

The Apyrase Gene of the Vector Mosquito, *Aedes aegypti*, Is Expressed Specifically in the Adult Female Salivary Glands

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SMARTT, C. T., KIM, A. P., GROSSMAN, G. L., AND JAMES, A. A. 1995. The Apyrase gene of the vector mosquito, *Aedes aegypti*, is expressed specifically in the adult female salivary glands. *Experimental Parasitology* 81, 239–248. The yellow fever mosquito, *Aedes aegypti*, expresses a gene, Apyrase (Apy), that encodes an ATP-diphosphohydrolase. The product of this gene is a secreted enzyme that facilitates hematophagy by preventing platelet aggregation in the host. Apy gene expression is limited to the cells of the distal-lateral and medial lobes of the adult female salivary glands. Apyrase protein levels, detectable by antibodies, peak in the salivary glands about 4 days after adult emergence and remain high after a blood meal. Primary sequence analysis of a genomic clone encoding apyrase reveals a unique TAAATA sequence and seven introns, as well as other conserved features of eukaryotic genes. The temporal, sex- and tissue-specific expression of the Apy gene is consistent with its role as encoding a platelet anti-aggregation factor that functions to facilitate hematophagy and decrease probing time. © 1995 Academic Press, Inc.

INDEX DESCRIPTORS AND ABBREVIATIONS: Apyrase; *Aedes aegypti*; mosquito; gene sequence; salivary gland; ADP, adenosine diphosphate; AMP, adenosine monophosphate; ATP, adenosine triphosphate; cDNA, "copy" DNA; SDS, sodium dodecyl sulfate.

INTRODUCTION

Many pathogens are transmitted by arthropod vectors to vertebrate hosts through salivary secretion that occurs while the vectors are feeding on blood. In addition to providing a fluid medium in which pathogens pass into their hosts, the saliva of hematophagous arthropods also has been proposed to facilitate infection (Titus and Ribeiro 1990). The study of salivary glands and their secretory products has revealed many aspects of the process of hematophagy and provided new perspectives for evaluating transmission dynamics of pathogens (James 1994).

Hematophagous arthropod saliva contains proteins that inhibit vasoconstriction, coagulation, and platelet aggregation, all of which are

important components of vertebrate hemostasis (Ribeiro 1987; James 1994). Antihemostatic components in saliva result from the evolutionary selection for active substances to counter host mechanisms that prevent excessive bleeding from wounds. The salivary glands and saliva of all hematophagous arthropods studied to date contain an apyrase, an ATP-diphosphohydrolase (EC 3.6.1.5), that appears to function as a platelet anti-aggregation component and is required for efficient feeding (Ribeiro *et al.* 1991). In addition, levels of apyrase activity are correlated positively with decreased probing time during feeding, leading investigators to suggest that the enzyme facilitates the speed of locating blood (Ribeiro 1987). Recently, we were able to demonstrate that the salivary gland apyrase of *Aedes aegypti* is a member of a protein family that includes 5'-nucleotidases (Champagne *et al.* 1995). We report here that the gene Apyrase (Apy) is expressed specifically in the adult female salivary glands of *A. aegypti*. Expression of this gene is confined to those lobes of the salivary glands that secrete female-enriched products. Apyrase protein levels increase after adult emergence and are not af-

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fects greatly by bloodfeeding. Analysis of a genomic clone encoding apyrase demonstrates that the gene is a distinct member of the 5'-nucleotidases family of genes.

MATERIALS AND METHODS

Animal maintenance. *A. aegypti* mosquitoes of the Rockefeller strain were reared in a 27°C incubator at 80% relative humidity using an 18L-6D light cycle. Unless otherwise specified, the adults used in the following procedures were 3–5 days old after emergence.

Nucleic acid isolation and labeling. Total mosquito RNA was isolated from whole adults and isolated tissues using the method of Chirgwin *et al.* (1979). ³⁵S-labeled sense and antisense RNA for hybridization *in situ* were prepared as in James *et al.* (1989). Radioactively labeled cDNA probes were produced using the random priming kit supplied by Boehringer-Mannheim. End-labeling of oligonucleotides used in primer-extension reactions was carried out as described in the protocol supplied with the AMV reverse transcriptase primer extension system (Promega).

