Title

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Authors

Muñoz, Delia
Jimenez, Alyssa J.
Marinotti, Osvaldo
et al.

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SHORT NOTE

The *AeAct-4* gene is expressed in the developing flight muscles of female *Aedes aegypti*

D. Muñoz*†‡, A. Jimenez†‡, O. Marinotti† and A. A. James†§

**Departamento de Producción Agraria, Universidad Pública de Navarra, Campus Arrosadía s/n, Pamplona, Spain; †Department of Molecular Biology & Biochemistry; and §Department of Microbiology & Molecular Genetics, University of California, Irvine, CA, USA*

Abstract

Population reduction of mosquitoes is an effective method for controlling dengue fever and malaria transmission. Recent developments in control techniques include proposals to construct transgenic strains of mosquitoes carrying dominant, conditional-lethal genes under the control of sex- and stage-specific promoters. In order to identify such promoters, subtractive cDNA libraries derived from male and female pupal mRNA of the yellow fever mosquito, *Aedes aegypti*, were constructed and screened. A cDNA clone, F49, corresponds to a gene expressed specifically in female pupae. Sequence analyses revealed that this gene belongs to the actin gene family, and therefore was designated *Aedes Actin-4* (*AeAct-4*). Transcription analyses demonstrated that this gene is expressed predominantly in the indirect flight muscles and, to a lesser extent, the legs of developing female mosquitoes. The promoter of this gene may be a useful tool for developing conditional lethal strains of mosquitoes.

Keywords: muscle actin, female-specific, pupal expression, RIDL.

Introduction

The extensive and frequent application of broad-spectrum pesticides for control of insect vectors of diseases has resulted in the selection of resistant vector populations as well as raising environmental concerns stemming from their toxicity to non-target organisms (World Health Organization, 1995; Schinas *et al*., 2000; Luo & Yu, 2001; Roos *et al*., 2001). The development of species-specific methods for insect control could diminish these hazards and make available population reduction protocols for integrated programs of vector control. One such method is sterile insect technique (SIT), which targets the reproductive potential of an insect population by the mass release of radiation-sterilized males meant to mate with wild females. Successful SIT programs have been conducted against several agricultural, veterinary and medically important insect species (Tan, 2000), but the tedious and costly production of single-sex populations for release in combination with the reduced fitness of irradiated insects has encouraged the development of new approaches. Recently, Thomas *et al*. (2000) and Heinrich & Scott (2000) elaborated a transgenic system to induce repressible female-specific lethality. This technique, termed the 'release of insects carrying a dominant lethal' (RIDL), requires that a strain of the target organism carry a dominant, sex-specific lethal gene whose expression can be repressed during the mass-rearing of the insect. RIDL has proven feasible in laboratory experiments with the vinegar fly, *Drosophila melanogaster*, in which it was demonstrated that it had advantages to conventional SITs by having less of an impact on the fitness of adult males. Additionally, the use of a sex-specific promoter driving the expression of the repressible lethal gene can be used to halt the survival of one of the sexes (the one with the active sex-specific promoter) making automatic the separation of males and females and thus potentially reducing production costs.

Mosquito vectors of human disease are candidates for RIDL because many of the components of the system designed for *D. melanogaster* could be adapted to them. However, sex-specific promoters need to be identified in order to develop further this approach. In this work, our aim was to identify sex-specific genes of *Ae. aegypti*, the mosquito vector of dengue virus to humans, that are expressed in larval or pupal stages of the insect life cycle, and whose product is needed in essential tissues. The screening of subtractive cDNA libraries resulted in the identification of a gene, *Aedes Actin-4* (*AeAct-4*), that is expressed

Received 31 March 2004; accepted following revision 19 July 2004. Correspondence: Dr Anthony A. James, Department of Molecular Biology & Biochemistry, 3205 McGaugh Hall, University of California, Irvine CA 92697–3900, USA. Tel.: +1 949 824 5930; fax: +1 949 824 2814; e-mail: aajames@uci.edu

‡These authors contributed equally to this work

abundantly in the indirect flight muscles of developing female pupae and to a lesser extent in the developing legs.

