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Forkhead transcription factors regulate mosquito reproduction

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Abstract

Forkhead-box (Fox) genes encode a family of transcription factors defined by a 'winged helix' DNA-binding domain. In this study we aimed to identify Fox factors that are expressed within the fat body of the yellow fever mosquito *Aedes aegypti*, and determine whether any of these are involved in the regulation of mosquito yolk protein gene expression. The *Ae. aegypti* genome contains 18 loci that encode putative Fox factors. Our stringent cladistic analysis has profound implications for the use of Fox genes as phylogenetic markers. Twelve *Ae. aegypti* Fox genes are expressed within various tissues of adult females, six of which are expressed within the fat body. All six Fox genes expressed in the fat body displayed dynamic expression profiles following a blood meal. We knocked down the 'fat body Foxes' through RNAi to determine whether these 'knockdowns' hindered amino acid-induced vitellogenin gene expression. We also determined the effect of these knockdowns on the number of eggs deposited following a blood meal. Knockdown of FoxN1, FoxN2, FoxL, and FoxO, had a negative effect on amino acid-induced vitellogenin gene expression and resulted in significantly fewer eggs laid. Our analysis stresses the importance of Fox transcription factors in regulating mosquito reproduction.

Keywords: Forkhead; *Aedes aegypti*; Fat body; Vitellogenin; Yolk protein; RNAi

1. Introduction

The class of proteins termed forkhead-box (Fox) transcription factors are a large and diverse group of transcriptional regulators characterized by a conserved 110-amino acid 'Fox' DNA-binding domain, also referred to as the 'winged-helix' domain. This domain consists of three alpha-helices connected by two polypeptide loops, or 'wings,' which are related to the 'helix-turn-helix' domain found in homeobox-containing proteins. Fox-containing proteins have been identified in eukaryotic organisms from yeast to humans, and have been shown to play important roles in various biological processes, including development, metabolism, immunoregulation, development of cancer, and aging (Carlsson and Mahlapuu, 2002; Coffer and Burgering, 2004; Jonsson and Peng, 2005; Kaufmann and Knochel, 1996; Lehmann et al., 2003). The nomenclature for the chordate forkhead transcription factors has been revised, and these genes, now termed 'Fox' (after 'Forkhead box'), are divided into seventeen subclasses, or clades, (A–Q), based on the amino acid (AA) sequence of their conserved forkhead domain (Kaestner et al., 2000). Invertebrates share several Fox genes that can be subgrouped according to the vertebrate subfamilies (Mazet et al., 2003).

The first Fox protein, forkhead (FoxA), was identified as a homeotic gene in the fruit fly *Drosophila melanogaster* (Weigel et al., 1989), followed by a description of its Fox DNA-binding domain (Weigel and Jackle, 1990). Several additional Fox transcription factors have been described in *Drosophila*, with greater emphasis placed on their regulation of embryonic development. *crocodile* (FoxC) is an early patterning gene expressed in the head anlagen of the *Drosophila* embryo, and is responsible for establishing the development of head structures (Hacker et al., 1995). *biniou* (FoxF) plays a key role in

Abbreviations: AA, amino acid; BM, blood meal; Fox, forkhead box; PBM, post blood meal; PV, previtellogenic; RNAi, RNA interference; TOR, Target of rapamycin; vg, vitellogenin; YPP, Yolk protein precursor

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the development of the visceral mesoderm and the derived gut musculature (Zaffran et al., 2001). The two FoxG genes in *Drosophila*, *sloppy paired* 1 and *sloppy paired* 2 (Grossniklaus et al., 1992) are both involved in the regulation of early embryo segmentation (Cadigan et al., 1994). *domina* (FoxN) is expressed mainly within the central and peripheral nervous systems, and *domina* mutants display multiple phenotypes including diminished vitality and fertility (Strodicke et al., 2000). The best-characterized invertebrate Fox gene is FoxO (*Caenorhabditis elegans*, DAF16), a target of the insulin-signaling pathway. dFoxO regulates insulin signaling in the brain and fat body, along with controlling fertility and lifespan (Hwangbo et al., 2004a). FoxO manifests this effect through exhibiting transcriptional feedback control of the insulin receptor (Puig and Tjian, 2005). Together, these studies stress the importance of Fox transcription factors for regulating development and homeostasis in *Drosophila*. However, the function for a majority of Fox transcription factors of *Drosophila* and other insects are yet to be described.