Library construction and screening. An *A. aegypti* genomic library was constructed using partial *Bam*HI-digested total DNA. The restriction fragments were cloned into the Lambda DASH vector using the Lambda DASH II/*Bam*HI cloning kit (Stratagene). Packaging was carried out using the Gigapack II packaging extract (Stratagene). A labeled cDNA clone, λ SGG12, encoding amino acids 153–564 of the conceptual translation product (Champagne *et al.* 1995), was used to screen the genomic library. The apyrase genomic clone, designated *Apy*, was analyzed by restriction digestion and Southern analysis performed as described in Ausubel *et al.* (1992). All hybridizations were performed at 42°C, with 50% formamide.

Northern transfer analyses. Total RNA from mosquito embryos, larvae, pupae, female salivary glands, female carcasses (adult mosquitoes with salivary glands removed), and adult females and males was isolated and electrophoresed on 1.2% agarose/formaldehyde gels (Ausubel *et al.* 1992). Radioactively labeled cDNA was used to probe filters to determine sex-, tissue-, and stage-specific transcription of *Apy* RNA. Hybridizations to filters were performed as outlined in Ausubel *et al.* (1992) using 50% formamide at 42°C. Filters that were reprobed were stripped first in boiling water for 3 min and hybridized with a clone, λ AE1g, with sequences homologous to the rRNA (James *et al.* 1989).

Spatial analysis of *Apy* expression. Hybridization reactions *in situ* were performed on thoraces that were fixed in alcoholic Bouin's solution, dehydrated, embedded in paraffin, sectioned, and processed as described in Grossman and James (1994). Both antisense and control sense-strand RNA probes were used.

Nucleotide sequence determination and analysis. Genomic fragments were sequenced by the method of Sanger *et al.* (1977) using a kit supplied by US Biochemicals. Oli-

gonucleotide primers were obtained commercially to complete sequence analysis of clones in both directions. Sequence data were confirmed by multiple readings in both directions. Sequence analysis was performed using LaserGene for Windows provided by DNASTAR.

Primer extension analysis. End-labeled primer G12C19 (5'-CTATATACGGGCGATTATTCCTACA-3'), a 25-base oligonucleotide primer complementary to the 5'-end of the CDNA clone, λ SGG12-5, encoding amino acids 1–152 of the conceptual translation product (Champagne *et al.* 1995) was annealed to 10 μ g of total female salivary gland RNA at 58°C. All primer extension reactions followed the protocol of Sambrook *et al.* (1989) with the AMV reverse transcription primer extension system kit provided by Promega. The same primer was used to sequence the *Apy* genomic clone and both reactions were run on an 8% 7 M urea polyacrylamide gel.

Immunoblot analysis of the Apyrase protein. Proteins were prepared by homogenizing dissected tissues or whole animals in sample loading buffer as specified by the Bio-Rad denaturing gel protocol. Samples were centrifuged briefly to pellet insoluble material. Protein samples were subjected to SDS-PAGE analysis and transferred in modified Towbin buffer (Towbin and Gordon 1984) to nitrocellulose filters, and immunoblots were performed using antibodies raised to recombinant apyrase peptide (Champagne *et al.* 1995). Varying concentrations of protein, 10–20 μ g, were separated by 10% SDS-PAGE. Blocking was performed overnight with 1% polyvinyl-pyrrolidone in Tris-buffered saline with 0.1% Tween 20. Immunoblot detection was performed as described in the ECL-Western blotting protocols provided by Amersham. The secondary antibody, goat anti-rabbit conjugated to horseradish peroxidase, was obtained from Sigma Chemical Company. Both primary and secondary antibodies were used at a dilution of 1:10,000.

RESULTS

Isolation of a Genomic Clone Homologous to the Apyrase Gene

A cDNA clone, λ SGG12, was found previously to contain a conceptual translation product, portions of which exactly matched sequenced peptides from the isolated apyrase protein (Champagne *et al.* 1995). This cDNA clone was used to isolate the corresponding genomic clone from an *A. aegypti* Lambda DASH genomic library. Primary sequence analysis of the genomic clone revealed that it contains a conceptual translation product that most likely represents the complete coding sequence of apyrase (Fig. 1). This gene has been given the name *Apyrase* (*Apy*). The *Apy* genomic clone contains

