GATA Factor Translation Is the Final Downstream Step in the Amino Acid/Target-of-Rapamycin-mediated Vitellogenin Gene Expression in the Anautogenous Mosquito Aedes aegypti*

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Ingestion of blood is required for vector mosquitoes to initiate reproductive cycles determining their role as vectors of devastating human diseases. Nutritional signaling plays a pivotal role in regulating mosquito reproduction. Transcription of yolk protein precursor genes is repressed until mosquitoes take blood. Previously, we have shown that to signal the presence of blood in the gut, mosquitoes utilize the target-of-rapamycin (TOR) pathway. The TOR signaling pathway transduces the amino acid signal activating the major yolk protein precursor gene, vitellogenin (Vg). Here we report the identification of a GATA factor (AaGATAa) that is synthesized after a blood meal and acts as a transcriptional activator of Vg. We showed that AaGATAa bound specifically to GATA-binding sites in the proximal promoter region of the Vg gene and positively regulated Vg expression in transfection assays. RNA interference-mediated knock down of AaGATAa resulted in a reduction in Vg expression in both fat-body tissue culture and blood-fed mosquitoes. AaGATAa mRNA accumulated in the fat body prior to blood feeding. However, translation of GATA was activated by blood feeding because the GATA protein increased dramatically in the fat body of blood-fed mosquitoes. This increase was also reproduced in the fat-body culture stimulated with amino acids. GATA translation was inhibited by rapamycin and cycloheximide as well as by RNA interference-mediated knock down of S6 kinase. These experiments have revealed that the TOR signaling pathway induced by nutritional signaling regulates the translation of a GATA factor, which is the specific transcriptional activator of the Vg gene.

Anauthogenous mosquitoes are effective disease vectors because adult females require vertebrate blood for their reproductive cycles. The need for blood brings mosquitoes into repeated contact with multiple host organisms, making mosquitoes an effective vehicle through which pathogens can be spread from host to host.

Initiation of vitellogenesis by blood feeding is a key event in the reproductive cycle of anautogenous mosquitoes. This process involves the synthesis and secretion of yolk protein precursors (YPPs) on a massive scale by the fat body, a tissue analogous to vertebrate liver and white fat tissue. The YPPs accumulate in developing oocytes (1, 2). The main YPP genes activated during vitellogenesis are vitellogenin (Vg), vitellogenic carboxypeptidase (VCP), vitellogenic cathepsin B (VCB), and lipophorin (Lp). The Vg gene is the most highly expressed and best characterized YPP; it is controlled by the combined inputs of the steroid hormone 20-hydroxyecdysone (20E) cascade and nutritional signaling.

The 2.1-kb upstream regulatory region of the Vg gene contains three regulatory units with binding sites for the ecdysoid receptor complex (EcR-USP), the products of the 20E-stimulated early genes E74 and E75, GATA-type transcription factors, and several factors determining specificity of fat-body expression (3). These regions are responsible for tissue- and stage-specific expression and hormonal enhancement of the Vg gene expression. The hormonal cascade triggered by blood feeding leads to the elevation of the endogenous steroid hormone 20E, which acts upon the Vg gene through the nuclear hormone receptor heterodimer EcR (ecdysone receptor)/USP (ultraspiracle, an insect homologue of the mammalian retinoid X receptor). The binding of EcR-USP ecysdane at an EcR-USP ecysdane-response element directly activates Vg gene transcription (4, 5). 20E stimulation also works through a hierarchy of 20E-activated intermediate genes (E74 and Broad) called early genes that code for transcription factors that bind in another unit of the regulatory region (6, 7). In addition to hormone-specific gene regulation, transcriptional activation of the Vg gene is regulated by GATA factors. The Vg regulatory region contains 12 putative GABA-binding sites, which lead to the hypothesis that GATA factors are important for tissue specificity as well as for high levels of Vg expression (3).

GATA factors are a ubiquitous family of transcription factors, conserved from yeast to vertebrates, which regulate a variety of developmental processes (8, 9). GATA factors have been shown to play critical roles in development, differentiation, and control of cell proliferation. GATA factors typically consist of either one or two conserved zinc finger DNA binding domains with characteristic C2H2, C2HC, C2HC motifs, followed by a basic region, whereas the rest of the protein is typically not conserved. The nonconserved regions share little or no sequence similarity, and their functions are poorly defined (8). These factors recognize and bind with high affinity to the DNA consensus motif (A/T) GATA(A/G) (10).

The abbreviations used are: YPP, yolk protein precursor; 20E, 20-hydroxyecdysone; AA, amino acid; DAPI, 4′,6-diamidino-2-phenylindole; EcR, ecdysone receptor; PBM, post-blood meal; RT, reverse transcription; TOR, target-of-rapamycin; USP, ultraspriacle; Vg, vitellogenin; EMSAs, electrophoretic mobility shift assays; RNAi, RNA interference; S6K, S6 kinase; AaS6K, A. aegypti S6K; TUNEL, terminal deoxynucleotidyltransferase-mediated dUTP nick-end labeling; PBS, phosphate-buffered saline; dsRNA, double-stranded RNA; GST, glutathione S-transferase.

