

# Target of Rapamycin (TOR) Mediates the Transduction of Nutritional Signals into Juvenile Hormone Production\*

Received for publication, September 10, 2008, and in revised form, December 23, 2008. Published, JBC Papers in Press, December 23, 2008, DOI 10.1074/jbc.M807042200

José L. Maestro<sup>†1</sup>, Juliana Cobo<sup>‡2</sup>, and Xavier Bellés<sup>†3</sup>

From the <sup>†</sup>Institut de Biologia Evolutiva (CSIC-UPF), Passeig Marítim de la Barceloneta 37-49, 08003 Barcelona, Spain and the

<sup>‡</sup>Instituto de Genética e Bioquímica, Universidade Federal de Uberlândia (UFU), 38400-902 Uberlândia, Brazil

Anautogeny is a reproductive strategy by which females do not reproduce until they feed. Therefore, nutritional signals must inform the reproductive tissues, and cells that the organism has reached a nutritional status suitable for triggering reproductive processes. One of the possible pathways involved in anautogeny is the “target of rapamycin” (TOR) pathway, which has been described as connecting the nutritional status with growth, proliferation, and cancer. The German cockroach, *Blattella germanica*, is an anautogenous species whose vitellogenesis is governed by juvenile hormone. In the present report, we describe the cloning of TOR cDNA from *B. germanica* (BgTOR). Expression studies showed that BgTOR is expressed in adult female corpora allata and fat body. BgTOR knockdown using systemic RNAi *in vivo* produced a severe inhibition of juvenile hormone synthesis in adult female corpora allata, together with a reduction of mRNA levels corresponding to 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) synthase-1, HMG-CoA synthase-2, and HMG-CoA reductase. In addition, there was a reduction of vitellogenin mRNA in the fat body, and ovaries did not grow. Analysis of TOR expression in corpora allata of fed and starved females suggested that TOR is not regulated at the transcriptional level. Nevertheless, there was a reduction in HMG-CoA synthases and reductase mRNA in corpora allata (but not in the fat body) of starved females, together with a dramatic reduction of juvenile hormone production and ovary development. Taken together, our results indicate that TOR knockdown mimics starvation in terms of corpora allata activity, and suggest that nutritional signals that activate juvenile hormone biosynthesis and vitellogenin production are mediated by the TOR pathway.

How is food transformed into eggs? The answer is not easy, because it involves a myriad of factors, not only metabolic but also hormonal, connected to each other in a complex regulatory

network. Good models to illuminate this subject might be anautogenous insects, first because insects are especially suitable for experimental manipulation, then because anautogenous species do not trigger reproductive processes until the female has taken a meal, which also facilitates experiments. In these species, there must be factors informing cells in tissues and organs related to reproduction that the animal has already fed, and these factors must belong to signaling pathways involved in sensing the nutritional status and in activating physiological processes, such as growth, cell proliferation, reproduction, and cancer. In this frame, a classical factor related to nutritional signaling is “target of rapamycin” (TOR).<sup>4</sup> The present report describes our studies on the role of TOR in the transduction of nutritional signals into endocrine and reproductive output in the anautogenous insect *Blattella germanica*, whose reproductive processes are mainly governed by juvenile hormone (JH).

The adult female of *B. germanica*, the German cockroach, undergoes marked reproductive cycles regulated by JH, a sesquiterpenoid hormone produced by the retrocerebral glands known as corpora allata (CA). In the fat body, JH induces the synthesis of vitellogenin (1), which is the main precursor of egg yolk, which is released to the hemolymph and then incorporated to the growing oocytes. In adult females of *B. germanica*, JH biosynthesis shows a cyclic pattern, with a rapid increase shortly after the adult moult and a sudden decrease at oviposition time, remaining at low levels during the period of ootheca transport, after which a new gonadotrophic cycle is initiated (1). *B. germanica* is a typical anautogenous species, and the female reproductive cycle starts only after feeding (2). Studies on the biosynthesis of JH have shown that the first steps of its biosynthetic pathway are common with those of the mevalonate pathway and cholesterol production. In this frame, the enzymes 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) synthase and HMG-CoA reductase are key in the mevalonate pathway and in the synthesis of JH, and both have been cloned in *B. germanica* (3).

TOR is a S/T kinase reported in a variety of eukaryotic systems (4), which is activated in the presence of amino acids and has an essential role in regulating cell growth and size by modulating gene transcription, mRNA translation, and ribosomal biogenesis, among other cell processes (5, 6). The activation of the TOR pathway in *Drosophila melanogaster* produces an increase in cell size, whereas its repression leads to an inhibition

\* This work was supported by the Spanish Ministry of Science and Technology (Projects BFU2006-01090 (to J. L. M.) and AGL2005-00773 and CGL2008-03517/BOS (to X. B.)). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

<sup>1</sup> To whom correspondence may be addressed: Institut de Biologia Evolutiva (CSIC-UPF), Passeig Marítim de la Barceloneta 37-49, 08003 Barcelona, Spain. E-mail: joseluis.maestro@ibe.upf-csic.es.

<sup>2</sup> Recipient of a research grant from CAPES (Brazil).

<sup>3</sup> To whom correspondence may be addressed: Institut de Biologia Evolutiva (CSIC-UPF), Passeig Marítim de la Barceloneta 37-49, 08003 Barcelona, Spain. E-mail: xavier.belles@ibe.upf-csic.es.

<sup>4</sup> The abbreviations used are: TOR, target of rapamycin; JH, juvenile hormone; CA, corpora allata; HMG-CoA, 3-hydroxy-3-methylglutaryl-coenzyme A; RACE, rapid amplification of cDNA ends.

of growth and cell proliferation (6). Similarly, TOR is related to sensing amino acid levels, and its activation is required, for example, to synthesize vitellogenin in the mosquito *Aedes aegypti* (5, 7). Exceptionally, the endocrinology of reproduction in *D. melanogaster* and *A. aegypti*, as in other dipterans, is governed by 20-hydroxyecdysone, whereas in most insects, including cockroaches, the gonadotrophic hormone is JH.

As a first step to study the link between nutrition and reproduction, and to clarify how nutritional signals may lead to JH production and activation of reproductive processes, we have analyzed TOR expression and function in adult females of *B. germanica*.

