SHORT COMMUNICATION

Isolation of a fragment homologous to the *rp49* constitutive gene of *Drosophila* in the Neotropical malaria vector *Anopheles aquasalis* (Diptera: Culicidae)

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The constitutive ribosomal gene rp49 *is frequently used as an endogenous control in* Drosophila *gene expression experiments. Using the degenerate primer PCR technique we have cloned a fragment homologous to this gene in* Anopheles aquasalis *Curry, a Neotropical vector of malaria. In addition, based on this first sequence, a new primer was designed, which allowed the isolation of fragments of* rp49 *in two other species,* Aedes aegypti *(Linnaeus) and* Culex quinquefasciatus *Say, suggesting that it could be used to clone fragments of this gene in a number of other mosquito species. Primers were also designed to specifically amplify* rp49 *cDNA fragments in* An. aquasalis *and* Ae. aegypti*, showing that* rp49 *could be used as a good constitutive control in gene expression studies of these and other vectorially important mosquito species.*

Key words: *Anopheles aquasalis* - *Aedes aegypti* - *Culex quinquefasciatus* - *Drosophila* constitutive gene - *rp49* - *rpL32* - mosquitoes

The ribosomal protein 49 gene (*rp49*) of the fruitfly *Drosophila* (O'Connell & Rosbash 1984), also known as *rpL32* (http://flybase.bio.indiana.edu), has been widely used as an endogenous constitutive control in gene expression studies (e.g. Glossop et al. 1999, Goodwin et al. 2000, Kurapati et al. 2000, Stanewsky et al. 2002). The sequence of *rp49* is also available for the mosquito *Anopheles gambiae* (Holt et al. 2002), the most important Afrotropical malaria vector, but until recently this gene had not been sequenced from other species of mosquitoes. As part of our molecular studies of insect vectors of tropical diseases, we attempted to clone a *rp49* homologous fragment in *An. aquasalis*, a widespread Neotropical malaria vector that is associated with coastal habitats (Consoli & Lourenço-de-Oliveira 1994, Fairley et al. 2002, Forattini 2002).

Specimens used in this work were derived from a laboratory colony of *An. aquasalis* established in 1993 with around 200 females collected in a farm in Paracambi, Rio de Janeiro, Brazil (Carvalho et al. 2002). Using kits supplied by Amersham Biosciences, genomic DNA was isolated by means of the GenomicPrep^{TM} Cells & Tissue DNA isolation kit; mRNAs with the QuickPrep^{TM} Micro mRNA purification kit and cDNAs were synthesized using the First-Strand cDNA synthesis kit. PCR was performed in 40 µl using Tth DNA polymerase (Biotools) according to manufacturer's directions using various cycling conditions (see below) and the primers listed in the Table. PCR products were purified using either the Micro Spin S-400 HR Column (Amersham Biosciences) or the Wizard SV Gel and PCR Clean-up System (Promega), and cloned using the pGEM-T Easy Vector Kit (Promega). DNA sequencing was carried out in an ABI377 Sequencer using the Big Dye 3.1 Kit (Applied Biosystems).

The first *rp49* fragment from *An. aquasalis* was obtained using cDNA as template and degenerate primers based on conserved regions identified by comparison between the putative protein sequences of *D. melanogaster* and *An. gambiae*. Initially, we conducted PCR using primers 5rp49deg1 and oligo-d(T)20 and the following cycling conditions: 94ºC for 5 min; 15 cycles at 94ºC for 1 min, 55ºC (minus 1ºC each cycle) for 1 min and 72ºC for 2 min; then 20 more cycles at 94ºC for 1 min, 50ºC for 1 min and 72ºC for 2 min. Although no amplification products could be detected by 2% agarose gel electrophoresis, a fragment of ~320 base pairs was observed after reamplification of 1 µl of the first reaction using primers 5rp49deg1 and 3rp49deg3 (Fig. 1, Table), and 35 cycles at 94ºC for 1 min, 50ºC for 1 min, and 72ºC for 2 min. This product was purified, cloned and sequenced as described above. Homology to *rp49* was confirmed by comparison to the *Drosophila* sequence database using BlastX (http://www.ncbi.nlm.nih.gov/).

