DNA Sequence, June 2006; 17(3): 223–230

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FULL LENGTH RESEARCH PAPER

Isolation and characterization of the *RanGAP* gene in the mosquito *Aedes aegypti*

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Abstract
A duplicated 3′-truncated version of *RanGAP* was previously identified as *Segregation distorter* (*Sd*), the meiotic drive gene in *Drosophila melanogaster*. Here we report the cloning and characterization of the complete gene sequence for the *RanGAP* homolog from the mosquito *Aedes aegypti*. The 1995 bp cDNA sequence consists of a 113 bp 5′ UTR and 130 bp 3′ UTR, and encodes a 583 amino acid protein with high sequence identity with RanGAP homologues of several species. A 20,125 bp genomic DNA sequence contains the complete *RanGAP* gene, consisting of three exons and two introns. Intron 2 comprises 18,082 bp and contains multiple repetitive elements as well as putative coding regions. The *RanGAP* locus was mapped to the q-arm of chromosome 2. Because the meiotic drive gene (M^D) in *A. aegypti* was previously shown to be tightly linked with the sex determining locus on chromosome 1, *RanGAP* is likely not the M^D gene.

Keywords: *Segregation distorter, meiotic drive, sex ratio distortion, linkage mapping, genomic organization, repetitive elements*

Introduction

Ran is a GTP-binding protein that is a member of the Ras-related GTP-binding protein superfamily (Drivas et al. 1990). It is involved in cell cycle control, mitotic spindle formation, post-mitotic nuclear envelope assembly, and is an important component of the nuclear transport machinery (Sazer and Dasso 2000, Dasso 2001, Moore 2001). It is an abundant protein localized mainly in the nucleus, and was first cloned from a human tetratocarcinoma cDNA library (Drivas et al. 1990). Ran has GTPase activities and cycles between a GTP and a GDP bound form. It tightly binds guanine nucleotides and slowly hydrolyses GTP (Klebe et al. 1995). The products of two major cofactors of Ran regulate its GTPase activities. These include RanGEF (Ran guanine nucleotide exchange factor; also known as RCC1) (Bischoff and Ponstingle 1991) and Ran-GTPase activating protein (*RanGAP*) (Bischoff et al. 1994). RanGEF is localized inside the nucleus, whereas RanGAP accumulates in the cytosol. Cytoplasmic localization of RanGAP and the nuclear localization of RanGEF establish a concentration gradient of Ran-GTP across the nuclear envelope that is critical for proper Ran-mediated nuclear transport (Kusano et al. 2002).

A mutated *RanGAP* gene has been shown to be a primary component of the well-characterized meiotic drive system (Segregation distorter or SD) in *Drosophila melanogaster* (Merrill et al. 1999). The SD complex has been resolved into three major components: the distorter gene (*Sd*), an enhancer of distortion (*E*(*Sd*)) and the responder locus (*Rsp*). These reflect strong linkage disequilibrium due to suppression of recombination within the peri-centromeric region of *D. melanogaster* chromosome two (Palopoli and Wu 1996). *Sd* individuals carry the wild type *RanGAP* (6.5 kb *Eco*RI fragment) and a 3′ truncated tandem duplication (5 kb *Eco*RI fragment) (Merrill et al. 1999). The duplicated copy

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ISSN 1042-5179 print/ISSN 1029-2365 online
DOI: 10.1080/10425170600805540

is expressed and encodes a truncated RanGAP missing 234 amino acids at the C-terminus, yet retains essentially normal enzymatic activity (Kusano et al. 2001). Transgenic studies showed that the 3′ truncated version of *RanGAP* is the effector gene for the SD system (Merrill et al. 1999). Males heterozygous for an *Sd* carrying chromosome and a sensitive *Rsp* carrying chromosome show sperm dysfunction. Dysfunction is evidenced as the failure of proper chromatin condensation in sensitive *Rsp* bearing spermatids, leading to subsequent defects in spermatid elongation and maturation (Tokuyasu et al. 1977). Kusano et al. (2001) suggested that the 3′ truncated *RanGAP* is missing one of the two normal nuclear export sequences (NES), thus disrupting its exportation from the nucleus. The truncated *RanGAP* is mislocalized to the nucleus, which increases Ran-GTP hydrolysis and disrupts normal Ran-dependent functions. The aberrant nuclear accumulation of even wild type *RanGAP* results in segregation distortion, although the exact mechanisms for distortion are unclear (Kusano et al. 2002).