## EXPERIMENTAL PROCEDURES

**Insects**—Specimens of *B. germanica* (L.) were from a colony reared on dog chow and water, at  $30 \pm 1$  °C and 60–70% rh. Dissections of CA and abdominal fat body were carried out on carbon dioxide-anesthetized specimens. Fat body tissue adhered to the abdominal sternites was dissected out, except in the case of incubations *in vitro*, where fat body together with the abdominal sternites and epidermis was used. For starvation experiments, animals received only water after the adult moult. Fed controls in these experiments and animals from all other treatments were fed *ad libitum* with dog chow and water.

**Cloning of BgTOR cDNA**—Degenerate primers based on conserved regions of insect and vertebrate TOR sequences were used to obtain a *B. germanica* homologue cDNA fragment by RT-PCR. The first PCR amplification was carried out using cDNA generated by reverse transcription from RNA from UM-BGE-1 cells (derived from early embryos of *B. germanica*) as template, as described (8). Primer sequences and PCR conditions are available upon request. We amplified a 176-bp fragment, which was subcloned into the pSTBlue-1 vector (Novagen) and sequenced. This was followed by 3'-RACE and several 5'-RACEs (5'- and 3'-RACE System Version 2.0; Invitrogen) using different specific primers to complete the sequence. As templates, we used again cDNA from UM-BGE-1 cells. PCR products were subcloned into the pSTBlue-1 vector and sequenced in both directions.

**Phylogenetic Analyses**—We used the following sequences: from the insects *D. melanogaster* (GenBank<sup>TM</sup> Accession Number: NP524891), *D. pseudoobscura* (XP001355903), *A. aegypti* (AAR97336), *Anopheles gambiae* (XP317619), *Tribolium castaneum* (XP971819), *Nasonia vitripennis* (XP001602345), *Apis mellifera* (XP625130), and *B. germanica* (EU926975); the nematode *Caenorhabditis elegans* (Q95Q95); the vertebrates *Danio rerio* (NP001070679), *Gallus gallus* (XP417614), *Homo sapiens* (NP004949), *Mus musculus* (NP064393), and *Rattus norvegicus* (NP063971); and the protein TOR1 from *Saccharomyces cerevisiae* (NP012600). The tree was rooted using the *Arabidopsis thaliana* sequence (NP175425) as outgroup. Protein sequences were aligned using ClustX (9). Poorly aligned positions and divergent regions were eliminated using Gblocks 0.91b (10), which resulted in 2022 final positions. The obtained alignment was analyzed by the PHYML program (11), based on the maximum-likelihood principle. Four substitution rate categories optimizing the gamma shape parameter were used. The data sets were bootstrapped for 100 replicates.

**RT-PCR/Southern Blot Analyses**—RT-PCR followed by Southern blotting was used to determine mRNA expression patterns. cDNA was synthesized from RNA as previously described (12). 1  $\mu$ g of total RNA was used for reverse transcription in the case of fat body, whereas in the case of CA, the whole RNA from one or two glands was used. To estimate mRNA levels semi-quantitatively, a non-saturating number of cycles in the PCR system were used. Primer sequences to amplify BgTOR, vitellogenin, HMG-CoA synthase-1, and HMG-CoA synthase-2, HMG-CoA reductase, and Actin 5C (used as a reference) are available upon request. cDNA probes for Southern blot analyses were generated by PCR with the same primer pairs and labeled with fluorescein, using the Gene Images random prime-labeling module (Amersham Biosciences) (13). RT-PCR followed by Southern blotting of total RNA without reverse transcription did not result in amplification, indicating that there was no genomic contamination.

**RNA Interference**—Systemic RNAi *in vivo* in females of *B. germanica* was performed as previously described (12). Two different fragments, a 506-bp dsRNA fragment (dsTOR) and a 547-bp fragment (dsTOR II), spanning positions 4257 to 4762 and 2071 to 2617, respectively, of the BgTOR cDNA, were used to generate two different dsRNA. As a control, a non-coding sequence of 92 bp from the pSTBlue-1 vector (dsControl) was used. A dose of 2  $\mu$ g of dsRNA diluted in sterile saline was injected into the abdomen of females that had just moulted into fifth nymphal instar, and again when they moulted into sixth (last) instar. Dissections were carried out 5 days after the adult moult. In another set of RNAi experiments, and following an approach designed by O. Maestro and D. Martín,<sup>5</sup> adult females in the first day of ootheca transport were treated with a single 2- $\mu$ g dsRNA dose. Twelve days later, the ootheca was removed, which induced the onset of the second gonadotrophic cycle, and dissections were made 5 days later. BgTOR, vitellogenin, and Actin 5C mRNA levels were determined by RT-PCR using appropriate primers (sequences available upon request).

**Real-time PCR analyses**—The CA and fat body expression levels of HMG-CoA synthase-1 and -2, HMG-CoA reductase, BgTOR, and Actin 5C from the RNAi and starvation experiments were analyzed using real-time PCR. cDNA was obtained as described above. cDNA levels in the different samples were quantified by using iQ SYBR Green supermix (Bio-Rad) in an iQ cyclor and iQ single color detection system (Bio-Rad). Primer sequences to amplify HMG-CoA synthase-1 and -2, HMG-CoA reductase, BgTOR, and Actin 5C (used as a reference), are available upon request. Total reaction volume was 20  $\mu$ l. All reactions were run in duplicate or triplicate. The program used to amplify the reaction was as follows: (i) 95 °C for 3 min; (ii) 95 °C for 10 s; (iii) 60 °C for 1 min (fluorescence recorded); and (iv) repeat to step ii for 50 cycles. Real-time data were collected by iQ5 optical system software v. 2.0 (Bio-Rad).

**Quantification of JH Synthesis and Cell Number in the CA**—JH III synthesis by CA incubated *in vitro* was quantified according to the method previously reported (14). Essentially, individual pairs of CA were incubated in 100  $\mu$ l of TC 199 medium

<sup>5</sup> Unpublished data.

## TOR Transduces Nutritional Signals into JH

(Flow) containing L-methionine (0.1 mM), Hank's salts, Hepes buffer (20 mM) plus Ficoll (20 mg/ml), to which L-[<sup>3</sup>H-methyl]-methionine (Amersham Biosciences) had been added, to achieve a final specific activity of 7.4 GBq/mmol. CA were incubated for 2 h, after which JH III was quantified in the medium plus homogenized glands. Total cell number of a single corpus allatum was determined by hemocytometric sampling, as previously described (15).