approximately 2.1 kb of coding sequence corresponding to the putative primary transcription product. Seven introns, I–VII, are evident when the genomic clone is compared with the primary sequence of the cDNA. Each intron is approximately 60 bp long and contains a consensus splice-junction acceptor and donor sequence (Hartl 1991). The start of transcription was defined by comparing primer extension products generated using RNA as a template with sequence of the *Apy* genomic clone. Using the primer, G12C19, 95- and 96-bp fragments were produced (Fig. 2). The corresponding nucleotides A and G were designated nt +1 and +2, respectively. A unique TATA-like sequence, TAAATA, at nt –27 is the most likely site for the TFIID recognition site. A polyadenylation signal sequence, AATAAAA, is located at nt 2165, at the 3' end of the coding region, and in an area with sequence similarity to the cDNA clone. Translation start and stop codons are evident at nt 34 and 2156, respectively. Sequence comparison of the genomic clone of apyrase and the complete cDNA clone revealed one nucleotide change and a 3-nucleotide insertion in the genomic clone (data not shown). The basepair change at nt 121 in the genomic sequence caused a transversion, C to G, which resulted in an amino acid change, proline to alanine. The addition of three basepairs at nt 1341–1343 inserted the amino acid glycine. All other nucleotides were identical. These differences in genomic and cDNA sequences indicate that the clones are most likely alleles.

Expression of the Gene Encoding Apyrase

Total mosquito RNA isolated from different developmental stages and salivary glands was probed using the ³²P-labeled cDNA clone, *λSGG12*. The cDNA clone hybridized to a 1.8-kb RNA species expressed specifically in the adult female salivary gland samples (Fig. 3). No corresponding signal was detected even after long exposure times in males, female carcasses, embryos, larvae, or pupae. The *Apy* gene appears to express its product in significant abundance as can be seen by comparing the intensity

of its signal with that of the ribosomal RNA in the salivary gland samples.

In situ hybridizations of RNA probes to sections of adult thoraces containing salivary glands was performed to determine the spatial accumulation of the *Apy* transcription products. Positive signals were detected in the cells of the distal–lateral and medial lobes (Fig. 4). No specific localization in male salivary gland sections was detected and sections hybridized with sense-strand RNA probes did not show signals (data not shown).

Immunodetection of Apyrase Protein during Development

Protein samples were prepared from second-, third-, and fourth-instar larvae and early and late pupae and were analyzed by immunoblot procedures using antibodies prepared to recombinant apyrase (Fig. 5). No specific signal corresponding to apyrase protein was detected in the subadult stages. The samples prepared from pupae showed low-molecular-weight, nonspecific binding of the antibodies.

Antibody detection of apyrase protein in dissected salivary glands revealed that the apyrase protein is not detectable in females on the first day after adult eclosion (Fig. 5C), but is detectable on the second day and is maximally expressed by Day 4. Interestingly, apyrase levels remain detectable and high following a blood-meal (Fig. 5D).

DISCUSSION

Hematophagous arthropods have a number of proteins present in their saliva that counteract host hemostasis and increase the efficiency of blood ingestion. Every vector arthropod that has been examined has a component in its saliva that prevents or reduces vasoconstriction, platelet aggregation, and coagulation (Law *et al.* 1992; James 1994). In *A. aegypti*, sialokinins are small peptides present in saliva and function as vasodilatory agents (Ribeiro 1992; Champagne and Ribeiro 1994). In this species, a protein with an apparent molecular weight of 35 kDa present in the salivary glands inhibits coagulation by interacting with Factor Xa (Stark