Meiotic drive has previously been reported in two mosquito species, *Aedes aegypti* and *Culex pipiens* (Sweeny and Barr 1978, Hickey and Craig 1996a,b). The meiotic drive gene (M^D) in *A. aegypti* is tightly linked with the male-determining allele (M) at the sex locus on chromosome 1, and its product acts to cause fragmentation of female determining gametes (m) carrying a susceptible responder allele (m^s). Because of the potential for effecting population replacement and similarities to the SD system of *D. melanogaster* (Wood and Newton 1991; Lyttle 1993), we have initiated efforts to identify and characterize the *A. aegypti RanGAP* homolog. That is, due to the general inability to control mosquito-borne disease transmission, mosquito population replacement strategies using genetic manipulation techniques to produce incompetent mosquito vectors are actively being evaluated (Adelman et al. 2002, Moreira et al. 2002). Population replacement with mosquitoes carrying anti-pathogen effector genes will require the identification and employment of strong gene drive systems to rapidly sweep effector genes into natural populations (Braig and Yan 2001, James 2005). Here we report the complete cDNA and genomic DNA sequences, and the genetic map position of the *RanGAP* gene.

Materials and methods

Degenerate primer design

We performed a multiple sequence alignment of *RanGAP* homologues from six species (*Anopheles gambiae*: GenBank accession no. XM317204, *D. melanogaster*: GenBank accession no. AF143860, *Homo sapiens*: GenBank accession no. BC041396, *Mus musculus*: GenBank accession no. AAH14855, *Xenopus laevis*: GenBank accession no. O13066, *Sacchromyces cerevisiae*: GenBank accession no. CAA90206) to find conserved sequence domains for designing degenerate primers. The most prominent features of the *RanGAP* proteins are the leucine-rich repeats (LRR) that constitute the major part of the protein sequence. LRRs have been found in proteins with different functions and intracellular localizations, and participate in protein–protein interactions (Kobe and Deisenhofer 1994). We selected two LRR regions for designing degenerate primers flanking the 5′- and 3′-ends (Aa-*RanGAP*_F 5′-ACRCTSGGCG-TKGARGCSGCCAA-3′, Aa-*RanGAP*_R 5′-GTTT-TSAKCARRCAGTCKCCGAAA-3′), for which the maximum degeneracy was 32.

cDNA isolation and characterization

Total RNA was isolated from 10 (*A. aegypti* Liverpool SB strain: five males and five females) mosquitoes in 1 ml of TRIZOL™ reagent (Invitrogen) following the supplier's protocol. RT-PCR was performed using the Access RT-PCR system (Promega) following the supplier's protocol with 40 pmol of forward and reverse degenerate primers and 500 ng of total RNA per 50 μl reaction. The first strand cDNA was synthesized with 5 U of AMV reverse transcriptase at 48°C for 45 min. The AMV reverse transcriptase was inactivated by 2 min incubation at 94°C, and then PCR reactions were performed: 95°C for 30 s, 58°C for 1 min, 68°C for 2 min for 40 cycles. The final cycle had an extension time of 7 min at 68°C.

The RT-PCR products were cloned using the TOPO TA cloning® Kit (Invitrogen) following the supplier's protocol. Cycle sequencing was performed using the ABI Prism Big Dye Terminator Kit v. 3.1 (PE Applied Biosystems) and an ABI PRISM 3700 Genetic Analyzer (Biosystems) with M13 primers. Sequence data were submitted to the TBLASTX program (Altschul et al. 1997) to verify identification as the *A. aegypti RanGAP* homolog. The 5′- and 3′-end sequences were determined by 5′- and 3′- RACE (rapid amplification of cDNA end), respectively. Full-length cDNA was synthesized using the GeneRacer™ RACE Ready cDNA Kit (Invitrogen) following the supplier's protocol. 1000 ng of total RNA was treated with calf intestinal phosphatase (CIP) to remove 5′ phosphates, and then with tobacco acid pyrophosphatase (TAP) to remove the 5′ mRNA cap structure, which leaves a 5′ phosphate required for ligation to the GeneRacer RNA Oligo (5′-CGACUGGAGCACGAGGACACU-GACAUGGACUGAAGGAGUAGAAA-3′). The GeneRacer RNA Oligo was ligated to the 5′ end of the decapped mRNA using T4 RNA ligase. The ligated mRNA was reverse-transcribed using SuperScript II reverse transcriptase and the GeneRacer Oligo dT