**Incubation of Fat Body in Vitro**—Fat body tissue adhered to abdominal tergites and epidermis was dissected from freshly emerged adult females. Fat body tissue was then incubated in 1 ml of Grace's medium, with L-glutamine and without insect hemolymph (Sigma) at 30 °C in the dark, as previously described (16). It was incubated for 4 h with 500 nM rapamycin or the same volume of DMSO (the rapamycin solvent), and then transferred to a medium containing, in addition to the previous treatment, 800 nM JH III or the same volume of acetone (the JH solvent) and incubated for another 4-h period. After the incubation, tissue was frozen in liquid N<sub>2</sub> and stored until RT/PCR Southern blot analysis.

### RESULTS

**Cloning of BgTOR, Sequence Comparison, and Phylogenetic Analysis**—Using degenerate primers and cDNA from UM-BGE 1 cells as template, a 176-bp fragment of a presumed TOR homologue of *B. germanica* (BgTOR) was obtained. To complete the cDNA sequence, we followed 3'-RACE and 5'-RACE methods, and we obtained a sequence of 7921 bp (GenBank™ accession number EU926975), which encoded a protein of 2470 amino acids with a predicted molecular mass of 280.6 kDa. The putative start codon was preceded by an in-frame stop codon and the final stop codon was followed by a poly(A) sequence, suggesting that it was a full-length ORF. BLAST data base search indicated that the protein was the *B. germanica* homologue of TOR. The amino acid sequence is remarkably conserved, showing high percentages of identity with respect to other TOR proteins, like that of *Apis mellifera* (80% identity), *Tribolium castaneum* (67%), *D. melanogaster* (59%), or *H. sapiens* (61%). BgTOR shows the typical organization of TOR proteins, containing N-terminal HEAT repeats, followed by a FAT domain, a rapamycin-FKBP12-binding domain, a kinase domain, and a C terminus FATC region (17).

Maximum-likelihood analysis including the BgTOR sequence and other representative TOR sequences available in databases, gave the tree shown in Fig. 1. Using *A. thaliana* TOR as outgroup, the topology of the tree closely matches the current phylogeny of the included species, and indicates that the *B. germanica* sequence corresponds to a TOR protein, being the first TOR sequence obtained from a hemimetabolous insect. The long branch length corresponding to *C. elegans* TOR suggests a rapid rate of divergence with respect to other Metazoa. The tree also shows that vertebrates cluster in a node separated from insects, and that vertebrate branches are remarkably short, thus indicating the great conservation of these sequences, whereas insect branches show intermediate lengths.

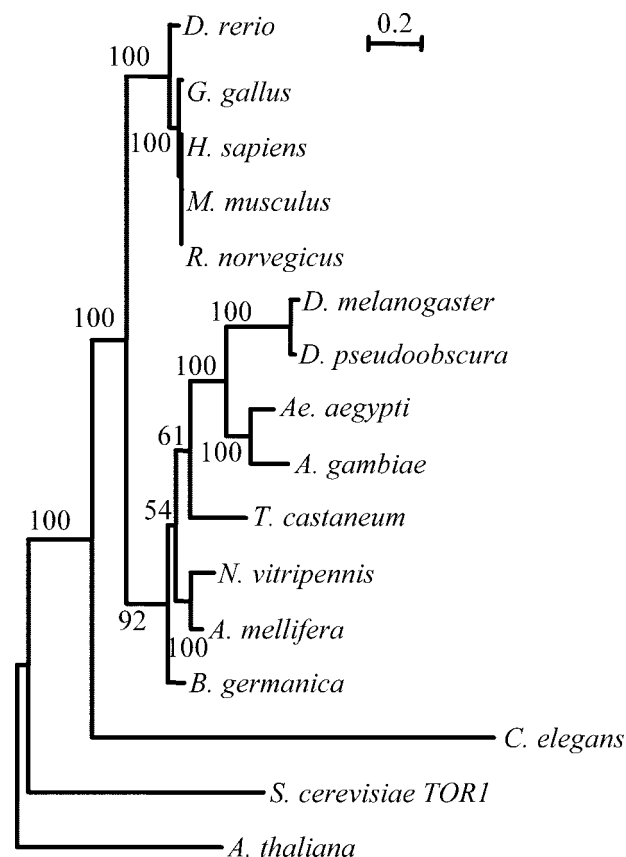
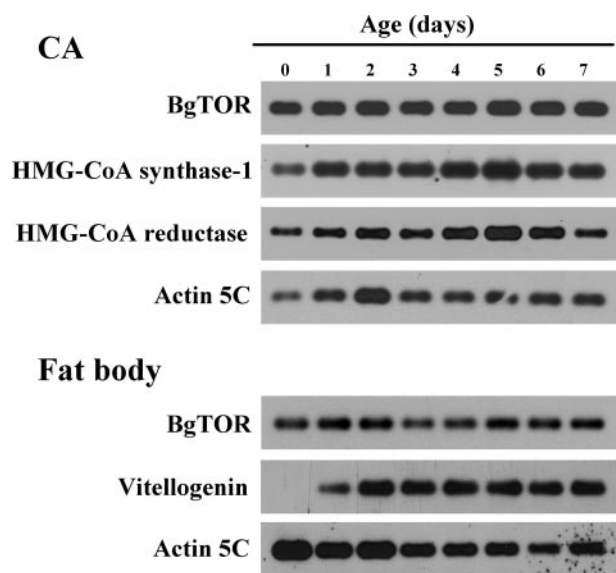


FIGURE 1. **Phylogenetic relationships of *B. germanica* TOR.** The tree was constructed using the maximum-likelihood approach. Branch lengths are proportional to sequence divergence. The bar represents 0.2 substitutions per site. Bootstrap values (100 replicates) are shown in the nodes. The sequence from *A. thaliana* was used as outgroup. The binomial name of the species is indicated under "Experimental Procedures."