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GATA Translation in Mosquito Gene Activation

In previous work, we cloned a two-zinc finger GATA factor (AaGATAa) that has the ability to repress transcription and specifically inhibit 20E-mediated activation of the Vg gene (11). It has been hypothesized that another GATA factor may be involved in positive regulation of Vg gene expression.

Nutrients have long been suspected of playing a significant role in the regulation of egg development in mosquitoes. The total amino acid (AA) concentration in the mosquito hemolymph is significantly increased within 8 h after a blood meal (12). A number of AAs are essential for oogenesis, and a steady infusion of a balanced AA mixture into the hemolymph can stimulate egg development in a variety of mosquito species (13). We have shown that AAs induce transcription of the Vg gene in the fat body. Furthermore, we have discovered that a conserved pathway (target-of-ramapycin or TOR pathway) is utilized by mosquitoes to signal the presence of blood in their gut. The TOR signaling pathway transduces the AA signal to regulate Vg gene expression and activate egg development after blood feeding. We have demonstrated that RNA interference-mediated knock down of TOR resulted in blockage of these blood meal-activated events (14).

Recently, we have established that the downstream step that is controlled by TOR is the phosphorylation of S6 kinase (15). This finding strongly suggests that underlying the AA/TOR nutritional signaling and the TOR signaling pathway transduces the AA signal to regulate gene expression (target-of-rapamycin or TOR pathway) is utilized by mosquito species (13). We have shown that AAs induce transcription of the Vg gene in the fat body. Furthermore, we have discovered that a conserved pathway (target-of-ramapycin or TOR pathway) is utilized by mosquitoes to signal the presence of blood in their gut. The TOR signaling pathway transduces the AA signal to regulate Vg gene expression and activate egg development after blood feeding. We have demonstrated that RNA interference-mediated knock down of TOR resulted in blockage of these blood meal-activated events (14).

In this work, we cloned the AaGATAa factor and demonstrated that it is an activator of Vg gene translation. Furthermore, we show that translation of the AaGATAa factor is the specific downstream step in the AA/TOR pathway that is mediated by S6 kinase.

EXPERIMENTAL PROCEDURES

Animals and Fat-body Culture—The Aedes aegypti mosquito strain UGAL/Rockefeller was reared according to the method described by Hays and Raikhel (16). Vitellogenesis was initiated by allowing mosquitoes to feed on an anesthetized white rat 4–6 days after eclosion. The abdominals walls with adhering fat bodies (hereinafter referred to as the fat body) were incubated in an organ culture system as described previously (17).

Cloning and Sequencing of AaGATAa—Two degenerate oligonucleotides were designed from highly conserved AA sequences of the AaGATAa C-terminal zinc finger region. The sequences of the oligonucleotides were as follows: sense oligonucleotide, 5′-GAT ATG TCA TGY DCS AAC TGG; and antisense oligonucleotide, 5′-AAT GGT GTC CTT YYY CAT CG, where Y is C or T, and D is A, T, or G. A 140-bp PCR product was obtained by degenerate RT-PCR using the above two oligonucleotides and cDNA made from previtellogenic fat-body total RNA isolated from the previtellogenic fat body. The 5′- and 3′-ends of the cDNAs were amplified by means of rapid amplification of cDNA ends PCR using a Generacer Core kit (Invitrogen). The full-length cDNA was amplified using RT-PCR with total RNA prepared from previtellogenic fat bodies and was cloned into the pCR4-TOPO vector (Invitrogen). DNA sequences were analyzed by Chromas (version 2.22) and BLAST (National Center for Biotechnology Information, National Institutes of Health).

RNA Extraction, Reverse Transcription, and Real Time PCR—Total RNA from mosquitoes or dissected mosquito tissues (fat body, ovary, gut) was extracted using the Trizol method (Invitrogen) after homogenization with a motor-driven pestle pestle mixer. Aliquots of 2 μg of total RNA treated with amplification-grade RNase-free DNase I (Invitrogen) were used in cDNA synthesis reactions using an Omni-script reverse transcriptase kit (Qiagen, Valencia, CA). Reverse transcription was carried out according to the manufacturer’s protocol in 40-μl reaction mixtures containing random primer or oligo(dT) primer at 37 °C for 1 h. PCR products were obtained from the PCR using a HotStarTaq Master Mix kit (Qiagen), and 2 μl of cDNA was subjected to PCR by using specific primers. PCR products were separated on a 1% agarose gel.