Primer (5′-GCTGTCAACGATACGCTACGTAACGGCATGACAGTG(T)18-3′) to synthesize complete cDNA carrying GeneRacer kit-specific oligonucleotide sequences at the 5′-and 3′-ends. The 5′-, 3′-cDNA ends were amplified with gene specific primers: 5′-primer (Ae-*RanGAP*_5′-RACE-R 5′-ATGCTTC CGACAGGGCCGTAATG-3′), 3′-primer (Ae-*RanGAP*_3′-RACE-F 5′-GCCTACGAGT ACGCGAAAGCCAACA-3′) and GeneRacer primers: 5′-primer (5′-GCACGAGGACACUGACAUGGACUGA-3′: position 9–33 of GeneRacer RNA Oligo), 3′-primer (5′-GCTGTCAACGATACGCTACGTAACG-3′: position 1–25 of GeneRacer Oligo dT primer). After 20-fold dilution of full length RACE cDNA, 1 μl was added to a 25 μl PCR reaction (35 cycles of 94°C for 1 min, 63°C for 1 min and 72°C for 2 min) with 5 min preheating at 94°C and 10 min final extension at 72°C. Amplified 5′- and 3′-RACE DNA was cloned using the TOPO TA cloning Kit (Invitrogen) following the supplier's protocol. Cycle sequencing was performed using the ABI Prism Big Dye Terminator Kit v. 3.1 (PE Applied Biosystems) and an ABI PRISM 3700 Genetic Analyzer with M13 primers. The 5′- and 3′-cDNA sequences were confirmed by sequence alignment to the *A. aegypti* genomic DNA sequence and by BLASTN hits against the TIGR *A. aegypti* gene index, v. 4.0 (http://www.tigr.org/tigrscripts/tgi/T_index.cgi?species = a_aegypti). Translate tool (http://us.expasy.org/tools/dna.html) was used to predict the amino acid sequence for the complete *RanGAP* cDNA. The complete cDNA sequence and putative polypeptide sequence were submitted to the TBLASTX and BLASTP (http://www.ncbi.nlm.nih/gov) programs for homology searches. To identify conserved sequence domains, we performed a multiple sequence alignment with other *RanGAP* homologues using ClustalX (ftp://www.ftp-igbmc.u-strasbg.fr/pub/ClustalX/).

Genomic DNA library screening and DNA sequence analysis

We used the partial *RanGAP* cDNA as a radiolabelled probe to screen an *A. aegypti* BAC library (Jiménez et al. 2004) using our standard procedures (Severson 1997). A *ca.* 100 kb BAC clone containing the *RanGAP* sequence was isolated. Purified BAC plasmid was prepared as previously described (Jiménez et al. 2004), and was subjected to direct cycle sequencing by primer walking with gene specific primers. Exon/intron junctions were determined by the pair-wise alignment of the complete cDNA sequence against the genomic DNA sequence using ClustalX 1.81.

Several bioinformatics tools were used to annotate the *RanGAP* genomic sequence. For EST queries, the complete genomic sequence was submitted to the BLASTN program against the TIGR *A. aegypti* gene index (v. 4.0) with cut off expectation value of e^{-100}. To predict putative genes, the complete genomic sequence was submitted to two gene finding programs: GENSCAN (http://genes.mit.edu/GENSCAN.html) using the vertebrate database and FGENESH (http://www.softberry.com/berry.phtml?topic = fgenesh&group = programs&subgroup = gfind) using the *A. gambiae* database. Predicted genes were submitted to Pfam (http://www.sanger.ac.uk/Software/Pfam/) for detection of conserved domain families. To detect transposable elements, the complete genomic sequence was submitted to the CENSOR SERVER (Jurka et al. 1996; http://www.girinst.org/Censor_Server.html) using the invertebrate database.

Genetic mapping

To identify informative strains for mapping, the partial *RanGAP* cDNA clone was radiolabelled and hybridized to *Eco* RI-digested genomic DNA from several *A. aegypti* strains. After selecting two informative strains, strain-diagnostic RFLP markers (Severson et al. 2002) were identified on each chromosome. F_1 intercross mapping populations were then prepared as previously described (Severson et al. 1993). From one mapping population, 48 males and 48 females were subjected to individual genomic DNA extractions and Southern transfer following our standard protocols (Severson 1997). Linkage associations were determined using Mapmaker/Exp (3.0b) with a minimum LOD threshold of 3.0 (Lander et al. 1987). Map distances were calculated as Kosambi centiMorgan distances (Kosambi 1944).

Results

Full-length RanGAP *cDNA characterization*

We successfully amplified a 656 bp fragment using degenerate primers and RT-PCR. TBLASTX analysis showed that the PCR product had 63 and 80% sequence identities to the *RanGAP* homolog in *H. sapiens* and *A. gambiae*, respectively.