GTCTTTTAAAGTAATTAATAGTAAATAAGAAAATTTTCATTACAAGTAGAATTACATGCCAGTAGGCCGCTAAATTAATTAATA
 -378
 -288
 TCACGTCGTTTTGATTGCATCGTCGTTGATTATGCATAATATTACATTCAAAATTATGTAATAACATGGACTACGGACGCTGTATA
 -198
 CATGCTACTGTGACTTTGCAAGCTTTTGAAGTCTGTTTCGATTTCGGTTCCAAATTGGCTCTGATTCAAACCTGGAAATTTACTGAGATT
 -108
 ACCAGCAGGAGTAAACTCGATTTCGAGGTAAGTCTGCCATTATTCTTTTGAATAATTTCTGCTCTCTGTTGTGAAGCCTTCTTTGAAA
 -18
 AATGATCACAGCACTCCACGTCGATGATTACAATCAACAGAGCCGATGGACAAAACAAACAGCGCAAGTTGCTTAAATAGAGA
 +1
 CGTTGGTGGGCTCTCAAGTCAAGTGGGCAAAATTTGTTAGTTAAGAAAACATGGCTGGAAGACCGGGTTACAGTGCAGTGATTTTCTAT
 73
 H A G R P G Y S A V I F L
 163
 ACGTAGTGAGTGGCGGTGATTGCAAGGCCACGGATAATATGCCCGTAATAAGGATGTATCAAAGCTGTTTCCCTCAGTTGATT
 Y V V S V A V I A R A T D N M P A N K D V S K L F P L T L I
 253
 ACATAAAGCACTTGCATGCAAGGTTGAGGCTGAGAAAGTTTAAAGCAAACTGTGATCTCAAAATTTGCTATTTTGGTTTGA
 H I N D L H A R I F E
 343
 AGAGACCAATGAAGTCCAAAGCTTGTACCCAGAAGGATCAATGCATTGCCGAATCGCAAGAGTTTATCAAAGATCAAGGATTTC
 E T N M K S N A C T Q K D Q C I A G I A R V Y Q K I K D L L
 433
 CAAAGAGTATGAGAGTAAATCCGATCTATCTCAATGCCGGTGATACTTCCAGGAACTCTGTGTTACAATCTTCTAAGGTGGAATGT
 K E Y E S K N P I Y L N A G D N F Q G T L W Y N L L R W N V
 523
 GACGGCGGATTTTATTAAGAGCTTAAACCGGTCGCCATGTAAGTTTTGGTACTATTTTCAAGATTTTATTAAGAACTAATTTATATTA
 T A D F I K K L K P A A M II
 613
 TCAAAACAGACTCTAGGAAACCAAGGTTTGTATCATACACGAAAGGATTGGCGCTTATCTGGCAGAACTGAATAAAGAGGGAATTC
 T L G N H E F D H T P K G L A P Y L A E L N K E G I P
 703
 AACCATTGTGCAATCTAGTAATGAACAACGACCCGATTTGAAAGTTCAAAATTCAAATCAATAAACTTACTGTTGGTAAGAG
 T I V A N L V M N N D P D L K S S K I P K S I K L T V G K R
 793
 GAAGATAGCTATAATGTTGCTCTGTATGATAAAACGCAATGTAAGTCTACCGATCTCATGAGCTAAGAAATTAATTTGATTAAAGAT
 K I G I I G V L Y D K T H III
 883
 TTCTTTAGAGATAGCCCAACCGGAAAGTTACTTTATCGAATGCCGTAGAACGGTGAGAGGAGGCGGCTGCAATGAAGAAAGATA
 E I A Q T G K V T L S N A V E A V R R E A A A L K K D
 973
 AAATCGATATTATTGTTGCTTGTCCCACTGTAGCTACGAAGAGGACAAAGAAATTCAGCTGAAGCCGCTGACGATATCGATGTAATG
 K I D I I V V L S H C S Y E E D K K I A A E A G D D I D V I
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 V G A H S H S F L Y S P D S K Q P H D P K D K V E G P Y P T
 1153
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 I V E S K N K R K IV I P I
 1243
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 V Q A K S F G K Y V G R L T L Y F D D T G E V Q H W E G Y P
 1333
 TGTGTTTCATCGACCACAAGGTTCAACAAGATCCACAATTTTGAAGATCTGGTCCCTGGCGGAGAGGTTGAAGCAATCGGATCAAC
 V F I D H K V Q Q D P Q I L K D L V P W R E K V E A I G S T
 1423
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 V V G G E T K I E L D R D S C R D Q E C T L G V L Y A D G F
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 A D Q V Y T N D T F
 1603
 GACCGTTTGAATCATTCAAGCAGGCAATTTTCGGAATCCGATCAAGTTGGAAGTATGCTCCCTTCTGCTAACTAATGATATGATCA
 R P F A I I Q A G N F R N P I K V G VI
 1693
 AATTTCAGTAAGCAATTTCTTACGACGAGATTACCAATCTGTGACATTATCGAGGCCGACCATTCGGTTCCACAGCGGATCTGATT
 X I T N G D I I E A A P F G S T A D L I
 1783
 CGATTGAAGGGTGTGACATATGGGACGTTGGCCGAGCATTTCATTCGCGCTGGAAGATGAGGGTGTACAAAATGCTTGAAGTATCGGGA
 R L K G A D I W D V A E H S F A L D D E G R T N C L Q V S G
 1873
 CTGAGGATTGTTATCGATATCAGTAAGCCGATTAGGAGTGGGTAAGAAAATGAAGTTATGACATACAAATCCCAAGCTCTGATGA
 L R I V I D I S K P I R S R V K K I E V M D Y T N P K S D E
 1963
 TTGAACCATTTGGATAAACAAGCAGACTACTACATAGTTGTCCTCATCATATCTGGCCGATGGAAGATGGATTCTTCTGAATGAAAGG
 L K P L D K Q A E Y Y I V V P S Y L A D G K D G F S A M K R
 2053
 GCGACGGCAAGCGGTAAGTATTTTCTGTTTTTTAGTATCCAACGTTTACTTCAATGTCATTATTTTATACAGAACTGGT
 A T A R R VII T G
 2143
 CCTTTGGATTCCGATGTTTCAAAAATATGTGGAATAAAGAAAGTAGATAACCTTAAGTTGGGTAGAGTAATAGTTTGTGAAGGT
 P L D S D V F K N Y V E K I K K V D N L K L G R V I V C K G
 2233
 TCGAATGACTTAGTGACCAATAAAGACGTTACGCTGTATATTCGACATAAGTTAGGTTTATTTTCACTTTTAAATGGTCATTGC
 S K C T *
 2279
 TCTCTGGGAATACATTGTACATGTTTAACACGACAATTTAGTACG