Real time PCR was performed using the iCycler iQ system (Bio-Rad), and reactions were performed in 96-well plates with a QuantiTect SYBR PCR kit (Qiagen). Quantitative measurements were performed in triplicate and normalized to the internal control of β-actin mRNA for each sample. Primers were as follows: actin forward, 5′-AGC GAC ATG TCT CCT TAA TGT CAC; Vg forward, 5′-GCA GGA ATG TGT CAA CAG TGA AG; Vg reverse, 5′-ACG AGG AGC AAG AAT CCG AGG AG; AaGATAa forward, 5′-GAC GGC GCT TCT CCA CAA GTG TAC; and AaGATAa reverse, 5′-GCT GGG AGT GTG CAA CGA ATT G. All reactions were run with 2 μl of cDNA and 0.5 μM primers per reaction. Standard curves used to quantify relative concentrations were made from 10-fold serial dilutions of cDNA pools containing high concentrations of the gene of interest or were from a plasmid standard. Real time data were collected and analyzed using iCycler iQ real time detection system software version 3.0.

In Vitro Transcription and Translation—PCR product containing the entire AaGATAa cDNA was cloned into pcDNA3.1/Zeo(+) plasmid. The TnT® system (Promega, San Luis Obispo, CA) was used for in vitro transcription and translation of AaGATAa in rabbit reticulocyte lysate, utilizing the T7 promoter. To monitor the in vitro reaction, the synthesized proteins were labeled with [35S]methionine (1,200 Ci/mmol), and the radiolabeled proteins were visualized by means of electrophoresis and autoradiography.

Electrophoretic Mobility Shift Assay—Electrophoretic mobility shift assays (EMSA)s were carried out as described previously (22), with some modification, in a 10-μl volume containing 2 μl of TnT-expressed AaGATAa, 5× gel shift-binding buffer (Promega), and the indicated amount of competitor DNA oligonucleotides obtained from the regulatory region of the Vg gene. After 10 min of incubation at room temperature, 32P-labeled DNA probe was added, and the incubation was continued for another 10 min at room temperature. Free and protein-bound DNA was separated on a 5% polyacrylamide gel, and the gel was then dried and autoradiographed. DNA probes for EMSA were made by annealing together complementary oligonucleotide, and they were end-labeled by T4 polynucleotide kinase using [γ-32P]ATP (PerkinElmer Life Sciences).

Site-directed Mutagenesis—GATA sequences present in the Vg gene regulatory region of the p0.6Vg-Luc vector were changed to AATA using a QuickChange site-direct mutagenesis kit (Stratagene, La Jolla, CA). For site-directed mutagenesis, complementary oligonucleotides carrying the designed nucleotide changes were used to amplify the entire p0.6Vg-Luc template. The amplified DNA mixture was treated with DpnI to digest the parental, methylated DNA; the DpnI-digested DNA was used to transform Escherichia coli XL-1 Blue, and mutated GATA-binding sites were checked by sequencing.

In Vitro Transfection Assay Using Drosophila Cells—The AaGATAa expression plasmid, pAc5.1-AaGATAa, was created by cloning the full-length AaGATAa cDNA into pAc5.1/V5/His(A) (Invitrogen) under the control of the actin 5C promoter. The 0.6- or 2.1-kb regulatory region of the Vg gene was inserted into pGL3/firefly luciferase vector (Promega) to form the reporter construct p0.6Vg-Luc or p2.1Vg-Luc, respectively (4). Transfections were carried out using Drosophila Kc cells (LS7-3-
11), as described by Wang et al. (18), with minor modification. The Drosophila L57-3-11 cell line was maintained at 27 °C in Drosophila-SFM (Invitrogen) containing 5% fetal bovine serum (19). Transfection was conducted with Cellfectin reagent (Invitrogen) with a DNA-to-lipid ratio of 1:4 (w/w). Luciferase enzyme activity was assayed with a dual-luciferase reporter assay system (Promega) and detected with Lumi- mark (Bio-Rad). β-Galactosidase enzyme activity was analyzed using a β-galactosidase enzyme assay system (Promega).

RNA Interference—For production of AaGATAa-specific, double-stranded RNA (dsRNA), an AaGATAa-specific cDNA region (~130 bp) was cloned into the pLitmus 28i vector, and AaGATAa dsRNA was produced by means of in vitro transcription with T7 RNA polymerase using the Hiscrise RNAI transcription kit (New England Biolabs). By using the same technique, a 500-bp cDNA fragment cloned in pLitmus 28i was used for the production of dsRNA against AaS6K. 0.8-kb Mal dsRNA was produced from the pLitmus 28i-Mal (containing a nonfunctional portion of the MalE gene) and used as a control in the RNA interference experiment. Approximately 1 μg of dsRNA in 0.3 μl of distilled H2O was injected into the thorax of CO2-anesthetized female mosquitoes 1 or 2 days after emergence. The dsRNA-injected mosquitoes were allowed to recover for 3 days and then were dissected for fat-body culture or fed blood to induce vitellogenesis.

