA ATC TAC GTG CGG AAG
61 G N Y Q A C N V D Y T M P T G I Y V R K

241 GAC ATC ACC GGC CAG CTG GAG GAT AAC ATT GCG CAC GCT TTC GTG GCG CTG TCG GAT AAG
81 D I T G Q L E D N I A H A F V A L S D K

301 TTT GGA CAC GGA GCC AAA AAC GAA GTG GTG TTC GAG ATG TTC GGC GAG TTC AAA GCG GGA
101 F G H G A K N E V V F E M F G E F K A G

361 GAG AAG AAT GTT CTG TTC CAC GAT CGT GCT GCC AAG TTC GGA GTG GCC GGC AAC AAG ATC
121 E K N V L F H D R A A K F G V A G N K I

421 GGC GAA GTG GAC CCG GCC AAC TAT CGG CGG TTG ATG TGC TTG TAG ATA ACA GTT CTG GAC
141 G E V D P A N Y R R L M C L *

481 ATC CGT AGT TGA TTT AGA CAA AAC TGC TTG GAA ATA TAG ATA CCA AAA TGA AAA AAA AAA

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FIG. 5. Nucleic acid and translated protein sequences of the D6.12 clone. The 15-amino-acid sequence, previously obtained by protein microsequencing, is underlined. The asterisk represents a stop codon.

84-kDa polypeptide that has been shown to be differentially expressed in mosquitoes that are undergoing melanotic encapsulation reactions or wound healing responses.

Production of this 84-kDa polypeptide in the hemolymph seems to be associated with situations, like parasite encapsulation and wound healing, in which melanotic materials are required. In a situation where a parasite is present but is not melanotically encapsulated, levels of this polypeptide are low and reflect levels seen following saline inoculations, which induce a healing process that involves the laying down of melanotic materials at the site of the wound (Lackie 1986). Thus, the presence of a parasite alone, without melanotic encapsulation reactions directed against it, does not cause a significant increase in levels of the 84-kDa polypeptide. However, when filarial worms are treated in a manner that elicits melanotic encapsulation reactions, levels of this polypeptide increase considerably (Fig. 1). Because these results, coupled with previous data (Beerntsen and Christensen 1990), suggest that the 84-kDa polypeptide plays a role, directly or indirectly, in the melanotic encapsulation process, efforts were made to obtain protein sequence data.

While we were able to obtain two small internal amino acid sequences, analysis of these sequences against known sequences in a protein database (SwissProt) did not reveal any sequence identities, possibly due to the limited amount of sequence data available. However, based upon these amino acid microsequence data, we designed PCR primers that enabled us to eventually isolate a larger clone (D6.12). Northern analysis with D6.12 showed hybridization to a differentially expressed 2.0-kb message in mosquitoes undergoing melanotic encapsulation reactions, suggesting that a biologically relevant clone was selected. The mechanism that regulates this increased message level is not known. It may be due to increased transcription of the

appropriate mRNA or increased message stability in the immune-activated mosquitoes.

The presence of one of the previously determined internal amino acid sequences in the deduced protein sequence of the D6.12 clone provided additional proof that an appropriate clone was selected. A search of the protein and nucleic acid databases with the D6.12 clone was not successful in determining its identity. Either a high percentage of identity, but only over a very small region, or a much lower identity over a larger region was noted with many different database sequences. For example, in the protein sequence comparisons, the translated protein had a 57% identity in a 14-amino-acid region with a neural cadherin from chicken, cow, or human. In another example, 20% identity in a 60-amino-acid overlap was observed for an epidermal growth factor receptor precursor from *Drosophila melanogaster*. With either of these two examples, the level of identity is not significant considering that the translated protein is 154 amino acids in length. Additionally, analysis of the deduced protein with a motifs program that searches for specific patterns (e.g., enzyme active sites, protein binding sites) did not result in any matches.

In summary, efforts to begin the identification and further characterization of a differentially expressed 84-kDa hemolymph polypeptide in mosquitoes undergoing melanotic encapsulation reactions against filarial worms have been successful despite a limited amount of experimental material. Sensitive biochemical techniques made it possible to obtain protein sequence data from small sample sizes. These data, in turn, enabled us to use molecular techniques to select a biologically relevant clone from a cDNA library. To date, the sequence analysis data suggest that the D6.12 clone might represent an undescribed gene; therefore, the complete sequence of a full-length clone will be neces-

sary to elucidate the role, either direct or indirect, of this 84-kDa polypeptide in destroying filarial worms in mosquitoes by melanotic encapsulation reactions.

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