Based on the *An. aquasalis rp49* cDNA sequence obtained, a specific primer (5aquaRP1, Fig. 1, Table) was designed and used with degenerate primer 3rp49deg3 in a new PCR (94ºC for 5 min, followed by 35 cycles of 94ºC for 30 s, 50ºC for 30 s, and 72ºC for 1 min) to amplify an *An. aquasalis rp49* 400 bp genomic sequence. This fragment was cloned and sequenced. Comparison with the

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cDNA sequence confirmed the presence of an 88 bp intron, located in the same position as that of *D. melanogaster*.

Because primer 5aquaRP1 is in a conserved region, we tried to see if it could be used to isolate fragments of *rp49* in other mosquito species. *Aedes aegypti* is a highly competent vector of dengue and urban yellow fever in Brazil and elsewhere (Lourenço-de-Oliveira et al. 2004); this mosquito originated from Africa and is now synanthropic (Alonso et al. 2003, Braga et al. 2004, Cunha et al. 2005) throughout the tropics (within the 20ºC isotherm) between latitudes 45º N and 35º S approximately (Christophers 1960, Forattini 2002). The tropical house mosquito *Culex quinquefasciatus* is the urban vector of lymphatic filariasis (White & Nathan 2002) and transmits arboviruses such as St. Louis and West Nile (Service 2001). The primer 5aquaRP1 was used, together with oligo-d(T)20, to amplify *rp49* cDNA sequences from these two species as described above. As before, products were observed only after a reamplification reaction, using primers 5aquaRP1 and 3rp49deg3. Fragments of ~ 300 bp obtained from *Ae. aegypti* and *Cx. quinquefasciatus* were purified, cloned and sequenced. Homology to *rp49* was again confirmed by comparison to the *Drosophila* sequence database.

Fig. 1 gives the alignment of RP49 proteins from *D. melanogaster* and *An. gambiae* compared to the deduced amino acid sequences obtained from the fragments we isolated from *An. aquasalis, Ae. aegypti* and *Cx. quinquefasciatus* (sequences submitted to the GenBank, accession numbers AY539746 to AY539748). As shown, the amplified region is highly conserved with only a few substitutions observed among the five sequences.

To illustrate the use of *rp49* as an endogenous control in mosquitoes we designed a primer (5aquaexpRP) across the intron-exon boundary to specifically amplify cDNA sequences of *An. aquasalis* (Fig. 1, Table). Fig. 2 shows the results of PCR carried out using this and other primers. Lanes 1 and 2 show the amplification products obtained for *An. aquasalis* cDNA and genomic DNA, respectively, using the primers 5aquaRP1 and 3aeaquaRP1, flanking the intron that accounts for the size difference between the two fragments. Lane 3 is the negative control for these reactions. Lanes 4 and 5 show the results of PCR using primers 5aquaexpRP and 3aeaquaRP1 with the same templates and lane 6 is their respective negative control. Note that although some primer-dimer formation is observed in all three lanes, a 190 bp fragment corresponding to the expected size is amplified when cDNA, but not

TABLE

Degenerate and specific primers used to amplify the *rp49* fragments of *Anopheles aquasalis, Aedes aegypti,* and *Culex quinquefasciatus*

Name	Sequence (5' —— 3')
5rp49deg1	GNCCNAARATHGTNAARAA
3rp49deg3	TCYTTNGCNCKYTCNACDAT
5aquaRP1	GTGAAGAAGCGGACGAAGAAGTT
5aquaexpRP	GCTATGATAAGCTCGCTCCTGC
3aeaquaRP1	TGCATCATCAGCACCTCCAGC
5aeexpRP	GCTATGACAAGCTTGCCCCCA
3aeaquaRP1b	TCATCAGCACCTCCAGCTC