**Expression Patterns of BgTOR and Other mRNAs Related to Reproductive Processes**—mRNA levels of BgTOR and other transcripts related to reproductive processes were studied in CA and fat body of adult females throughout the first gonadotrophic cycle. We used semi-quantitative RT/PCR followed by Southern blot, and results showed that BgTOR mRNA levels in the CA were practically constant throughout the gonadotrophic cycle (Fig. 2). As expected, mRNA levels of HMG-CoA synthase-1 and HMG-CoA reductase, two key enzymes in the JH biosynthetic pathway, showed a pattern resembling that of JH production (1) (Fig. 2). The pattern of HMG-CoA synthase-2 was similar to that of HMG-CoA synthase-1 (results not shown). As in the CA, levels of BgTOR mRNA in the fat body showed a practically constant pattern. In the fat body, expression of vitellogenin was activated 1 day after the adult moult, and reached full mRNA levels at mid vitellogenesis (Fig. 2).

**Effects of BgTOR RNAi**—To study the function of BgTOR, its expression was lowered using systemic RNAi. A dose of 2 μg of a 506-bp dsRNA fragment encompassing part of the FAT domain (dsTOR, Fig. 3A) was injected into the abdomen of freshly emerged fifth (penultimate) instar female nymphs, and the treatment was repeated after the next moult (BgTOR RNAi group). Specimens treated equivalently with a nonspecific dsRNA were used as negative controls (control group). RNAi-mediated BgTOR knockdown females moulted normally into



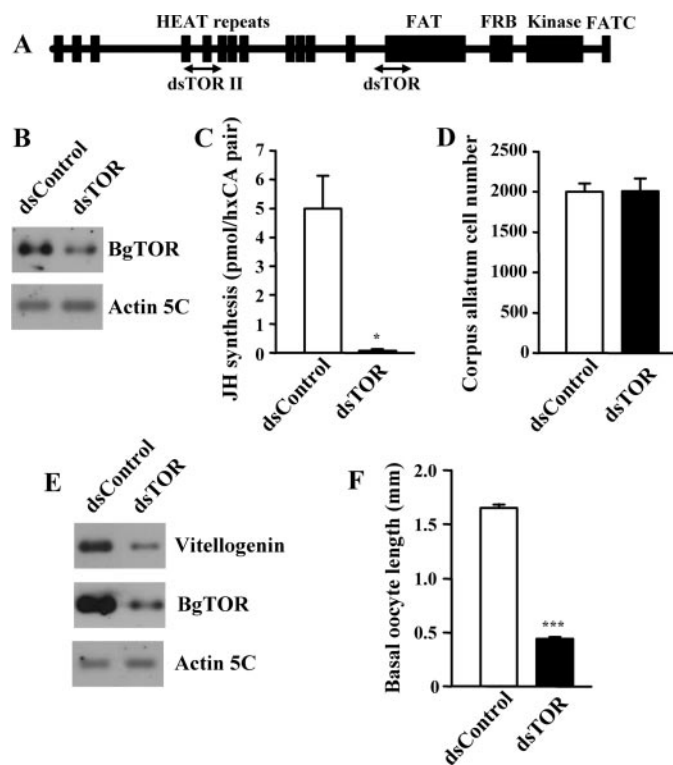
**FIGURE 2. Expression patterns of BgTOR mRNA compared with other transcripts in the CA and the fat body of *B. germanica* adult females.** mRNA levels of BgTOR, HMG-CoA synthase-1, HMG-CoA reductase, and vitellogenin were analyzed using semi-quantitative RT-PCR/Southern-blot in CA and fat body during the 7 days of the first gonadotrophic cycle. Actin 5C levels were used as a reference. Blots shown are representative of 3–5 replicates.

the sixth nymphal instar and later into adults. Differences between both treatments were examined 5 days after the adult moult.

BgTOR mRNA levels in CA from 5-day-old BgTOR RNAi group were lower than those of control group (Fig. 3B). Importantly, JH synthesis by the CA from BgTOR knockdown females was dramatically reduced with respect to that measured in controls (Fig. 3C). Given that TOR has been related to growth and developmental processes, we considered the possibility that low JH production in the females treated with dsTOR might be due to disfunctions related to cell proliferation in the CA. However, cell quantification in 5-day-old females showed that the CA from the BgTOR RNAi group had practically the same number of cells than CA from the control group (Fig. 3D).

Interestingly, total RNA extracted from the abdominal fat body was significantly lower in the BgTOR RNAi group than in the control group ( $2.23 \pm 0.51 \mu\text{g}$ ,  $n = 5$ , versus  $6.74 \pm 0.89 \mu\text{g}$ ,  $n = 5$ ;  $p = 0.0023$ , Student's *t* test). Conversely, total protein levels were higher in the BgTOR RNAi group than in controls ( $576.1 \pm 110.0 \mu\text{g}$ ,  $n = 4$ , versus  $257.7 \pm 24.3 \mu\text{g}$ ,  $n = 6$ ;  $p = 0.0086$ , Student's *t* test). As observed in the CA, fat body tissues from BgTOR knockdown animals had lower levels of BgTOR mRNA than in controls (Fig. 3E). Moreover, a clear reduction in vitellogenin mRNA was observed in the knockdown group with respect to the control group (Fig. 3E). Consequently, whereas ovaries from the control group grew and developed normally, those from the BgTOR RNAi group did not grow at all and the basal oocytes were as small as those of freshly emerged adult females (Fig. 3F).

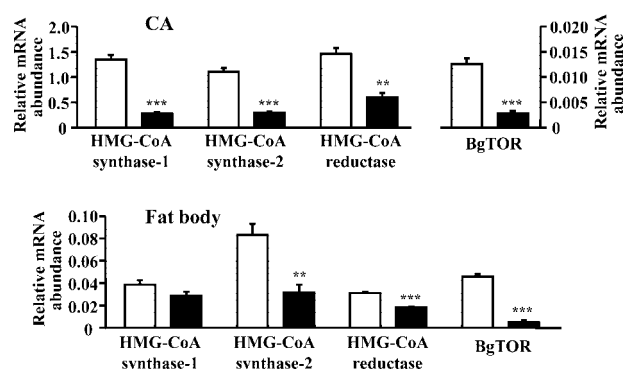
**Effects of BgTOR RNAi on HMG-CoA Synthase and HMG-CoA Reductase**—HMG-CoA synthase and HMG-CoA reductase are considered key enzymes in the mevalonate pathway and for JH synthesis (3). Given that BgTOR RNAi treatment resulted in a dramatic reduction of JH production, we studied