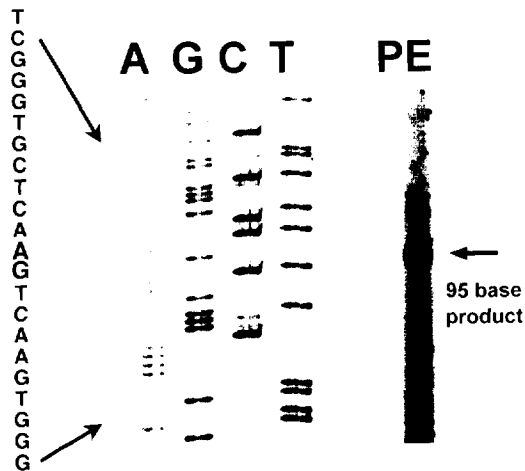


FIG. 2. Primer extension analysis of the 5' end of the *Apyrase* transcription product. Primer extension was carried out on 15 μ g of total female salivary gland RNA using the 5'-end *Apy* (G12C19) oligonucleotide primer. PE represents the primer extension product lane and the arrow points to the product of 95 bp. The primer also was used in dideoxy sequencing of the *Apy* genomic clone (lanes A, G, C, and T). The transcriptional start site is indicated by the doublet corresponding to nucleotides A and G.

and James 1995; James 1994). In addition, the salivary gland apyrase that functions as a platelet anti-aggregation factor has been isolated from *A. aegypti* and shown to be a member of a family of proteins that includes the 5'-nucleotidases (Champagne *et al.* 1995). Thus, the biochemical and molecular characterization of the three major salivary gland anti-hemostatic proteins is almost complete for this vector species.

Apyrase is an ATP-diphosphohydrolase that cleaves specifically di- and triphosphonucleotides into monophosphonucleotides and inorganic phosphate (Ribeiro *et al.* 1984). The enzyme is defined further by its inability to use

AMP as a substrate (Zimmerman 1992). Apyrase in animals has been found in the bovine aorta (Côté *et al.* 1992), rat uterus and mammary glands (Valenzuela *et al.* 1992), human placenta (Kettlun 1993), and the tegument of schistosomes (Vasconcelos *et al.* 1993). All of these enzymes have been implicated as inhibitors of platelet aggregation.

Previous work on apyrases in mosquitoes has been limited to *A. aegypti* (Ribeiro *et al.* 1984, 1985; Marinotti *et al.* 1990; Champagne *et al.* 1995) and members of the genus *Anopheles* (Ribeiro *et al.* 1985; Cupp *et al.* 1994). In *A. aegypti*, apyrase enzymatic activity was detected in female salivary glands with the majority of activity in the distal-lateral and medial lobes (Ribeiro *et al.* 1984). In addition, apyrase activity was detected in male mosquitoes, but at levels 20-fold lower than in females. Apyrase levels are correlated positively with the speed with which mosquitoes obtain a bloodmeal (Ribeiro *et al.* 1985) and Cupp and colleagues (1994) have shown a relationship in the *Anopheles gambiae* species complex between high apyrase levels and preference for feeding on humans.