Results and Discussion

Isolation and primary characterization of AeAct-4 *cDNAs and genomic clone*

Duplicate filters spotted with samples of cDNAs derived from subtractive libraries were screened with probes made from either male- or female-specific pupal mRNA. These procedures resulted in the identification of a female-specific cDNA, F49, that was 455 bp in length (Fig. 1). Sequence analysis revealed that the cDNA was incomplete, and 5′- and 3′-RACE procedures were used to generate a full-length product of 1787 bp. A 200 bp gene-specific probe (*AeAct-4* GSP) based on F49 was used to screen a genomic library, and a clone, A5, identified. Sequencing of A5 revealed that the complete gene is ~3.6 kb in length. The gene contains three exons and two introns. The first intron is ~1.8 kb in length and is located in the 5′-end untranslated region (5′-UTR). The second intron is 60 bp in length and interrupts the translated portion of the coding sequence.

Features of the AeAct-4 *gene*

Sequence analysis of the 1128 bp open reading frame (ORF) of the cDNA revealed the coding potential for a 376 amino acid protein with a predicted M_r = ~41 600 and sequence similarity with previously characterized insect actins (Fig. 2A). Based on the sequence similarity and the order in which *Ae. aegypti* actin genes have been named (Ibrahim *et al*., 1996; Vyazunova & Lan, 2004), the corresponding gene was designated *Aedes Actin-4* (*AeAct-4*).

BLASTX analysis of the *AeAct-4* showed that its amino acid sequence has 95% identity to numerous insect actins, including *D. melanogaster Actin 88F* and *D. virilis Actin E2*, which were identified previously as encoding 'adult' actins (Mounier & Sparrow, 1993). *AeAct-4* contains five of the seven typical amino acid residues of insect-specific muscle actins: D3, A232, V279, I326 and G369. Two additional amino acid residues, A5 and I76 are typically found in insect adult muscle actins and only in insect larval and adult muscle actins, respectively (Mounier & Sparrow, 1993). The exon–intron structure of the gene is similar to the *D. melanogaster Act88F* and *D. virilis ActE2* genes. Each of the fruit fly genes contains an intron in the 5′-UTR and an intron in the protein encoding sequence. The relative locations of the introns are preserved in particular for the latter intron.

Nucleotide sequence analysis shows that *AeAct-4* is not identical to any of the three previously described *Ae. aegypti* actin genes showing only 65%, 66% and 62% identity with *AeAct-1*, *AeAct-2*, *AeAct-3*, respectively (Ibrahim *et al*., 1996; Krebs *et al*., 2002; Vyazunova & Lan, 2004). Although actin cDNAs are highly conserved in their coding regions, they are markedly different in the 3′-UTRs and the four *Aedes* genes, which all code for muscle actins, can be readily differentiated based on the nucleotide sequences of those regions. The distinction of *AeAct-4* from other *Aedes* actins and its relationship with other insect actins is further demonstrated in a phylogenetic analysis by using ClustalW (Thompson *et al*., 1994) of deduced amino acid sequences of *Aedes* actins and related actins from *An. gambiae*, *D. melanogaster*, *D. virilis*, and *B. mori* (Fig. 2B; Mounier *et al*., 1987; Ibrahim *et al*., 1996; Krebs *et al*., 2002; Vyazunova & Lan, 2004). Southern blot analysis of genomic DNA probed with *AeAct-4* GSP demonstrates that *AeAct-4* is a single copy gene (Fig. 3).

Expression profile of the AeAct-4 *gene*

AeAct-4 GSP was used to study the expression pattern of *AeAct-4* throughout the life cycle of *Ae. aegypti*. Total RNA