The 5′- and 3′-end sequences of the cDNA were isolated using 5′-, 3′-RACE and sequenced using M13 primers. The complete cDNA sequence consisted of 1,995 bp and included a 113 bp 5′ untranslated region (UTR) and a 130 bp 3′ UTR (Figure 1). BLASTN analysis against the *A. aegypti* TIGR EST database identified one tentative consensus sequence (TC31112), that showed 99% sequence identity with our *RanGAP* cDNA clone and contained a polyadenylation signal near the 3′-end. No significant match with the 5′-cDNA end sequence was detected.

The complete *RanGAP* cDNA was deduced to encode a 583 amino acid protein (Figure 1). BLASTP

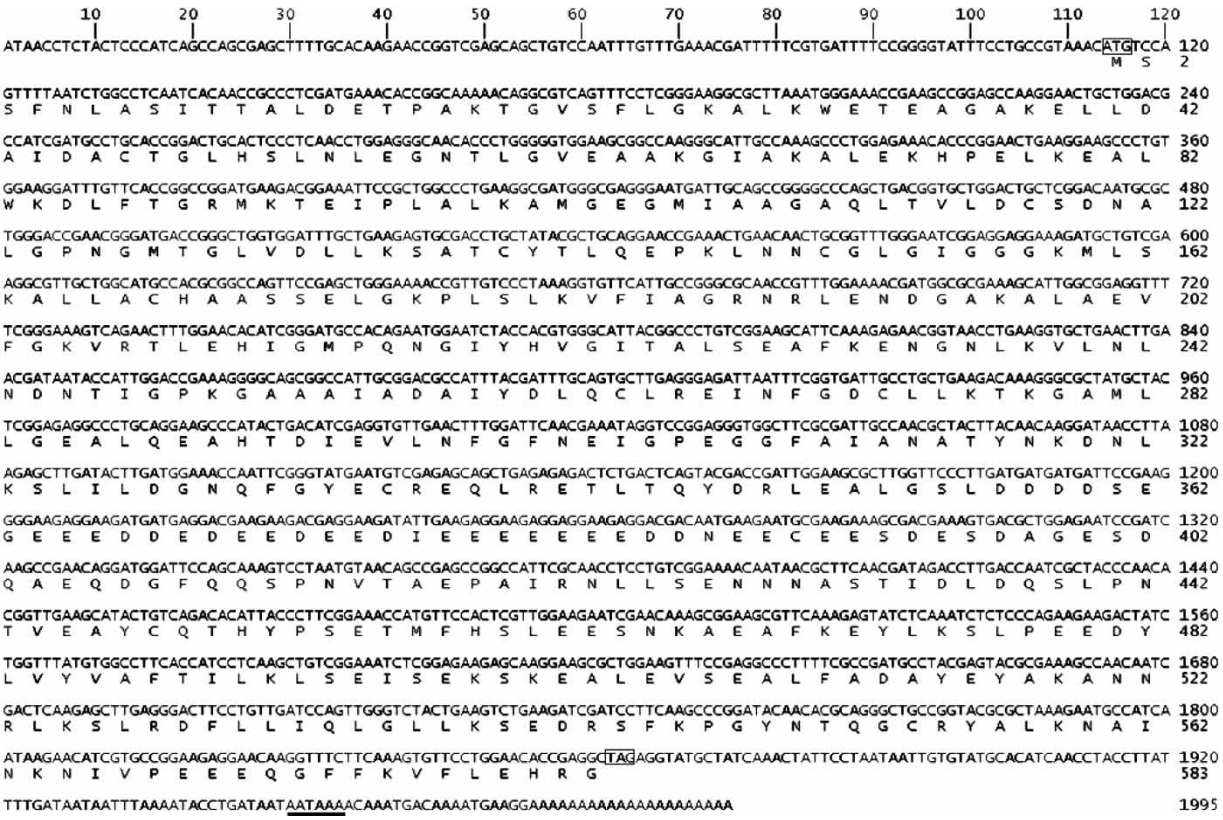

Figure 1. cDNA sequence of the *RanGAP* gene. The predicted 583 amino acid sequence is shown below the nucleotide sequence. The start codon (ATG) and the stop codon (TAG) are enclosed by a box and the polyadenylation signal (AATAAA) is underlined.