The spatial and temporal expression patterns of the *Apy* gene are consistent with its role in facilitating hematophagy. Only adult females feed on blood and Northern analyses demonstrate *Apy* expression only in this stage and sex of the mosquito. The previous report of low-level activity in males (Ribeiro *et al.* 1984) was based on a biochemical assay and may reflect the activity of another apyrase-like gene whose product does not cross-hybridize with the *Apy* cDNA probes. Low-stringency probing of restriction enzyme-digested mosquito genomic DNA with the cDNA clone failed to reveal frag-

FIG. 1. Primary nucleotide sequence of a genomic DNA fragment containing the *Apyrase* gene. A 5.5-kb *EcoRI* fragment containing the complete coding region of the *Apy* gene was subcloned from a Lambda DASH phage clone, 10a2a. The primary sequence of a portion of this fragment is shown above the conceptual translation product. The numbering refers to the DNA sequence. Various features of the DNA sequence are shown underlined. A unique TAAATA and polyadenylation consensus sequence AATAAA are evident. The probable transcriptional start site (A) is underlined and the nucleotide marked by +1. Translation initiation (ATG) and stop (TAG) codons are also underlined. Amino acid changes as well as introns I-VII are underlined.

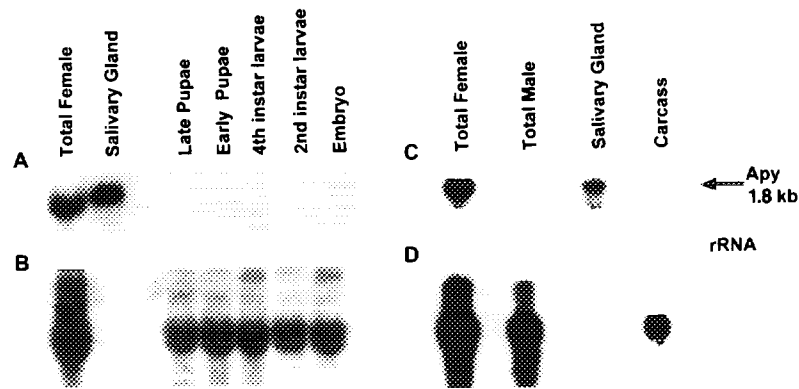


FIG. 3. Developmental expression of the *Apyrase* gene. RNA was isolated from different developmental stages and adult salivary glands, resolved on 1.2% agarose/formaldehyde gels, and transferred to nitrocellulose. The resulting Northern blot was probed with ^{32} P-labeled λ SGG12, the *aprase* cDNA clone. After exposure, the labeled probe was removed and the filters were reprobed with ^{32} P-labeled clone, λ AE1g, with sequence homology to mosquito rRNA. (A) Two micrograms of female salivary gland RNA and 7 μ g each of total female, late pupae, early pupae, fourth-instar larvae, second-instar larvae, and embryonic RNA were probed with λ SGG12. (B) is the same as (A), but reprobed with the ribosomal clone. (C) Five micrograms of total adult female and male RNA and 1 μ g of female salivary gland and carcass (whole body with salivary glands removed) probed with λ SGG12. (D) is the same as (C), reprobed with the ribosomal clone.

ments other than those expected from the *Apy* gene, but these experiments must be considered preliminary (C. T. Smartt and A. A. James, unpublished data). However, in our studies, neither Northern analyses nor analyses with immunological reagents revealed the presence of any product of the *Apy* gene in male mosquitoes.

The pattern of expression of the *Apy* gene in the hybridizations *in situ* to tissue sections extends the observations that the adult female salivary gland has region-specific gene expression. Analyses of four genes, two of which are expressed in both males and females [*Mall* (James *et al.* 1989) and *Amy* (Grossman and James 1994)] and two of which are expressed only in females [*D7* (James *et al.* 1991) and *Apy*], clearly shows that the medial and distal-lateral lobes of the female salivary glands express genes that are female-specific and presumed to be involved in hematophagy. The sialokinins were demonstrated by immunofluorescence to be localized in the medial lobe (Ribeiro 1992), but the pattern of gene expression has yet to be determined.