**FIGURE 3. Effect of BgTOR RNAi in the CA, fat body, and ovaries of *B. germanica*.** dsRNA targeting BgTOR (*dsTOR*) or a nonspecific dsRNA (*dsControl*) were administered in freshly emerged penultimate instar nymphs and again when they moulted into last instar. Dissections were carried out 5 days after the adult moult. mRNA levels were studied using semi-quantitative RT-PCR/Southern-blot. Actin 5C levels were used as a reference. *A*, BgTOR domain organization showing the regions used to generate the two dsRNAs: *dsTOR* and *dsTOR II*. *B*, BgTOR mRNA levels in CA; blots are representative of 7–8 replicates. *C*, rates of JH synthesis by CA incubated *in vitro*; results are expressed as the mean  $\pm$  S.E. ( $n = 4–8$ ). *D*, total cell number in a single corpus allatum; results are expressed as the mean  $\pm$  S.E. ( $n = 9$ ). *E*, mRNA levels of vitellogenin and BgTOR in fat body; blots are representative of three replicates. *F*, basal oocyte length; results expressed as the mean  $\pm$  S.E. ( $n = 25–31$ ). Asterisks indicate significant differences (Student's *t* test, \*  $p < 0.05$ ; \*\*\*  $p < 0.0001$ ).

the knockdown effects on the expression of HMG-CoA synthase-1 and -2 (18, 19) and HMG-CoA reductase (20) in CA and fat body. In this case, we were interested not only in the mere modification of mRNA levels, but also in the relative contribution of each of these enzymes in both tissues. Consequently, we quantified the mRNA levels with real-time quantitative PCR. We used CA and fat body tissues from 5-day-old control and BgTOR RNAi groups, and data were normalized by measuring mRNA levels of Actin 5C.

CA from the BgTOR RNAi group showed a significant decrease (77%) of BgTOR mRNA levels, concomitant with a marked decrease of mRNA levels of HMG-CoA synthase-1 (80%), -2 (73%), and HMG-CoA reductase (59%) (Fig. 4, upper panel). In the fat body, mRNA levels of BgTOR were also significantly reduced (89%), in parallel with those of HMG-CoA synthase-2 (62%) and HMG-CoA reductase (42%); mRNA levels of HMG-CoA synthase-1 in the BgTOR knockdown animals did not differ significantly from controls (Fig. 4, lower panel). Results also indicated that in CA from controls, the mRNA for the three enzymes showed similar levels, whereas in the fat body, mRNA levels of HMG-CoA synthase-2 were twice of

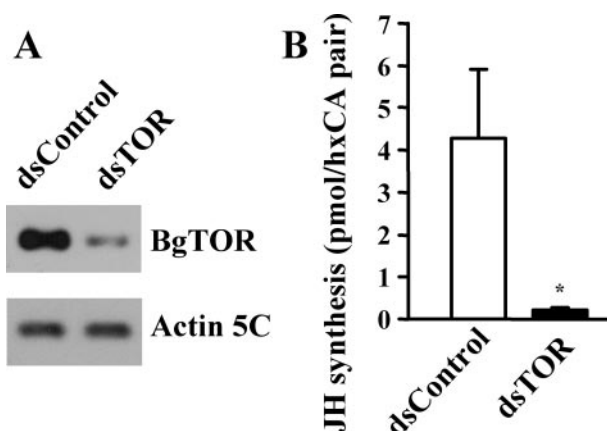


**FIGURE 4. Effect of BgTOR RNAi on mRNA levels of enzymes of the mevalonate pathway and BgTOR in CA and fat body of *B. germanica*.** dsRNA targeting BgTOR (*dsTOR*) or a nonspecific dsRNA (*dsControl*) were administered in freshly emerged penultimate instar nymphs and again when they moulted into the last instar. Dissections were made 5 days after the adult moult. Results show mRNA levels of HMG-CoA synthase-1, -2, HMG-CoA reductase, and BgTOR in CA (upper panel) and fat body (lower panel) quantified by real-time PCR analysis. White and solid bars indicate dsControl and dsTOR treatments, respectively. Y-axis indicates copies per copy of Actin 5C. Results are expressed as the mean  $\pm$  S.E. ( $n = 4-5$ ). Asterisks indicate significant differences (Student's *t* test, \*\*,  $p < 0.005$ ; \*\*\*,  $p < 0.0001$ ).

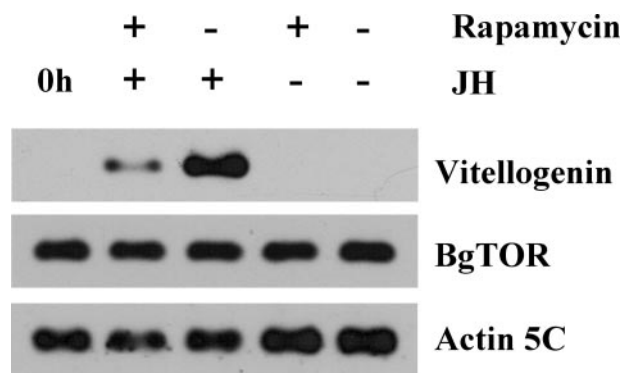
those of HMG-CoA synthase-1 and HMG-CoA reductase. Moreover, relative mRNA levels of HMG-CoA synthase-1, -2, and HMG-CoA reductase are around 30-fold higher in the CA than in the fat body (Fig. 4).

Equivalent RNAi experiments using a dsRNA based on a 547-bp fragment encompassing the HEAT repeats region (*dsTOR II*, Fig. 3A), resulted in similar phenotypes showing a JH-deficiency syndrome. For example, the basal oocyte did not grow in *dsTOR II*-treated females, with length values significantly lower than in controls at day 5 of adult life (control group:  $1.37 \pm 0.11$  mm,  $n = 13$ ; *dsTOR II*-treated group:  $0.43 \pm 0.01$  mm, ( $n = 12$ ); *t* test  $p < 0.0001$ ). BgTOR mRNA levels in the CA from *dsTOR II*-treated females were quantified with real-time quantitative PCR, and results indicated that they were significantly lower (77%) than in controls. Moreover, the effects of *dsTOR II* treatment were studied in the CA in terms of expression of the enzymes regulating JH synthesis. Results indicated that *dsTOR II*-treated females showed a significant decrease of mRNA levels of HMG-CoA synthase-1 (82%), -2 (80%), and HMG-CoA reductase (78%) ( $n = 3$  in all cases).