queries showed that *A. aegypti* RanGAP has high sequence identity with the RanGAP homolog in several other species: 73% with *A. gambiae*; 39% with *D. melanogaster*; 52% with *X. laevis*; 53% with *H. sapiens*; 31% with *S. cerevisiae* and 52% with *M. musculus*, respectively. In addition, BLASTP queries against the *D. melanogaster* and *A. gambiae* databases identified only a single best match in each organism with the *A. aegypti* sequence. Multiple RanGAP sequence alignments from 6 species were performed using ClustalX (Figure 2). Alignments show the conserved sequence domain of one putative nuclear localization sequence (NLS) and two NES critical for the cytosolic localization of *RanGAP*. The complete *RanGAP* cDNA and genomic sequences were submitted to GenBank (GenBank accession nos. DQ138953 and DQ177444).

Genomic structure of the RanGAP *gene*

Using the 656 bp degenerate RT-PCR product as a probe, we isolated a BAC clone containing the *RanGAP* gene and determined the complete gene sequence by primer walking. Pair-wise alignment of the cDNA and genomic DNA sequences indicated that the *RanGAP* gene consists of three exons and two introns. The exon–intron boundaries and the respective exon and intron sizes are shown in Table I. All exon–intron junctions contain the conserved GT/AG dinucleotide motifs. The complete genomic DNA sequence (20,125 bp) has similarity to six *A. aegypti* ESTs and to several putative transposable elements (Figure 3). Neither of the gene finding programs (GENSCAN and FGENESH) correctly identified the *RanGAP* coding sequence. Both resulted in sets of inaccurate predictions that overlapped the *RanGAP* open reading frame (ORF), the EST hits, as well as the transposon ORFs. Pfam searches of predicted genes confirmed that the gene finding algorithm in GENSCAN interpreted repetitive elements, likely associated with transposons, as putative genes. Of six ESTs that had significant similarity ($< e^{-100}$) to this genomic region, TC66147 matched the 3′ end of the *RanGAP* gene, while the other five were located within the *ca.* 18 kb second intron. Three overlapping consensus EST sequences (TC52230, TC51913 and TC51912) had significant similarity to only a portion of the *A. aegypti nanos* gene (AY878073), while TC57031 had low similarity to the 5′ end of an *A. gambiae* gene (ENS ANGG00000023523). The intron region showing partial similarity with TC52296 also included several repetitive sequences indicating that it is probably a degenerate region or pseudogene.