The analysis of apyrase protein accumulation using antibodies to recombinant protein re-

vealed that no enzyme is detectable in the salivary glands of newly emerged females. This is consistent with observations of gland morphology of young females in that they appear smaller and contain less material than do the glands of older females (Orr *et al.* 1961). On the second day following emergence, apyrase protein is detectable; it reaches a maximum level by the fourth day. This timing coincides with a behavioral change in the female from nonquesting to questing status (Christophers 1960). The expression of *Apy* is consistent with the hypothesis that female mosquitoes are incompetent for hematophagy until sufficient salivary gland products are present to ensure successful feeding.

The amount of apyrase protein in salivary glands following a bloodmeal did not appear to differ from the amount observed in nonfed females. This result appears to be in contrast to observations by Marinotti *et al.* (1990), who observed a 46% decrease in apyrase activity recovered from glands just after bloodfeeding. However, the apparent differences in the results may be accounted for by the greater sensitivity of the immunoblots. Interestingly, females in

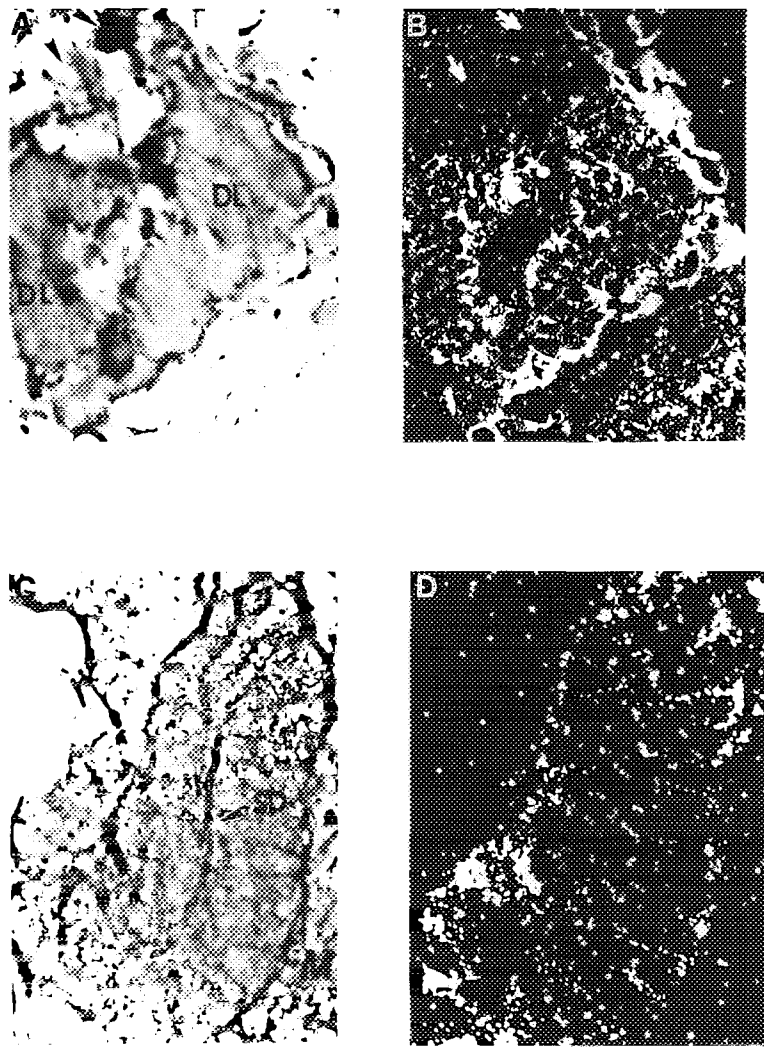


FIG. 4. Spatial expression of the *Apyrase* gene in the lateral and medial lobes of the adult female salivary glands. Female thoraces were prepared for hybridization and probed with ^{35}S -labeled λSGG12 antisense RNA. (A) Giemsa-stained, bright-field image showing the lateral lobes of the female salivary gland. (B) Dark-field image of (A) showing the silver grains corresponding to hybridization. Note that the distal-lateral (DL) lobes show a strong signal with antisense RNA. The proximal-lateral lobes (arrows) show no hybridization. (C) Giemsa-stained, bright-field image of the medial (M) lobe. (D) Dark-field image of (C). Note the specific hybridization corresponding to this lobe. SD, salivary duct. Magnification of (A) and (B) approximately 300 $\times$, and (C) and (D) approximately 500 $\times$.

both experiments did not completely deplete their salivary gland secretory products.