**BgTOR RNAi in Adult Females**—The treatment with *dsTOR* in last instar nymphs inhibited JH synthesis in the adult CA, but did not reduce the cell number in these glands. However, the possibility that reduced TOR levels had affected some process of CA development linked to the transition from nymph to adult, still remained. Thus, to see whether the effects of TOR knockdown upon the synthesis of JH are specific and independent of the metamorphic moult, we carried out RNAi experiments in the adult. Thus, a dose of 2  $\mu$ g of *dsTOR* was injected in adult females at the first day of ootheca transport, whereas specimens treated equivalently with the nonspecific dsRNA served as negative controls. Then a second gonadotrophic cycle was induced by removing the ootheca 12 days later. At day 5 of this second cycle, BgTOR mRNA levels in the RNAi group were clearly lower than in controls (Fig. 5A), and production of JH in the TOR interfered animals was dramatically reduced (Fig. 5B). Therefore, the results were similar to those obtained with the



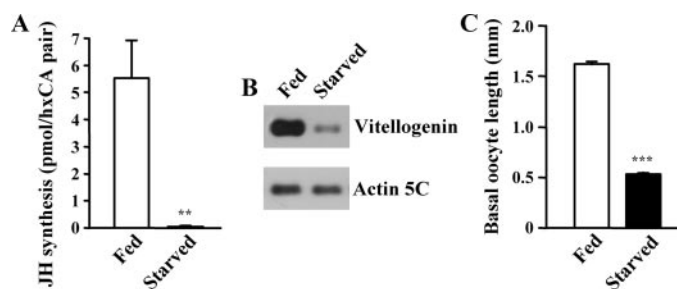
**FIGURE 5. Effect of BgTOR RNAi on adult females of *B. germanica*.** dsRNA targeting BgTOR (*dsTOR*) or a nonspecific dsRNA (*dsControl*) were administered in adult females at the first day of ootheca transport. Then a second gonadotrophic cycle was induced by removing the ootheca 12 days later. Results were examined at day 5 of this second cycle. A, mRNA levels of BgTOR in CA; mRNA levels were studied using semi-quantitative RT-PCR/Southern-blot; Actin 5C levels were used as a reference; the blot is representative of six replicates. B, rates of JH synthesis by CA incubated *in vitro*; results are expressed as the mean  $\pm$  S.E. ( $n = 9-10$ ). The asterisks indicate significant differences (Student's *t* test,  $p < 0.05$ ).



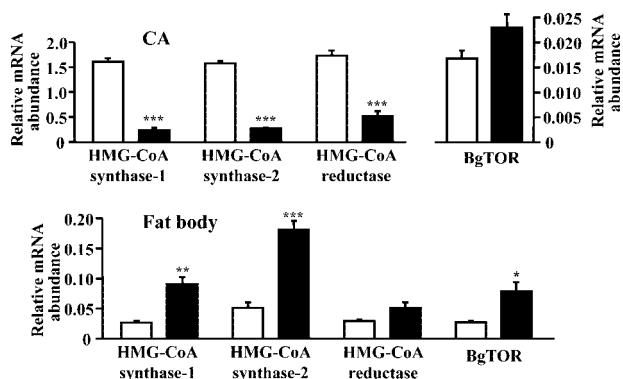
**FIGURE 6. Effect of rapamycin on JH induction of vitellogenin expression in *B. germanica*.** Fat bodies from freshly emerged adult females were incubated *in vitro* with different combinations of JH and rapamycin. After incubation, vitellogenin and BgTOR mRNA levels were analyzed using semi-quantitative RT-PCR/Southern-blot. Actin 5C levels were used as a reference. 0 h indicates the status of tissues before the incubation. Blots shown are representative of four replicates.

specimens treated as nymphs and analyzed in the first gonadotrophic cycle (Fig. 3).

**Effects of Rapamycin on Vitellogenin Transcription**—Our experiments indicated that BgTOR RNAi inhibited JH production in the CA, and also that fat body was affected as well, at least in terms of vitellogenin, HMG-CoA synthase, and HMG-CoA reductase expression (Figs. 3E and 4). In this context, we wondered whether TOR would play a role on the induction of vitellogenin transcription by JH. To answer that, we incubated fat body tissues *in vitro* in the presence of rapamycin, a specific inhibitor of TOR (17). Fat bodies from freshly emerged adult females (which do not produce vitellogenin because the CA are still inactive) were incubated for 4 h in Grace's medium containing 500 nM of rapamycin or the corresponding solvent alone. Then, fat bodies were transferred to a medium containing, in addition to the previous treatment, 800 nM of JH III or the corresponding solvent and incubated for 4 h. Results (Fig. 6) showed that fat body from freshly emerged females incubated



**FIGURE 7. Effect of starvation in the CA, fat body, and ovaries of *B. germanica*.** Starved females received only water after the adult moult and dissections were carried out 5 days later. *A*, rates of JH synthesis by CA incubated *in vitro*; results are expressed as the mean  $\pm$  S.E. ( $n = 7$ ). *B*, vitellogenin mRNA levels in the fat body analyzed with semi-quantitative RT-PCR/Southern-blot; Actin 5C levels were used as a reference; blots are representative of four replicates. *C*, basal oocyte length, expressed as the mean  $\pm$  S.E. ( $n = 5-6$ ). Asterisks represent significant differences (Student's *t* test, \*\*,  $p < 0.005$ ; \*\*\*,  $p < 0.0001$ ).



**FIGURE 8. Effect of starvation on mRNA levels of enzymes of the mevalonate pathway and BgTOR in CA and fat body of *B. germanica*.** Starved females were supplied only with water after the adult moult and dissections were made 5 days later. Results show mRNA levels of HMG-CoA synthase-1, -2, HMG-CoA reductase, and BgTOR in CA (upper panel) and fat body (lower panel) quantified by real-time PCR analysis. White and solid bars indicate fed and starved specimens, respectively. Y-axis indicates copies per copy of Actin 5C. Results are expressed as the mean  $\pm$  S.E. ( $n = 5-6$ ). Asterisks represent significant differences (Student's *t* test, \*,  $p < 0.05$ ; \*\*,  $p < 0.005$ ; \*\*\*,  $p < 0.0001$ ).

in media without JH do not express vitellogenin, as expected. Conversely, fat body incubated with JH produced vitellogenin mRNA, although the expression levels observed in rapamycin-treated fat body were lower than those observed in control medium with JH only (Fig. 6).