GATA Antibody—Antibody against a region present in both the AaGATAa and AaGATAr proteins was made using a bacterially expressed GST fusion protein. The GST fusion construct was created in the vector pGex-AT1 (GE Healthcare BioSciences Corp., Piscataway, NJ) by cloning in the region of the AaGATAa gene coding for AAs 601–861. This region is also shared by the AaGATAr protein; however, generation of an antibody specific to AaGATAa was impossible because of the short length of the sequence unique to AaGATAa. Transformed cells with GST fusion construct were tested for expression of the fusion protein by SDS-PAGE comparison of isopropyl β-D-galactopyranoside-induced and uninduced cell extracts. The expression of GST-fused AaGATA was tested by Western blot analysis using GST antibody and was purified by glutathione-agarose beads. The purified fraction was separated by means of SDS-PAGE, and the band containing the predicted protein was excised and sent to Cocalico Biologicals, Inc., for antibody production. Antibody was purified from antiseria produced in five guinea pigs by antigen affinity column purification using a GST orientation kit (Pierce).

Protein Extraction and Western Blot Analysis—Total protein extracts were prepared using an extraction method described elsewhere (20). Fifty fat bodies were ground in a cracking buffer (0.125 M Tris-HCl, pH 6.8, 5% β-mercaptoethanol, 2% SDS, 4 M urea). After 2 min of heating, the soluble protein fraction was separated by centrifugation. Preparation of nuclear extracts from fat bodies was carried out in accordance with the method described previously (21, 22), with minor modification. Fifty fat bodies frozen in liquid nitrogen were homogenized in 1 ml of 10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, and 1 mM dithiothreitol. The homogenate was kept on ice for 15 min before being lysed by the addition of 0.6% (v/v) Nonident P-40, and nuclei were pelleted by centrifugation at 13,000 rpm for 5 min. The nuclear pellet was resuspended in 50 μl of 20 mM HEPES, pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, and 10% (v/v) glycerol. Nuclei were lysed by shaking for 30 min at 4 °C. The lysate was centrifuged at 13,000 rpm for 15 min to remove cell debris, and a protease inhibitor mixture (Roche Applied Science) was added to all buffers in accordance with the manufacturer’s instructions. Total or nuclear protein extracts were separated on a 10% SDS-polyacrylamide gel and transferred to a polyvinylidene difluoride membrane (Bio-Rad). The membrane was blocked in 5% nonfat dry milk in PBST (137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 1.8 mM KH2PO4, pH 7.4, 0.05% Tween 20) and then incubated with GATA antibody (1:200 dilution). After the incubation of peroxidase-conjugated goat anti-guinea pig antibody (1:1000 dilution), a signal was detected using West Pico chemiluminescent substrate (Pierce).

Immunohistochemistry—Dissected fat bodies were fixed overnight at 4 °C with 4% paraformaldehyde in PBS. After multiple washes with PBS solution (PBS, 1% albumin, 0.5% Triton X-100), the samples were submitted to a graded ethanol series (20, 40, 60, 80, 100, 80, 40, and 20%, PBS for 15 min each). After multiple washes with PBS, the samples were treated with 3% goat serum in PBS solution for 2 h at room temperature and then washed three times with PBS. Subsequent incubation with GATA antibody (diluted 1:50) lasted 2 days at 4 °C. After multiple washes with PBS, the fat bodies were incubated overnight at 4 °C with Alexa Fluor 488 goat anti-guinea pig IgG (diluted 1:100; Invitrogen). Following thorough washing with PBS, the fat bodies were treated with DAPI (4′,6-diamidino-2-phenylindole) solution (10 μg/ml) for 1 h. After washing with PBS, the fat bodies were mounted with Gel/Mount (EMScience, Gibbstown, NJ) for microscopic analysis. Samples were examined under a Leica TCS SP2 confocal microscope (Leica Microsystems) at the Center for Plant Cell Biology/Institute for Integrative Genome Biology Core Facility.

Chromosomal DNA Fragmentation Assay—Apoptosis-induced nuclear DNA fragmentation was assayed via terminal deoxynucleotidyltransferase-mediated dUTP nick-end labeling (TUNEL) using the ApoAlert DNA fragmentation assay kit (Clontech), following the manufacturer’s protocol.