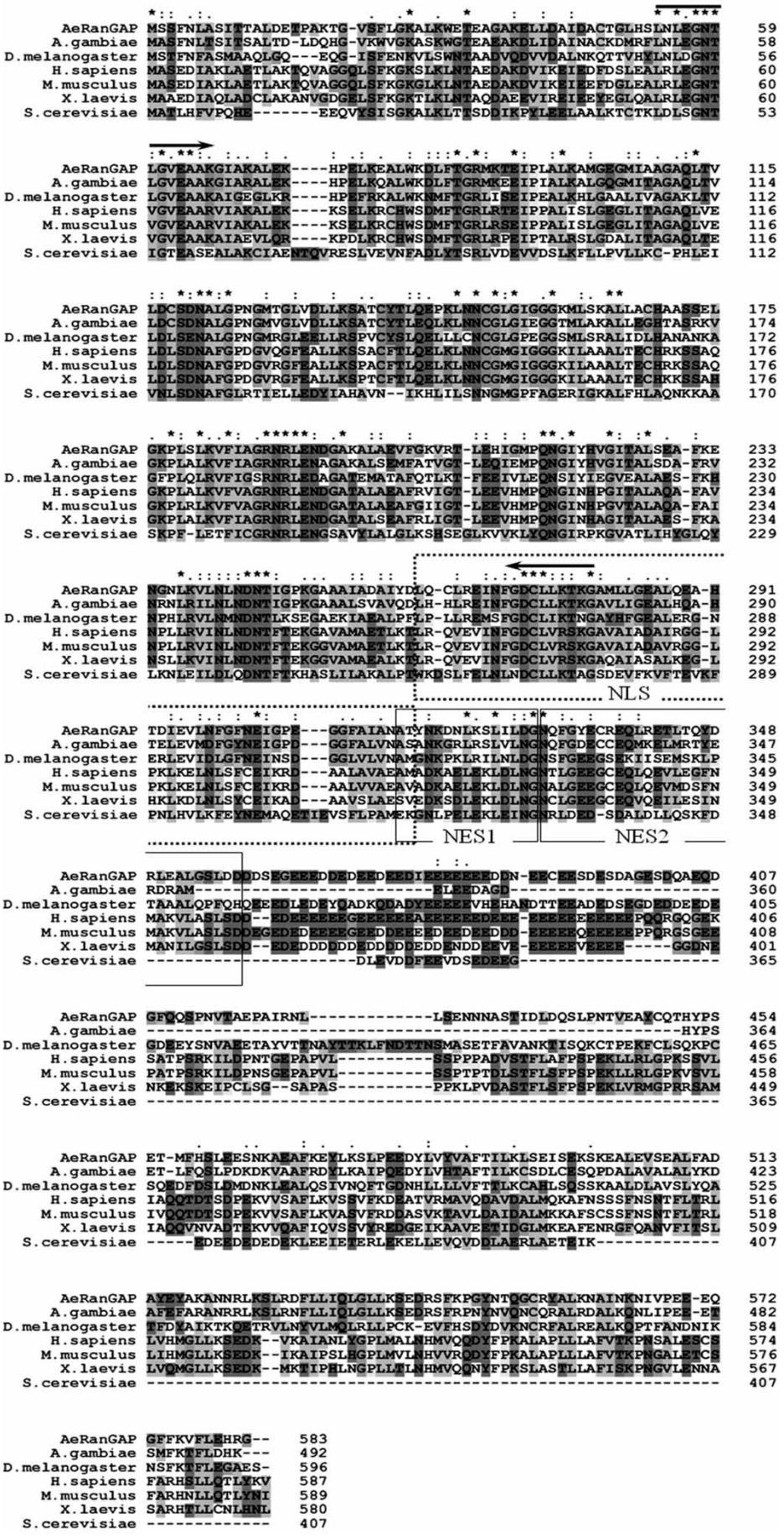
NLS
NES1
NES2

Table I. Exon–intron junctions of the *RanGAP* gene. Coding sequences are shown in uppercase letters and noncoding regions in lower case letters. The conserved GT/AG exon/intron junctions are shown in bold. Amino acid residues are indicated with respect to each boundary and the stop codon (TGA) is indicated by italics. Numbers refer to the corresponding positions in the *RanGAP* cDNA starting with +1 at the adenosine defining the initiation codon ATG.

No.	Exon size (bp)	Splice-donor	Intron size (bp)	Splice-acceptor
11	222	109 CCGGAG**gt**aagtggaa... Pro Glu	70	110 ...tatccgga**ag**CCAAGG Pro Arg
22	324	433 CTGCAG**gt**gagtttt t... Leu Gln	18082	434 ...ttttt tgt**ag**GAACCG Glu Pro
33	1429	1753 3 *TAG*aggtatgctatca...		

Chromosome location of the RanGAP *locus*

We selected two informative strains, Liverpool and RED strain, for preparing segregating populations to determine linkage associations between *RanGAP* and marker loci distributed across the genome. The parental cross was set up with one Liverpool male and five RED females. A single F_1 egg paper was hatched and mass mated to produce an F_2 generation. Liverpool and RED strain diagnostic markers were identified for each chromosome by screening bulked genomic DNA digests of each strain and included: LF397 and LF198 on chromosome 1; LF169, *RpL17A* on chromosome 2; and LF168 on chromosome 3. The individual F_2 progenies were then genotyped for each marker and for *RanGAP*, and the data were analysed using Mapmaker/Exp (3.0b). The *RanGAP* locus mapped to the q-arm of chromosome 2, about 25.5 cM from the *RpL17A* locus (Figure 4). Because the M^D locus is tightly linked with the sex-determining locus on chromosome 1 (Newton et al. 1978, Hickey and Craig, 1996a,b), the *RanGAP* gene is, therefore, likely not responsible for the meiotic drive phenotype in *A. aegypti*.

Discussion

We have identified and characterized a 1995 bp cDNA and 20,125 bp genomic sequence as the *RanGAP* homolog in *A. aegypti*, because of its potential as the meiotic driver gene M^D. Sequence alignment with *RanGAP* homologues in six other species indicates high sequence identities with the *RanGAP* homolog in *A. aegypti*. In addition, BLASTP analyses identified the *D. melanogaster* and *A. gambiae RanGAP* genes as the single best matches to the *A. aegypti* sequence. The conserved LRR_RI motifs are prominent features of the *RanGAP* proteins, and participate in protein–protein interactions (Kobe and Deisenhofer 1994). Both an NLS and two NESs are conserved, with identities of 72 and 61% with *A. gambiae*, respectively. Therefore, *A. aegypti RanGAP* has high functional domain sequence identities for *RanGAP* activities and its NLS/NES ratio predicts the cytoplasmic localization of the gene product critical for the *Ran* pathway (Kusano et al. 2002).