**Effects of Starvation**—Given that TOR is involved in nutritional signaling, we wondered about the effects of starvation on the same parameters analyzed in BgTOR knockdown animals. To study them, we used 5-day-old *B. germanica* females that had been starved from the adult moult. As expected, the CA from starved females produced JH at very low levels (Fig. 7A), vitellogenin mRNA levels were also severely reduced (Fig. 7B), and the ovaries remained as in freshly emerged specimens (Fig. 7C). The mRNA levels of HMG-CoA synthase-1, -2, and HMG-CoA reductase in CA from starved females were lower than those from fed females (Fig. 8, upper panel), whereas in the fat body, starved females showed higher mRNA levels for the three enzymes than fed females (Fig. 8). Concerning mRNA levels of BgTOR, differences between fed and starved were not statistically significant in the CA (Fig. 8), whereas in the fat body, BgTOR mRNA levels were somewhat higher in starved females (Fig. 8).

## DISCUSSION

We have cloned and characterized BgTOR as the TOR homologue in the German cockroach, *B. germanica*. It is the first TOR sequence described in a hemimetabolous insect species. BgTOR shows the characteristic organization of TOR proteins, with N-terminal HEAT repeats, followed by a FAT domain, a rapamycin-FKBP12-binding domain, a kinase domain, and a C terminus FATC region (17).

BgTOR mRNA levels in the CA and the fat body of *B. germanica* adult females are practically constant throughout the first gonadotrophic cycle, in spite that during it there is also a marked feeding cycle (2). It is worth remembering that CA are the glands synthesizing JH, the gonadotrophic hormone in *B. germanica*. In unfed adult females of the mosquito *A. aegypti*, AaTOR mRNA is present in carcass, fat body, midgut, and Malpighian tubules at low concentrations, and a blood meal did not result in significant expression changes in these tissues. The ovaries (which synthesize ecdysteroids, the gonadotrophic hormones in this mosquito) showed higher levels of AaTOR mRNA, and they increased 2-fold 24 h after a blood meal (7).

RNAi of BgTOR in penultimate and last instar nymphs inhibited almost completely the production of JH in the adult CA, and this was not related with cell proliferation in the glands, given that RNAi treatments did not influence CA cell number. Moreover, BgTOR RNAi treatment in the adult inhibited JH production by the CA in the second gonadotrophic cycle, which indicates that TOR is crucial for CA activation and JH biosynthesis, independently of metamorphic processes occurring in the adult moult. Concomitant with JH inhibition, BgTOR RNAi specimens showed low levels of vitellogenin mRNA in the fat body, and basal oocytes did not grow at all.

Moreover, expression studies demonstrated that mRNA levels of HMG-CoA synthase-1, -2, and HMG-CoA reductase are exceptionally high in CA from control adult females, and that these levels resulted remarkably reduced in CA from interfered for BgTOR specimens, which is in agreement with the low levels of JH synthesis measured in them. Identical phenotypes were obtained using two completely different dsRNA sequences (dsTOR and dsTOR II), which verifies that the obtained results do not derive from off target, unrelated to TOR knockdown, effects. The whole data indicate that TOR is needed to activate JH production in CA of adult females after feeding. A relationship between CA activation and the insulin/insulin-like growth factor signaling (IIS) pathway, a hormonal pathway related to nutritional signaling and growth, has been reported in *D. melanogaster*. In the fruit fly, insulin receptor (*InR*) mutant adult females showed a reduction in JH production, together with an increase of longevity, whereas treatment of these mutants with a JH analogue restored normal lifespan (21). In addition, RNAi of *InR* specifically in the CA, blocked HMG-CoA reductase expression in these glands (22). These results indicate that IIS pathway is important to regulate JH production in *D. melanogaster* by modulating the expression of enzymes of the mevalonate pathway, as occurs with the TOR pathway and the CA of *B. germanica*.

Our results also showed that RNAi of TOR decreases mRNA levels of HMG-CoA synthase and HMG-CoA reductase in the fat body. This suggests that TOR may be important for full expression of these enzymes of the mevalonate pathway in the fat body, and for the general functioning of this organ. Thus, we considered whether TOR would play a role in one of the major functions of the female adult fat body, that is, on the induction of vitellogenin transcription under the influence of JH. To answer this question, we incubated fat body tissue *in vitro* in the presence of rapamycin. Rapamycin is an immunosuppressive drug that binds to FKBP12, and this complex binds to TOR and inhibits its kinase activity (6). Results showed that fat body of freshly emerged *B. germanica* females with TOR protein inhibited by rapamycin were less responsive to JH activation of *vitellogenin* transcription. This suggests that TOR plays also a role in the induction of vitellogenesis by JH in the fat body, independently of its action on JH synthesis in the CA. Interestingly, in the mosquito *A. aegypti*, where the gonadotrophic hormone is 20-hydroxyecdysone instead of JH, rapamycin also inhibits vitellogenin gene expression in fat body *in vitro* (5).

A remarkable effect of RNAi experiments was that total RNA extracted from abdominal fat body was 3-fold lower in the BgTOR RNAi group than in the control group, whereas total protein contents were twice as high in that group compared with controls. Transcription initiation factor IA (TIF-IA), a conserved RNA polymerase I (Pol I) transcription factor, has been reported to be regulated by the TOR pathway in *D. melanogaster* (23). Pol I is the main enzyme involved in the synthesis of rRNA and, hence, of total RNA. Therefore, we presume that reduction of BgTOR activity in our knockdown group may have led to a decrease of activity of the *B. germanica* TIF-IA homolog, thus affecting rRNA transcription and total RNA. Indeed, comparison of the levels of Actin 5C mRNA in the fat body from BgTOR RNAi and control groups indicated that, despite the reduction of total RNA induced by the treatment, Actin 5C mRNA levels were similar in fat body of the two groups (results not shown), as would be observed if total RNA reduction was related to a decrease in rRNA. The higher levels of protein found in the fat body from BgTOR knockdown specimens with respect to controls may be related with the fact that control females were at the end of the vitellogenic period, when total fat body protein levels are decreasing (24).