RESULTS

AaGATAa and Its Expression in the Mosquito Fat Body—A GATA factor (AaGATAa) containing a single zinc finger motif was cloned by means of degenerate and rapid amplification of cDNA ends PCR, as described under “Experimental Procedures.” The sequence data have been published in the DDBJ/EMBL/GenBank™ database under accession number AY439009. The AaGATAa mRNA encodes a protein with 1,003 AAs and a relative molecular mass of 120 kDa. AaGATAa is an isoform of the two-zinc finger AaGATAr transcription factor and shares the majority of its sequence with it, with the exception of a short stretch of AAs that replace the N-terminal zinc finger normally found in AaGATAr. AaGATAa shares a high homology with DmGATAβ (serpent) from Drosophila melanogaster (22). In a semi-quantitative RT-PCR experiment using primers against the AaGATAa-specific region, AaGATAa mRNA was found in previtellogenic and vitellogenic mosquito fat bodies and ovaries; the levels expressed in the fat bodies were higher than those in the ovaries. After the blood meal, the mRNA levels decreased (data not shown).

Real time PCR was used to obtain a detailed expression profile of AaGATAa in the fat body of the adult female mosquito. Total RNA was obtained from mosquito fat bodies dissected at 24-h intervals during the previtellogenic state and at either 6- or 12-h intervals during the vitellogenic state. Equal amounts of total RNA obtained from groups of 10 fat bodies were used to synthesize cDNA. AaGATAa mRNA levels were determined using primers designed from the AaGATAa-specific region. During previtellogensis, the AaGATAa mRNA level gradually increased in the fat body (Fig. 1). In contrast, it decreased PBM, declining significantly between 3 and 24 h PBM, which is the time of active expression of the Vg gene. This AaGATAa expression pattern is different from that of AaGATAr, which functions as a repressor of Vg expres-
AaGATAa mRNA is expressed constitutively in previtellogenesis and increases at the end of vitellogenesis. AaGATAa binds specific GATA sites in the Vg gene regulatory region. The AaGATAa cDNA sequence was translated by a coupled in vitro transcription and translation system and labeled by [35S]methionine. A band of 110 kDa was detected by means of SDS-PAGE using 10% acrylamide gel followed by exposure to x-ray film (data not shown). This size is similar to the predicted AaGATAa molecular weight.

The TNT-expressed AaGATAa was used in EMSA to identify the binding specificity of AaGATAa to the GATA-binding sites present in the upstream regulatory region of the Vg gene. The binding specificity of AaGATAa to the GATA-binding sites in the upstream regulatory region of the Vg gene was confirmed by its competition with a 50-fold molar excess of box-A or GATA oligonucleotides.

The TNT-expressed AaGATAa was used in EMSA to identify the binding specificity of AaGATAa to the GATA-binding sites present in the Vg regulatory region. Nine GATA-like binding sites are located within the 2-kb upstream region of the Vg gene (Fig. 2A). Nine GATA oligonucleotides were designed from 12 putative GATA-binding sites within the regulatory region of the Vg gene (Fig. 2B). The box-A sequence from the D. mulleri alcohol dehydrogenase gene was labeled with 32P and used as a probe. This sequence had been shown to bind to the AaGATAa protein.
previously to bind a truncated version of the *Drosophila* GATA\(\beta\) protein (24). The TNT-expressed AaGATA\(\alpha\) formed a binding complex with the box-A probe (Fig. 2C, lane 2), and the specificity of this complex was confirmed by its competition with a 50-fold molar excess of the box-A DNA (Fig. 2C, 3rd lane).

To demonstrate the different relative affinities of AaGATA\(\alpha\) binding to different GATA-binding sites within the regulatory region of the *Vg* gene, double-stranded oligonucleotides containing GATA-binding sites presented in the regulatory region of the *Vg* gene were added as competitors in the EMSA. The binding complex formed by AaGATA\(\alpha\) and the box-A probe was competed away with the unlabeled GATA 1–3 oligonucleotides (Fig. 2C, 4th to 12th lanes). To confirm the binding specificity of AaGATA\(\alpha\) on the *Vg* gene regulatory region, TNT-expressed AaGATA\(\alpha\) was tested by means of EMSA using the box-A DNA and GATA oligonucleotides as probes for direct binding (Fig. 2D). Binding affinities between AaGATA\(\alpha\) and GATA oligonucleotide probes were highest in lanes in which GATA oligonucleotides 1 and 2 were used as probes. This result matched those obtained from the competitive EMSA (Fig. 2C). The GATA 1 and 2 oligonucleotides contained GATA-binding sites located at −115 to −118 and −161 to −164, respectively. The GATA 3 oligonucleotide was shown to contain two GATA-binding sites (−412 to −415 and −429 to −432). These results indicate that AaGATA\(\alpha\) specifically binds to GATA-binding sites located in the proximal region (−115 to −440) of the *Vg* gene regulatory region (Fig. 2A).