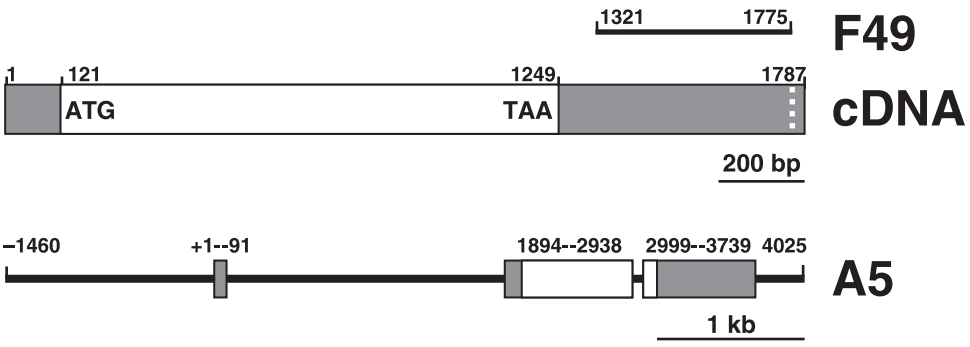

Figure 1. Schematic representation of the primary structure of the *AeAct-4* transcript and genomic DNA. The reconstructed F49 cDNA is represented as a single horizontal line (top). The numbers refer to its position relative to the reconstructed complete cDNA (middle). The cDNA is represented as boxed structures with the 5′- and 3′-end untranslated regions (UTRs) shaded grey and the open reading frame (ORF) depicted in white. 'ATG' and 'TAA' refer to the translation initiation and termination codons, respectively. The vertical dashed region represents the relative location of the polyadenylation signal sequence. The numbers above the figure represent the locations of the ORF with respect to the overall length of the cDNA. A portion of the genomic clone, A5 is depicted (bottom). Non-coding sequence and introns are represented as thick lines, the 5′- and 3′ UTRs are shaded grey, and the coding sequence is white. Numbers above the figure refer to nucleotide positions relative to the transcription initiation site. Scale bars for the cDNAs (in bp) and genomic clone (in kb) are represented by thin horizontal lines. GenBank accession numbers: *AeAct-4* cDNA, AY531222; A5 genomic clone, AY531223.