It has been suggested that Fox factors may be involved in the regulation of mosquito reproduction, for the vitellogenin (*Vg*) gene of *Aedes aegyptii* has a Fox-binding site at position −469 of its proximal promoter. *In vitro* expressed *Drosophila* FoxA can bind to the promoter of the *Vg* gene, and further, it has been suggested that a Fox transcription factor in combination with other transcriptional regulators may coordinate the tissue-specific expression of *YPP* genes in the fat body of mosquitoes (Kokoza et al., 2001). Interestingly, the Fox-binding site in the *Vg* promoter is in close proximity to a CCAAT/Enhancer-binding site. A similar arrangement of binding sites has been found in the proximal promoter of the human decidual prolactin gene. Human FoxO1 interacts directly with C/EBPβ and this complex binds to the composite Fox/C/EBPβ binding site to strongly activate decidual prolactin transcription (Christian et al., 2002).

In this study, we identified 18 putative Fox transcription factors from the genome of *Ae. aegypti*, along with predicting their phylogenetic relationships with other insect and human Fox factors. We determined whether the identified Fox factors are expressed within tissues of adult female mosquitoes both before and after a blood meal. The Fox factors determined to be expressed within the fat body were characterized further with a more extensive expression profile during the course of a gonotrophic cycle, and by determining effects of knockdown of these Fox factors on *Vg* gene expression. Using RNA interference-mediated knockdown, we identified several of these Fox factors as being involved in promoting vitellogenesis and mosquito reproduction. The results of our study lay the foundation for the study of the role of Fox factors in the regulation of reproduction in mosquitoes as well as other insects.

2. Materials and methods

2.1. Sequence identification

Predicted cDNA and deduced AA sequences of Fox transcription factors were identified using BLAST tools at two databases: Ensembl (http://www.ensembl.org/Aedes_aegypti/blastview), and VectorBase (http://www.vectorbase.org). Predicted Fox cDNAs were blasted against an EST-database at TIGR (TIGR (http://tigrblast.tigr.org/tgi/). The full length coding sequences of *Ae. aegypti* FoxL, and FoxO, and partial sequences of FoxN1, and FoxN2 were isolated and sequenced. Sequences were submitted to Genbank with the accession numbers: EF088380 (FoxO), EF088381 (FoxL), EF537041 (FoxN2). Nine Hymenoptera sequences (EF137264-EF137272) were provided by John Heraty. Human Fox transcription factor sequences were obtained from Kaestner et al. (2000).

2.2. Phylogenetic analyses of sequences

Stringent cladistic analyses were conducted on the data. Four alignment programs produced competing alignments, which were evaluated, and the best two scoring alignments then subjected to cladistic analyses using parsimony, maximum likelihood, and Bayesian methodologies (see Supplemental Methods for a complete discussion).

2.3. Ae. aegypti rearing and fat body culture

The *Ae. aegypti* mosquito strain UGAL/Rockefeller was maintained in laboratory culture as described by Hays and Raikhel (1990). The fat body culture system is described in detail elsewhere (Hays and Raikhel, 1990; Raikhel et al., 1997).

2.4. RNA isolation

For expression profiling, RNA was isolated from head (hd), thorax (tx), abdominal body wall (fb), midgut (gt), malpighian tubules (mt) and ovaries (ov) both before (PV = previtellogenic) and after a blood meal (24 h post blood meal (PBM)). Tissues of 10–50 individual mosquitoes were pooled. For the fat body expression time course, the fb was dissected from females at PV, 6, 16, 22, 24, 30, 48 and 65 h PBM. For the fb time course, quantitative PCR (qPCR) against three groups of three fat bodies were analyzed. Total RNA was isolated by means of a commercially available modification (TRIzol, Invitrogen, Carlsbad, CA) of the one-step phenol/guanidinium thiocyanate method (14). Genomic DNA contamination was removed by treatment with Rnase-free Dnase I (Invitrogen) prior to cDNA synthesis. Reverse transcription was carried out using an Omniscript reverse transcriptase kit (Qiagen, Hilden, Germany) with a final volume of 20-μl