**AaGATA\(\alpha\) Activates the Vg Regulatory Region in Cell Transfection Assays**—Cell transfection assays utilizing *Drosophila* cells were used to investigate the function of AaGATA\(\alpha\) in *Vg* gene expression. The *Drosophila* L57-3-11 cells used in these transfection experiments are EcR-deficient and are derived from the Kc 167 cell line via parahomologous targeting (19). We utilized luciferase reporter constructs driven by the 0.6- or 2.1-kb regulatory region of the *Vg* gene (4). Co-transfection of the p0.6Vg-Luc reporter with the AaGATA\(\alpha\) expression plasmid (pAc5.1-AaGATA\(\alpha\)) resulted in a 40-fold enhancement of the basal luciferase activity (Fig. 3A). When the p2.1Vg-Luc reporter was co-transfected with pAc5.1-AaGATA\(\alpha\), the resulting luciferase activity was the same as that of the p0.6Vg-Luc co-transfection. No additional enhancement in the same level of expression was observed when the 2.1-kb construct...
was used, because AaGATAa had an affinity for the GATA sites located within the 0.6-kb section of the Vg regulatory region (Fig. 3A). The 0.6-kb regulatory region of the Vg gene also contains an ecdysone-response element (3, 4). However, because we utilized an EcR-deficient cell line, no activation of the reporter was expected from EcR-USP. The AaGATAa expression in the transfected Drosophila cells was confirmed using Western blot analysis with an anti-V5-HRP antibody (Fig. 3B).

For the next transfection experiments, we used the p0.6Vg-Luc reporter. There are five putative GATA-binding sites in the 0.6-kb regulatory region of the Vg gene (Fig. 3C). We used site-directed mutagenesis to determine which GATA-binding sites in the proximal region of the Vg gene were necessary for its transactivation by AaGATAa. GATA sequences present in the 0.6-kb Vg gene regulatory region of p0.6Vg-Luc vector were individually converted to AATA sequences. The lack of AaGATAa binding to the point-mutated GATA-binding sites was confirmed by competitive EMSA. Binding of TnT-expressed AaGATAa and box-A probe was not competed away by a 50-fold molar excess of unlabeled, double-stranded oligonucleotides carrying the AATA motif. Nonmodified oligonucleotides 1–3, which contained the original GATA sequences, competed away binding of AaGATAa to the box-A probe (data not shown). Mutant constructs of p0.6Vg-Luc were then co-transfected into Drosophila cells with pAc5.1-AaGATAa (Fig. 3C), and luciferase activity was measured. The luciferase activity was not changed by co-transfection of p0.6Vg-Luc Δ4 and pAc5.1-AaGATAa. In the case of co-transfection with p0.6Vg-Luc Δ3 and pAc5.1-AaGATAa, luciferase activity was reduced by 50%. Co-transfection with either p0.6Vg-Luc Δ1 or -2 and pAc5.1-AaGATAa decreased luciferase activity by 80–90%. These results demonstrate that AaGATAa bound to GATA-binding sites 1–3 and that GATA-binding sites 1 and 2 were shown to be more important for transcriptional activation of the Vg gene by AaGATAa. These results are in good agreement with EMSA binding analyses (Fig. 2).

RNAi-mediated Knock Down of AaGATAa Results in Decreased Transcription of the Vg Gene—RNAi-mediated knock down of AaGATAa was used to determine the function of AaGATAa on Vg gene expression in vivo. RNAi causes the disruption of specific mRNAs in response to the presence of dsRNA. To obtain the AaGATAa-specific dsRNA, an AaGATAa-specific cDNA region (~130 bp) was used. Likewise, dsRNA specific for AaGATAr was obtained using AaGATAr-specific region of the first zinc finger. Female mosquitoes, 1 or 2 days after eclosion, were injected with 1 µg of either dsRNA. At 3 days post-injection, Vg gene expression was monitored in cultured fat bodies from RNAi-treated mosquitoes (Fig. 4A) and in live, treated mosquitoes (Fig. 4B). As shown in Fig. 4A, Vg gene expression in the fat bodies of previtellogenic female mosquitoes was induced by the addition of AAs in culture medium. Knock down of AaGATAa resulted in a 55% reduction of expression (Fig. 4A). In addition, Vg expression was analyzed in vivo at different time points after induction by a blood meal in the AaGATAa RNAi-treated mosquitoes. Measurements using real time PCR indicated that the levels of Vg transcripts in vitellogenic mosquitoes were significantly reduced after the injection of AaGATAa dsRNA (Fig. 4B). The specificity of knock down was confirmed by a Western blot showing the lack of AaGATAa protein in response to AaGATAa dsRNA treatment but not to AaGATAr or MAL control treatments (Fig. 4C).