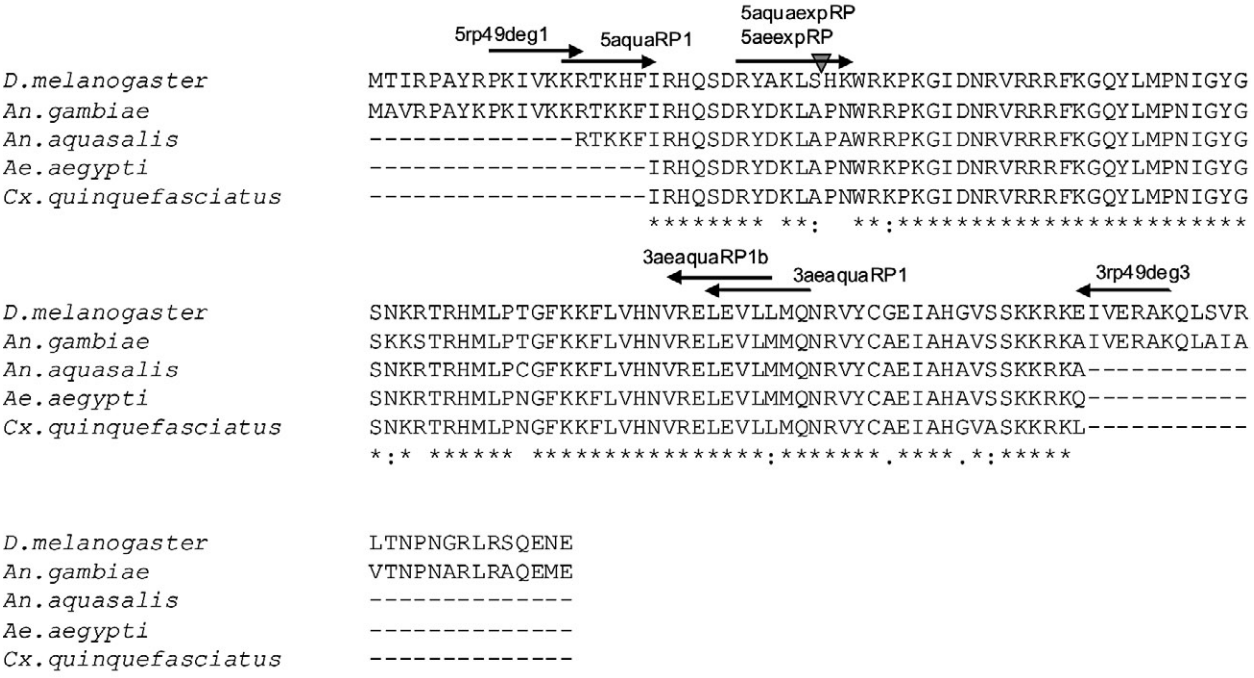

Fig. 1: alignment of the RP49 proteins from *Drosophila melanogaster* and *Anopheles gambiae* compared to the amino acid sequences encoded by the fragments obtained from *An. aquasalis, Aedes aegypti,* and *Culex quinquefasciatus.* Arrows point to the approximate location of the different primers used. The position of the intron found in the genomic sequences of *D. melanogaster, An. gambiae,* and *An. aquasalis* is indicated by an inverted triangle (▽).

genomic DNA, is used as template. Similar results were obtained with *Ae. aegypti* using primers 5aeexpRP and 3aeaquaRP1b (data not shown).

Since *rp49* has been frequently used as an endogenous constitutive control in gene expression studies in *Drosophila*, its homologues from *An. aquasalis*, *Ae. aegypti*, *Cx. quinquefasciatus,* and other vector species might provide useful tools in molecular studies of these medically important mosquitoes.

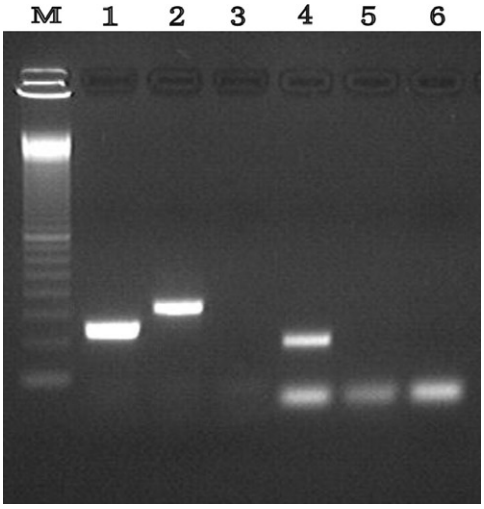

Fig. 2: electrophoresis (agarose 2%) of the *Anopheles aquasalis rp49* PCR products. M- 100 bp ladder; 1: cDNA, primers 5aquaRP1 and 3aeaquaRP1; 2: genomic DNA, primers 5aquaRP1 and 3aeaquaRP1; 3: negative control, primers 5aquaRP1 and 3aeaquaRP1; 4: cDNA, primers 5aquaexpRP and 3aeaquaRP1; 5: genomic DNA, primers 5aquaexpRP and 3aeaquaRP1; 6: negative control, primers 5aquaexpRP and 3aeaquaRP1.

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