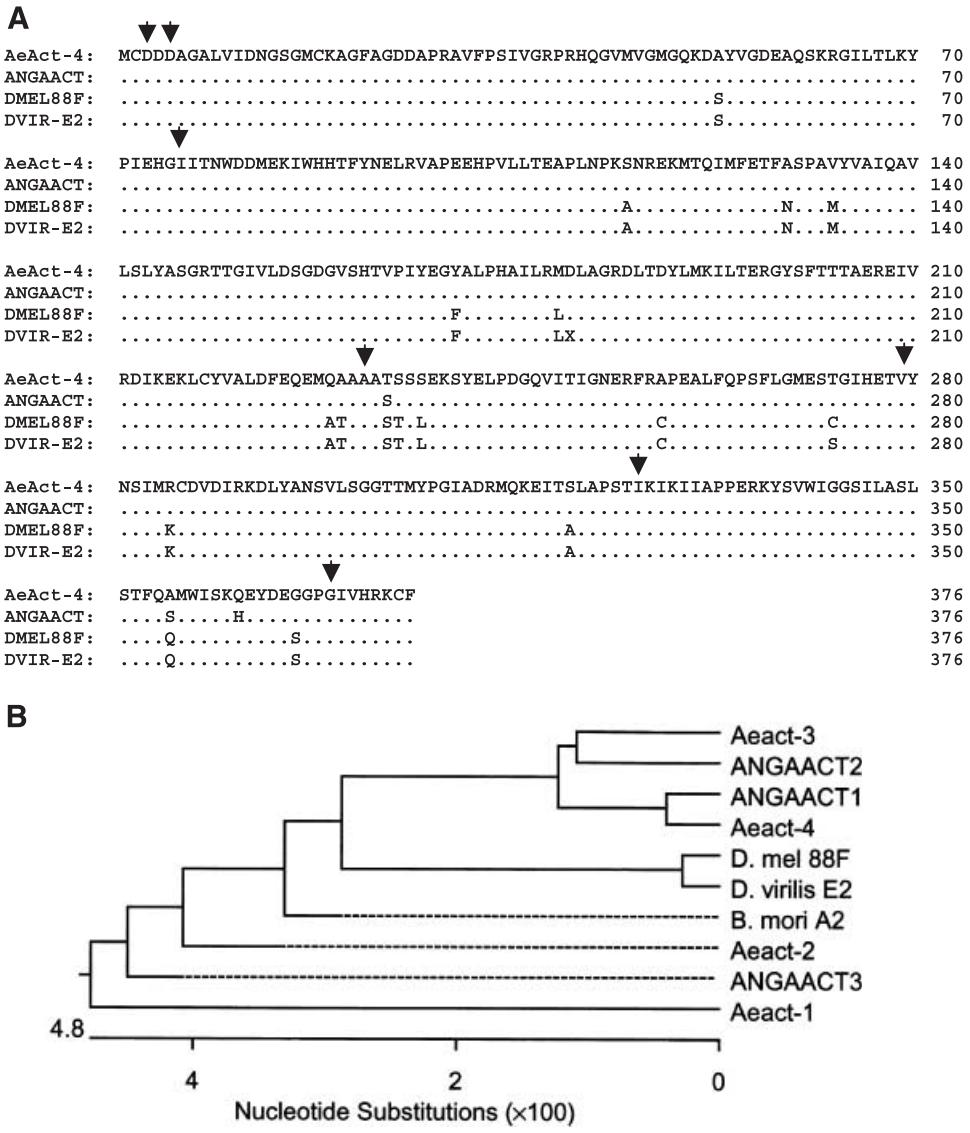

Figure 2. *AeAct-4* is a member of the actin gene family. (A) Amino acid alignments (Clustal-W) of the conceptual translation products of *AeAct-4* (AeAct-4), the *Drosophila melanogaster* Actin *88F* gene (DMEL88F), the *D. virilis* Actin *E2* gene (DVIR-E2), and an *Anopheles gambiae* Actin gene (ANGAACT1). The complete amino acid sequence encoded by *AeAct-4* is given on the top line and differences in the other proteins are listed below. Dots represent identity with the *AeAct-4* sequence. Vertical arrows highlight amino acids conserved in insect actins. Numbers refer to the positions of the amino acids. (B) Phylogenetic relationships of deduced amino acid sequences of *Ae. aegypti* actins and related insect proteins. Numbers refer to the number of nucleotide substitutions in the transcribed portions of the genes. GenBank accession numbers: *AeAct-1*, U20287; *AeAct-2*, AY289764; *AeAct-3*, AY289765; DMEL-88F, M18826; DVIR-E2, AF358263; BMORI-A2, P07837; ANGAACT1, XM315270; ANGAACT2, XP32140; ANGAACT3, XP314407.

was isolated from pools of third- and fourth-instar larvae, early and late male and female pupae, and male and female adults, and Northern blot analyses performed (Fig. 3). Specific hybridization to an ~1.8 kb RNA was evident only in RNA samples from the female pupae. This hybridization pattern confirms that *AeAct-4* is female and pupal-specific. The accumulation of *AeAct-4* mRNA is increased in the late pupal stage and is absent from fully developed 3-day-old mosquitoes. Hybridizations *in situ* were performed using the *AeAct-4* GSP probe and whole-mounts of female pupae to determine the location of *AeAct-4* expression (Fig. 4). *AeAct-4* mRNA accumulates in the longitudinal and dorso-ventral indirect flight muscles. Weak staining in the developing legs suggests that *AeAct-4* mRNA is also expressed here in lower levels. RT-PCR analysis confirmed the presence of mRNA in the legs. RT-PCR also showed that there is no expression in males (whole pupae) or in female abdomens. No signals were seen in control female preparations hybridized to sense probes or in males (data not shown).