GATA Factor Protein Is Increased in the Fat Body after a Blood Meal—Localization of the AaGATA protein and its levels was analyzed by using immunohistochemistry and Western blotting techniques, respectively.
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For these analyses, fat bodies were dissected from mosquito females at several points during the vitellogenic cycle. The fat-body tissue was treated with the anti-GATA antibody and DAPI solution, and it was examined by means of confocal microscopy. Weak positive staining for GATA was detected in the previtellogenic fat body (Fig. 5, A–C). The activation of vitellogenic events in the fat body by a blood meal was marked by a dramatic increase in intensity of the AaGATA-positive staining, which was located in both the cytoplasm and nuclei of fat-body cells (Fig. 5, D–F).

The AaGATA profile at the protein level was monitored by means of Western blot analysis at different time points of the vitellogenic cycle (Fig. 5G). For each time point, total and nuclear protein was extracted from 50 fat bodies, and then equal amounts of protein extract were loaded on the gel and subjected to Western blotting. The AaGATA protein level in the previtellogenic fat body was low, particularly in nuclei. After a blood meal, AaGATA levels in both the total and nuclear protein level increased considerably; the protein level was maximal at 12 h in the total extracts and at 12–24 h in the nuclear extracts.

Western blot analysis of fat bodies from mosquitoes with RNAi-induced knockdown using AaGATAa dsRNA showed a strong reduction in the AaGATAa protein after a blood meal when compared with Mal-treated controls confirming specificity of anti-AaGATA antibodies (Fig. 5H).

Thus, both the immunohistochemical staining and the Western detection in fat-body protein extracts clearly indicated a dramatic increase of GATA protein in the fat-body cells after a blood meal. The intensity of the signal was declining after 24 h PBM, when the Vg expression and protein translation were at their peak.

Rapamycin and Cycloheximide Inhibit AA-stimulated GATA Factor Translation—Previtellogenic fat bodies from 5 days post-eclosion were dissected and cultured for 12 h in the culture media with or without AAs. GATA protein level and localization were assayed by means of immunohistochemistry and Western blot analyses. Culturing fat bodies for 12 h in the medium without AAs changed neither the distribution nor the level of GATA protein (Fig. 6, A and B). In contrast, culturing in the presence of AAs resulted in a marked increase in GATA protein similar to that seen after blood meal stimulation (Fig. 6, C and D). The addition of rapamycin (200 nM) to an AA-containing medium blocked the rise in GATA protein level (Fig. 6, E–G).

To examine whether AAs and TOR were involved in the translational regulation of GATA, previtellogenic fat bodies were incubated in culture media with or without AAs in the presence or absence of cycloheximide (10^{-4} M), an inhibitor of translation. Immunohistochemistry and Western blot analysis of GATA protein confirmed that the AA-stimulated increase in GATA protein level was inhibited by the addition of cycloheximide in the culture medium in a manner similar to that of rapamycin (Fig. 7, A–G). This suggests that AAs likely stimulate the translation of GATA and that TOR signaling induced by AAs is required for this process. To further confirm that inhibition of GATA translation negatively affected the transcription of its target gene, Vg, its expression was examined (real time PCR) in previtellogenic fat bodies incubated in culture media with or without AAs and in the presence or absence of cycloheximide. Cycloheximide treatment inhibited the AA-stimulated Vg expression (Fig. 7H). Exposure to cycloheximide, however, did not induce apoptosis in fat-body cells, because no apoptotic bodies were detected in the DAPI-stained nuclei (Fig. 7E) and no DNA fragmentation was found using TUNEL staining (Fig. 7, I–L).

RNAi Knock Down of S6 Kinase Blocks GATA Factor Translation—To determine whether the translational regulator S6 kinase (S6K) was involved in control of GATA translation, we used RNAi to knock down this gene and determine the effect on GATA protein abundance. The phosphorylation state of S6K (and the resulting kinase activity) is regulated by TOR. The presence of AAs causes an increase in S6K phosphorylation, resulting in its phosphorylation of the S6 ribosomal subunit to promote translation (15). To obtain specific dsRNA, an AaS6K-specific cDNA region was cloned into the plitmus 28i vector, and dsRNA was produced as described previously (15). Female mosquitoes, 1–2 days post-eclosion, were injected with 1 μg of AaS6K dsRNA or dsMal RNA. At 3 days post-injection, fat bodies were dissected from RNAi-treated mosquitoes and placed in culture for 12 h, either with or without AAs. After this treatment, fat bodies were collected for protein analysis by Western blot using GATA antibodies and phosphorylated S6K antibodies. At 8 h PBM, the presence of AAs resulted in a marked increase in GATA protein, but this increase was inhibited by both RNAi and cycloheximide in a manner similar to that of rapamycin (Fig. 7, A–G). Thus, rapamycin, a specific inhibitor of TOR, inhibited AA-activated elevation of the GATA protein level.
presence of AAs. Under the same AA conditions, there was no AaS6K phosphorylation detected in the fat bodies of S6K dsRNA-treated mosquitoes. Western blot also showed no S6K protein in the fat bodies of S6K dsRNA-treated mosquitoes, indicating that the protein was successfully knocked down. The GATA level increased in the Mal-treated control in the presence of AA. However, the AaS6K knock down prevented an increase in the AaGATA protein level in the presence of AAs (Fig. 8). These results strongly suggest that AaS6K is located upstream of GATA factor in the AA/TOR pathway regulating its translation in response to AAs.