Previous analyses of corresponding cDNAs and conceptual translation products have shown that apyrase belongs to a family of proteins that includes 5'-nucleotidases (Champagne *et al.* 1995). No other genomic sequences for mem-

bers of this family are available currently, so that the number and position of introns and their potential influence on the evolution of the gene family remains to be determined. The mosquito apyrase lacks a carboxyl-terminal hydrophobic region that is present in membrane-bound 5'-nucleotidases (Champagne *et al.* 1995). There is

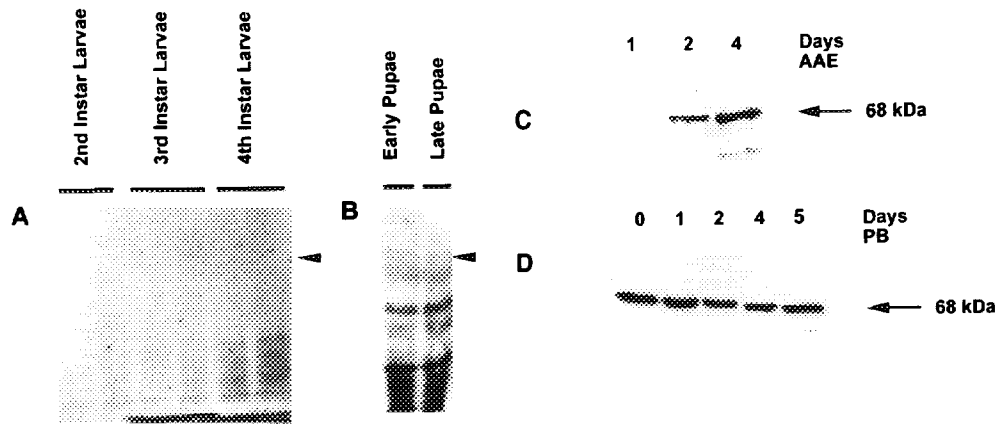


FIG. 5. Immunoblot of *A. aegypti* apyrase during different developmental stages and before and after blood-feeding. Two samples containing 20 and 40 μ g of protein isolated from second-, third-, and fourth-instar larvae were subjected to immunoblot analysis (A). Proteins isolated from single early and late pupae are shown in (B). Protein equivalent to a single pair of salivary glands from pre-blood-fed females 1, 2, and 4 days after adult emergence (AAE) are shown in (C). Protein equivalent to one salivary gland pair from females from 0 to 4 days post-blood-feeding (PB) are shown in (D). The arrows and arrowheads indicate known and predicted positions of the apyrase protein.

no evidence of an exon encoding this or a similar region near the 3' end of the coding region of the *Apy* gene, and the close juxtaposition of a consensus polyadenylation signal sequence appears to indicate that the apyrase product is not likely an alternatively spliced form of a 5'-nucleotidase gene.

The sequence polymorphisms that were observed between the genomic clone and cDNAs are minimal when compared to those observed in the alleles of other cloned genes (James *et al.* 1989, 1991; Grossman and James, 1994). Although the strain of mosquitoes (Rockefeller) used in these studies is inbred, our sequence analyses of genes and associated cDNAs indicate that the strain retains significant polymorphisms. The two polymorphisms between the genomic clone and the cDNA result in a same-charge substitution of a single amino acid, proline for alanine, and the insertion of a glycine residue. Neither of these changes are in regions conserved in the family of 5'-nucleotidases and therefore are not expected to affect the function of the apyrase enzyme.

The unusual TATA-like sequence, TAAATA, at nt -27 has not been observed in any of the

other cloned mosquito genes. Its position relative to the transcription initiation site and the lack of any other similar sequence nearby indicate that it is the most likely site for the TFIID binding domain. Analysis of the putative promoter regions of this gene is planned and should reveal how well this particular sequence functions.

The data presented here along with previous work on apyrase function and primary characterization provide a nearly complete picture of one of the most important salivary gland secretory products in hematophagous arthropods. It remains to be determined if apyrases in other arthropods and in animals in general share similar structural or expression features. However, because of different divalent cation requirements and differences in pH optima (Ribeiro *et al.* 1991), it may turn out that the designation apyrase includes proteins whose corresponding genes belong to different and distinct molecular families. The analysis of salivary glands and salivary gland secretory products will continue to reveal insights into the evolution of hematophagy in vector mosquitoes and how this has been exploited by pathogens to aid their transmission.

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