The gene organization, high amino acid identity and expression profiles of *AeAct-4* are similar to *Act88F* and

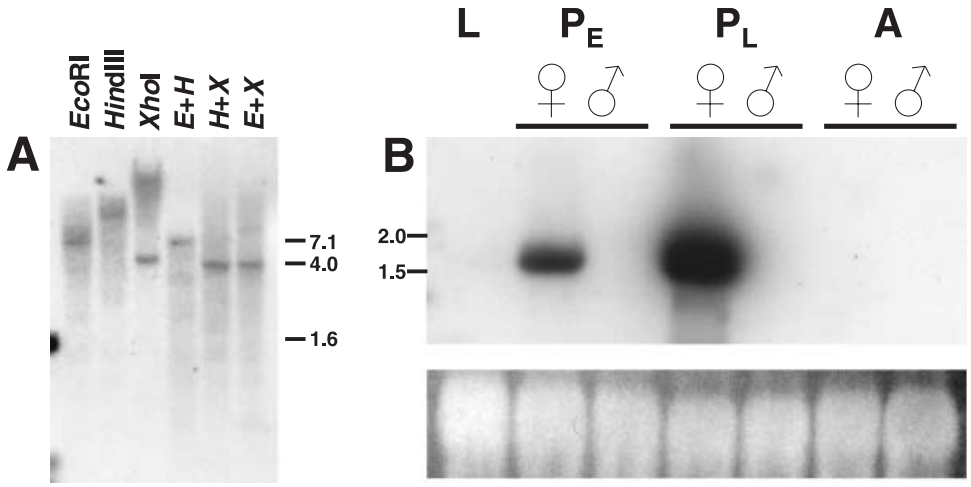

Figure 3. Southern and Northern analysis of the *AeAct-4* gene. (A) Southern analysis of genomic DNA using the *AeAct-4* GSP probe. Lanes represent single- and double-digestion products. Abbreviations: E + H, *Eco*RI and *Hin*dIII digestion products; H + X, *Hin*dIII and *Xho*I digestive products; E + X, *Eco*RI and *Xho*I digestion products. (B) Northern analysis of *Ae. aegypti* total RNA using the *AeAct-4* GSP. Top panel, samples prepared from 3rd and 4th instar larvae (L); early (= 3 h after pupation) pupae (P_E); late (> 24 h after pupation) pupae (P_L); 3-day-old adults (A). Sex-specific samples are indicated. Bottom panel: Ethidium-bromide stained ribosomal RNA photographed to demonstrate equal loading of samples in the gel. Numbers in both panels are fragment-length size markers in kb.

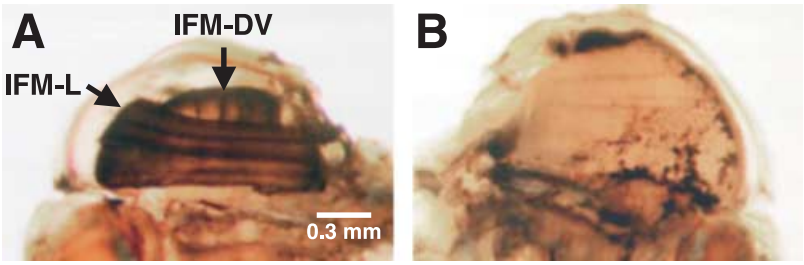

Figure 4. *AeAct-4* is expressed in the indirect flight muscles of pupal females. Hybridization *in situ* with antisense (A) and sense (B) F49 RNA probes. Heavy staining of the longitudinal (IFML) and dorso-ventral (IFM-DV) indirect flight muscles indicates significant *AeAct-4* mRNA accumulation.

ActE2, which are specialized actins found in the indirect flight muscles of *D. melanogaster* and *D. virilis*, respectively (Lovato *et al*., 2001). The data indicate that these *Drosophila* genes and *AeAct-4* are likely orthologous, however, expression of this member of the actin gene family exclusively in females is unique to *Ae. aegypti*. The biological significance of a female-specific muscle actin in *Ae. aegypti* is unknown. However, the expression pattern of *AeAct-4* makes its promoter a useful tool to direct female-specific gene expression in transgenic mosquitoes, which is a requirement for the development of *Ae. aegypti* strains to be used in RIDL programs to control mosquito populations.

Experimental procedures

Mosquitoes

Aedes aegypti mosquitoes (Rockefeller strain) were reared in the UCI insectary facility using standard protocols (Munstermann, 1997).