Finally, and given that TOR has been related to nutritional signaling (4, 6), we examined the expression of the different transcripts, the JH production and the ovary growth in starved adult females, to compare the starvation syndrome with that obtained in BgTOR knockdown specimens. CA from 5-day-old starved females showed very low JH production, and had reduced mRNA levels of HMG-CoA synthase-1, -2, and HMG-CoA reductase, these data being similar to those found in the BgTOR RNAi specimens. Conversely, BgTOR mRNA levels were similar in CA from fed and starved females. This contrasts with reports describing that starvation reduces TOR activity in other models, including animals and yeast (4, 6, 7) and suggests that in CA of starved *B. germanica* females, BgTOR activity in relation to feeding should be modulated at post-transcriptional level. Post-transcriptional regulation of TOR activity by direct interaction between TOR and the small GTP-binding protein

Ras homolog enriched in brain (Rheb), has been demonstrated in yeast (25) and mammals (26, 27). In addition, tuberous sclerosis complex (TSC1/TSC2) proteins negatively regulate Rheb activity (28).

Again, as it occurs in the BgTOR RNAi group, fat body from starved females expressed much less vitellogenin mRNA compared with fed females, and their ovaries did not grow. In the anaotogenous mosquito *A. aegypti*, 20-hydroxyecdysone is only released after a blood meal (29) and only the concomitancy of the gonadotrophic hormone and feeding or amino acid supply is able to increase vitellogenin transcription (5). Results also showed higher HMG-CoA synthases and HMG-CoA reductase mRNA levels in fat body from starved compared with fed females, contrarily to what occurs in CA. Given that the fat body is a tissue with basically metabolic functions, in comparison with a classical endocrine organ as CA, it is not surprising that an extended starvation condition could result in different regulatory responses in these two tissues.

In summary, the whole results indicate that BgTOR knockdown mimics the effect of starvation in CA of *B. germanica* females, and suggest that nutritional signals that activate JH biosynthesis in CA and vitellogenin production in fat body of adult cockroaches are mediated by the TOR pathway.

### REFERENCES

- Bellés, X. (2005) in *Reproductive Biology of Invertebrates* (Raikhel, A. S. ed), pp. 157–197, Science Publishers, Inc.
- Osorio, S., Piulachs, M. D., and Belles, X. (1998) *J. Insect Physiol.* **44**, 31–38
- Belles, X., Martin, D., and Piulachs, M. D. (2005) *Annu. Rev. Entomol.* **50**, 181–199
- Baker, K. D., and Thummel, C. S. (2007) *Cell Metab.* **6**, 257–266
- Hansen, I. A., Attardo, G. M., Park, J. H., Peng, Q., and Raikhel, A. S. (2004) *Proc. Natl. Acad. Sci. U. S. A.* **101**, 10626–10631
- Oldham, S., and Hafen, E. (2003) *Trends Cell Biol.* **13**, 79–85
- Hansen, I. A., Attardo, G. M., Roy, S. G., and Raikhel, A. S. (2005) *J. Biol. Chem.* **280**, 20565–20572
- Maestro, O., Cruz, J., Pascual, N., Martin, D., and Belles, X. (2005) *Mol. Cell Endocrinol.* **238**, 27–37
- Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F., and Higgins, D. G. (1997) *Nucleic Acids Res.* **25**, 4876–4882
- Castresana, J. (2000) *Mol. Biol. Evol.* **17**, 540–552
- Guindon, S., and Gascuel, O. (2003) *Syst. Biol.* **52**, 696–704
- Maestro, J. L., and Belles, X. (2006) *Arch. Insect Biochem. Physiol.* **62**, 73–79
- Mane-Padros, D., Cruz, J., Vilaplana, L., Pascual, N., Belles, X., and Martin, D. (2008) *Dev. Biol.* **315**, 147–160
- Piulachs, M. D., and Couillaud, F. (1992) *J. Insect Physiol.* **38**, 555–560
- Chiang, A.-S., Tsai, W.-H., Holbrook, G. L., and Schal, C. (1996) *Arch. Insect Biochem. Physiol.* **32**, 299–313
- Cruz, J., Mane-Padros, D., Belles, X., and Martin, D. (2006) *Dev. Biol.* **297**, 158–171
- Schmelzle, T., and Hall, M. N. (2000) *Cell* **103**, 253–262
- Buesa, C., Martinez-Gonzalez, J., Casals, N., Haro, D., Piulachs, M. D., Belles, X., and Hegardt, F. G. (1994) *J. Biol. Chem.* **269**, 11707–11713
- Martinez-Gonzalez, J., Buesa, C., Piulachs, M. D., Belles, X., and Hegardt, F. G. (1993) *Eur. J. Biochem.* **217**, 691–699
- Martinez-Gonzalez, J., Buesa, C., Piulachs, M. D., Belles, X., and Hegardt, F. G. (1993) *Eur. J. Biochem.* **213**, 233–241
- Tatar, M., Kopelman, A., Epstein, D., Tu, M. P., Yin, C. M., and Garofalo, R. S. (2001) *Science* **292**, 107–110
- Belgacem, Y. H., and Martin, J. R. (2007) *PLoS ONE* **2**, e187
- Grewal, S. S., Evans, J. R., and Edgar, B. A. (2007) *J. Cell Biol.* **179**, 1105–1113
- Martin, D., Piulachs, M. D., and Bellés, X. (1995) *Invert. Reprod. Dev.* **28**,

- 171–176
25. Urano, J., Comiso, M. J., Guo, L., Aspuria, P. J., Deniskin, R., Tabancay, A. P., Jr., Kato-Stankiewicz, J., and Tamanoi, F. (2005) *Mol. Microbiol.* **58**, 1074–1086
26. Long, X., Lin, Y., Ortiz-Vega, S., Yonezawa, K., and Avruch, J. (2005) *Curr. Biol.* **15**, 702–713
27. Long, X., Ortiz-Vega, S., Lin, Y., and Avruch, J. (2005) *J. Biol. Chem.* **280**, 23433–23436
28. Zhang, Y., Gao, X., Saucedo, L. J., Ru, B., Edgar, B. A., and Pan, D. (2003) *Nat. Cell Biol.* **5**, 578–581
29. Hagedorn, H. H., O'Connor, J. D., Fuchs, M. S., Sage, B., Schlaeger, D. A., and Bohm, M. K. (1975) *Proc. Natl. Acad. Sci. U. S. A.* **72**, 3255–3259