The *RanGAP* gene in *A. aegyp*ti is slightly greater than 20 kb, compared with only 6.5 kb in *D. melanogaster*. Although the length of *RanGAP* coding sequences in the two species are similar at ~2 kb, the largest intron in *A. aegypti* is ~4.5-fold larger than that observed in *D. melanogaster*. This phenomenon is not surprising given that genome size in *A. aegypti* is at least 4.8-fold larger than *D. melanogaster* (Severson et al. 2004b). Presence of a large 18 kb intron, harbouring several repetitive elements, negatively influenced the ability of GENSCAN and FGENESH gene finding programs to accurately predict the *RanGAP* coding sequence in the *ca.* 20 kb genomic sequence. In particular, the observed frequency of sequences with high identities to transposons reflects the highly repetitive nature of the *A. aegypti* genome, and indicates the expected complexity associated with efforts to correctly annotate the entire genome sequence. Of note, is the absence of the SINE element, *Feilai*, within the *RanGAP* gene as this element is ubiquitous throughout the genome with an estimated 59,000 copies (Tu 1999). Regions with high similarity to *A. aegypti* ESTs suggest the presence of possible transcripts within the large exon. However, these regions only partially match functional genes, indicating that they likely do not correspond to complete functional transcript units, but instead reflect the long term effects of transposon mobilization and movement that includes partial gene duplication.

Our genetic mapping results show that *RanGAP* is located on chromosome 2q in *A. aegypti*. This indicates that *RanGAP* is likely not responsible for meiotic drive in *A. aegypti*, because the M^D locus has

Figure 2. Sequence comparisons of the *Aedes aegypti* RanGAP protein with homologous sequences from 6 species by ClustalX (*Anopheles gambiae*: GenBank accession no. XM317204, *Drosophila melanogaster*: GenBank accession no. AF143860, *H. sapiens*: GenBank accession no. BC041396, *M. musculus*: GenBank accession no. AAH14855, *X. laevis*: GenBank accession no. O13066, *S. cerevisiae*: GenBank accession no. CAA90206). Shading of the alignments is based on the BLOSUM62 scoring matrix. Amino acid identity is designated by an asterisk (*) and deletions are represented by hyphens (-). The arrows show two conserved regions used for designing forward and reverse degenerate primers. The dashed box indicates putative NLS-like motifs of RanGAP homologues and the two solid boxes are the first and the second NES motifs, respectively.

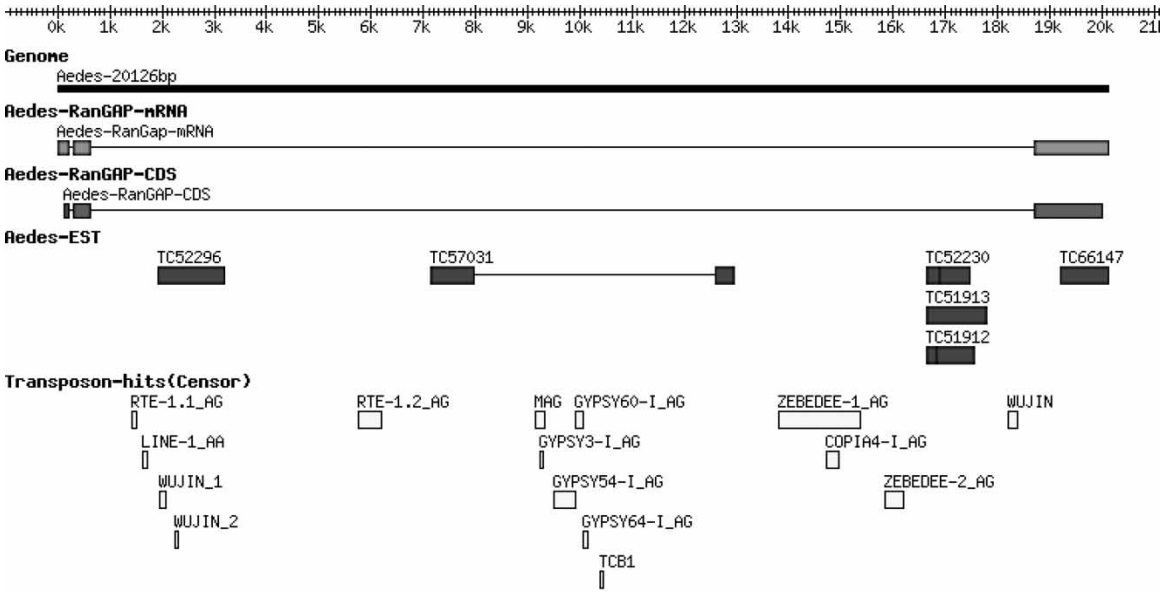

Figure 3. Genomic structure of the *RanGAP* gene. ESTs detected by the TIGR *Aedes aegypti* Gene Index and putative transposons predicted by CENSOR SERVER are drawn below the *RanGAP* mRNA structure. Each shaded box represents coding sequences and the solid lines are noncoding sequences.