Extraction and purification of RNA

Groups of ten pupae were collected in 1.5 ml disposable microcentrifuge tubes. Each group was homogenized with 1.0 ml Trizol (Life Technologies, Rockville, MD) and RNA extraction followed the manufacturer's protocol. Purification of poly(A)$^+$ RNA from total RNA was performed using the Oligotex kit and procedure (Qiagen, Valencia, CA).

Construction of subtracted female- and male-specific libraries

Subtracted cDNA libraries were constructed using the PCR-Select^{TM} cDNA subtraction kit (Clontech Laboratories, Palo Alto, CA). Two micrograms of female poly(A)$^+$ RNA was used as a template for the synthesis of the 'female tester' (F tester), and 2.0 μg of male poly(A)$^+$ RNA was used as a template for the synthesis of the 'male tester' (M tester). Similar samples containing 2.0 μg of female or male poly(A)$^+$ RNA were used as templates for the synthesis of the F-driver and M-driver, respectively. The subtraction protocol uses subtractive hybridization and suppression PCR to specifically amplify sequences that are expressed in the tester cDNA populations. Amplified fragments of most of the female-specific cDNAs were between 200 and 700 bp in length while the sizes of most of the male-specific cDNA fragments were slightly larger, between 250 and 900 bp (data not shown). The products of the suppression PCR were ligated into pGEM-T (Promega, Madison, WI), transformed by electroporation into DH10B cells, and plated on LB-agar containing 100 μg/ml ampicillin, 0.4% X-gal, and 0.5% IPTG.

Differential screening of the subtracted cDNA libraries

The identification of differentially expressed cDNAs from the male and the female subtracted libraries was performed using the PCR-Select™ differential screening kit (Clontech laboratories). The DNA inserts contained in the plasmids of 135 and 96 clones from the female and male libraries, respectively, were PCR-amplified (Israel, 1993). An aliquot of each amplification product was denatured and dot-arrayed in four identical Zeta-Probe® GT nylon membranes (Bio-Rad laboratories, Hercules, CA). Each of the four identical membranes then was hybridized for 12–16 h at 65 °C with one of the following ^{32}P-labelled probes: subtracted female cDNA, unsubtracted female cDNA, subtracted male cDNA, and unsubtracted male cDNA. Probes were labelled with ^{32}P-dATP by Random priming using the MegaPrime DNA labelling system (Amersham, Buckinghamshire, UK). Clones hybridized to the female probes but not to the male probes were considered for further analysis.

Rapid amplification of cDNA ends (RACE)

The 5′- and 3′-ends of the *AeAct-4* cDNA were synthesized using the primers 5′-TGAACGAACGCATAAAGGTG-3′ and 5′-AGGATATCACTGCAGCCGCGAGAAGAAC-3′, respectively, 1.0 μg of poly(A)$^+$ RNA extracted from early pupae using the Smart™ Race kit (Clontech Laboratories). Reverse transcription was performed as recommended by the supplier although amplification conditions were varied as follows to adjust them to Peltier Thermal Cycler 200 (MJ Research Inc., Watertown, MA) 5 cycles of 94 °C for 15 s and 72 °C for 3 min; and 30 cycles of 94 °C for 15 s, 70 °C for 30 s, and 72 °C for 3 min.

Isolation of genomic clones

An *Ae. aegypti* genomic library in Lambda DASH® II (Stratagene, La Jolla, CA), was screened initially using a PCR-based method as described (Israel, 1993). Gene-specific primers used for this screening were based upon the cDNA F49 clone: 5′-TGAACGAACGCATAAAGGTG-3′ and 5′-GCGAAAATCTGCGACTCCA-3′. Secondary and tertiary screenings were carried out by performing plaque lifts and hybridization with the ^{32}P-radiolabelled F49 probe. Library plating, filter lifting, probe-labelling, hybridization and exposure to X-ray film were performed using standard protocols (Sambrook *et al*., 1989).

DNA sequencing and analysis

DNA samples were submitted to automated sequencing (Davis sequencing LLC, Davis, CA). Protein and DNA sequence database searches were performed using BLAST (NCBI). The alignments were obtained by the Clustal-W function of the Megalign in the Lasergene software package (DNAstar Inc., Madison, WI).