DISCUSSION

In this study, we have characterized AaGATAa, a mosquito transcription factor belonging to the GATA family implicated in AA/TOR-mediated activation of Vg gene expression. AaGATAa contains one C-terminal C_{1}X_{1}C_{2}X_{1}C_{2} zinc finger region, a typical motif of the GATA transcription factors (10). AaGATAa is structurally similar to AaGATAr; however, it lacks the N-terminal zinc finger DNA binding domain present in AaGATAr that represses the Vg transcription (11, 23). Phylogenetic analysis shows that AaGATAa and AaGATAr have
**GATA Translation in Mosquito Gene Activation**

The role of GATA transcription factors in mediating nutritional signaling has been well established in *Saccharomyces cerevisiae* (29, 30). Nitrogen catabolic gene expression and genes responsible for autophagy are regulated by the GATA transcription factors Gln3 and Gat1. These factors serve as activators (31) and are retained in the cytoplasm by the URE2 protein when they are phosphorylated (32). The TOR kinase pathway maintains the phosphorylation state of these GATA factors. Inactivation of the yeast TOR kinase, by limiting nitrogen, results in dephosphorylation of Gln3 and Gat1 by a TOR-regulated phosphatase called SIT4, allowing them to separate from URE2, translocate to the nucleus, and activate nitrogen catabolic genes (33). Likewise, GATA factors have also been shown to be involved in the regulation of differentiation of pre-adipocyte cells to mature adipocytes in response to growth factors and nutrients in vertebrates (34). Furthermore, treatment of pre-adipocytes with rapamycin inhibits their differentiation into mature adipocytes (35). This suggests that nutrient-regulated control of GATA factors may work through the conserved TOR pathway. The molecular mechanisms regulating the TOR pathway-controlled GATA factors could be different. In the case of the mosquito fat body, our experiments have clearly shown that the downstream events are linked with the translational regulation of AaGATAa via S6K. A significant increase in GATA protein in fat-body nuclear extracts suggests that, in addition to AaGATAa translation during the vitellogenic period, the nuclear translocation of the factor also occurs (Fig. 5G).

TOR kinase is a member of the phosphatidylinositol 3-kinase superfamily, which regulates gene expression, protein biosynthesis, and cell differentiation via transcriptional and translational regulatory pathways (36). The TOR pathway is a nutrient-sensing signal transduction pathway stimulated by hormones and AAs, and it is conserved from yeast to mammals. Recent findings in *Drosophila* show that the fat-body tissue functions as an AA sensor and that nutritional signals are transduced by the TOR signaling pathway (37). An important mechanism by which TOR affects cell function is through translational regulation (38). We previously showed that in the mosquito *A. aegypti* the TOR signaling pathway mediated by AAs regulates Vg expression in the fat body and the activation of egg development after a blood meal (14). More recently, we have determined that the phosphorylation of S6 kinase is the key downstream event of the AA/TOR nutritional signaling in *A. aegypti* (15).

To determine whether TOR signaling in *A. aegypti* is involved in the translational regulation of AaGATAa, we used rapamycin and cycloheximide in the fat-body culture system. Rapamycin, a specific inhibitor of TOR, binds to FKBP12, a peptidyl-prolyl isomerase, and this complex combines with TOR to inhibit its kinase activity (39). Previous work has shown that rapamycin inhibits AA-stimulated Vg expression in fat-body culture (14). Cycloheximide, a global inhibitor of protein synthesis, is also capable of inhibiting this expression (Fig. 7H). This suggests that protein synthesis is required for Vg expression. AaGATAa protein levels were increased by the addition of AAs in the fat-body culture, and this enhancement was inhibited in the rapamycin- and cycloheximide-treated fat bodies (Figs. 6 and 7). Furthermore, knock down of AaS6K also resulted in blockage of AaGATAa translation (Fig. 8). These data indicate that the TOR pathway induced by AA-nutritional signaling regulates the translation of AaGATAa via S6K, which in turn activates transcription of the Vg gene.

In summary, we have identified that AaGATAa functions as an activator of Vg gene expression. Furthermore, based on isoform-specific *in vitro* and *in vivo* RNAi data, we believe that transcription and translation of AaGATAa are uncoupled and that the expression is regulated...
independently at previtellogenic and vitellogenic stages, respectively. Therefore, AaGATAa translation is the final step in the blood meal-activated AA/TOR signaling pathway mediating vitellogenic gene expression in anautogenous mosquitoes.

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