been mapped to chromosome 1 (Hickey and Craig 1966a,b, Newton et al. 1978).

Although the observed sperm dysfunction of the *A. aegypti* and *D. melanogaster* systems may look similar, the mode of action in each drive system can be different. Sperm dysfunction in SD flies is due to interference with proper chromatin condensation during spermatogenesis (Tokuyasu et al. 1977). Defective sperm fail to undergo the normal lysine-rich to arginine-rich histone transition and generally do not separate properly into individual sperm, which remain syncytial spermatids with tightly coiled tails (Lyttle 1993). With *A. aegypti*, the M^D phenotype is fragmentation of the female determining chromosome, wherein isochromatid breakage occurs on chiasmic arms of the bivalent at or before the diplotene stage (Wood and Newton 1991). However, it is reasonable to predict that M^D is a gene involved in cell division and chromosome assembly during sperm generation. Because the *Ran* signalling pathway is deeply involved in cell division and chromosome formation (Bischoff et al. 1994, Kusano et al. 2002), M^D may be a mutant version of another co-factor in the *Ran* signalling pathway, although it may be due to novel gene effects.

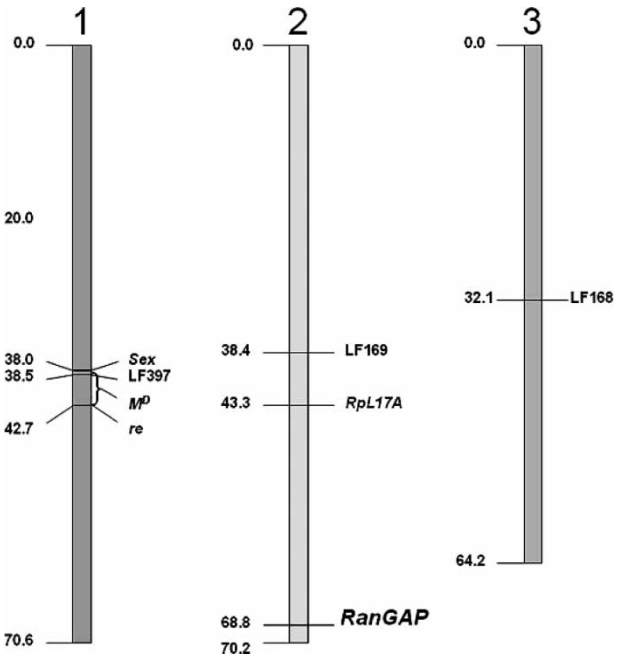

Figure 4. Chromosome location of the *RanGAP* locus on the *Aedes aegypti* genetic map. *MD* is located between sex and re (red-eye), as indicated by the bracket on chromosome 1 (Wood and Ouda 1987). Map distances are listed in Kosambi centiMorgans. Map positions are designated after Severson et al. (2002).

The complete genome sequences of the *D. melanogaster* and the African malaria mosquito *A. gambiae* have been reported (Adams et al. 2000, Holt et al. 2002). Using this genome information, an *in silico* comparative genome analysis to *A. aegypti* was performed and identified significant whole chromosome arm conservation between all three species (Severson et al. 2004a). Our studies showed that the *RanGAP* gene is located on chromosome 2q in *A. aegypti*, while gene database searches indicated that the homolog is located on chromosome 2L in *D. melanogaster* and chromosome 3R in *A. gambiae*. However, the M^D locus in *A. aegypti* is tightly linked with the sex determining locus on chromosome 1 (Newton et al. 1976). Therefore, the M^D homolog is most likely located on chromosome 3R or the X in

A. gambiae (Severson et al. 2004a). The anticipated availability of the complete *A. aegypti* genome sequence (Severson et al. 2004b) should facilitate our efforts to identify and characterize the M^D system.

Acknowledgements

This work was supported by grant PO1-AI45123 and contract HHSN266200400039C from NIH NIAID.

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