Northern and dot blot hybridizations

Fifteen μg of total RNA was separated on a 1.2% agarose/6% formaldehyde gel and transferred to a nylon GeneScreen membrane (NEN Research, Boston, MA) via capillary action using 10× SSC in an overnight transfer. The membrane was cross-linked at 1200 μW/cm^2 with a UV Stratalinker (Stratagene, La Jolla, CA). Blots were hybridized with the 200 bp *AeAct-4* GSP probe made from a gene amplification product using primers 5′-TGAACGAACGCATAAAGGTG-3′ and 5′- GCGAAAATCTGCGACTCCA-3′ and F49 as template. Hybridization was conducted at 60 °C in Church's buffer (0.5 M Na-phosphate buffer, pH 7.2, 1% BSA, 1 mM EDTA, 7% SDS) and washed twice at 60 °C in 2× SSC/0.1% SDS solution for 15 min and once in 0.1× SSC/0.1% SDS at 60 °C for 15 min. Membranes were exposed to Biomax X-ray film (Eastman Kodak, Rochester, NY) at −70 °C.

Southern blot analysis

Total genomic DNA was isolated from adult *Ae. aegypti* using GenomicPrep Cells and Tissue DNA Isolation kit from Amersham Biosciences. Approximately 12 μg DNA was used per digestion reaction. DNA was either singly or doubly digested with selected restriction endonucleases and probed with *AeAct-4* GSP. Standard protocols for gel electrophoresis, filter transfers, probing and exposures to film were used (Sambrook *et al*., 1989).

Hybridizations in situ *to whole-mount tissues*

Hybridization *in situ* to combined heads and thoraces was carried out using digoxigenin-labelled RNA probes and a commercially available DIG Nucleic Acid Detection Kit (Roche, Indianapolis, IN). The F49 cDNA was cloned into pGEM-T easy vector to provide a 3′-UTR-specific riboprobe (Promega, Madison, WI). Sense and antisense riboprobes were generated from these plasmids in the presence of digoxigenin-labelled UTP using either T7 or SP6 polymerase (Roche, Indianopolis, IN).

Pupal abdomens were separated and removed from thoraces before fixation. Pupae were fixed in 1× SSPE/4% formaldehyde at 4 °C for 3 h with gentle agitation, and rinsed in 1× SSPE overnight at 4 °C. Specimens were dehydrated in an increasing ethanol (EtOH) gradient. The subsequent prehybridization steps were carried out at 4 °C unless otherwise specified. Pupae were washed in 50/50 xylene/EtOH for 1 h, followed by five 3-min washes in EtOH. Pupae then were washed twice in methanol (MetOH) for 2 min each, one time in 50/50 MetOH/PBT (0.1% Tween-20 in PBS) + 5% formaldehyde for 5 min, and then gently agitated for 30 min in PBT + 5% formaldehyde at 4 °C. Pupae were washed in five 2-min PBT washes and then incubated for 10 min in PBT/Proteinase K (3.2 μg) at 37 °C. Pupae were washed five times in PBT for 2 min each, followed by a 5 min wash in 50/50 PBT/hybridization solution (50% deionized formamide, 5× SSC, 100 μg/ml herring sperm DNA, 50 μg/ml heparin, 0.1% Tween-20). At this time, pupal thoraces were dissected laterally and longitudinally to allow for optimal probe access to tissues. Dissected thoraces then were washed three times in hybridization solution before prehybridizing at 55 °C overnight. Subsequent hybridizations with either sense or antisense probes were conducted at 55 °C with gentle rotation. Following hybridization, washes were conducted as follows: one rinse in fresh hybridization solution, one wash in hybridization solution for 1 h at 55 °C, six 20-min washes in hybridization (Hyb) solution at 55 °C, followed by three 15-min washes: 3 : 1 Hyb solution/PBT, 1 : 1 Hyb solution/PBT, 1 : 3 Hyb solution/PBT. Thoraces were washed five times in PBT before incubation with α-DIG/AP antibody (Roche, Indianapolis, IN) and subsequent detection with NBT/BCIP stock solution (Roche, Indianapolis, IN) according to manufacturer's protocol.

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