Quantitative Trait Loci That Control Vector Competence for Dengue-2 Virus in the Mosquito *Aedes aegypti*

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Manuscript received November 30, 1999
Accepted for publication June 4, 2000

ABSTRACT

Quantitative trait loci (QTL) affecting the ability of the mosquito *Aedes aegypti* to become infected with dengue-2 virus were mapped in an F_1 intercross. Dengue-susceptible *A. aegypti aegypti* were crossed with dengue refractory *A. aegypti formosus*. F_2 offspring were analyzed for midgut infection and escape barriers. In P_1 and F_1 parents and in 207 F_2 individuals, regions of 14 cDNA loci were analyzed with single-strand conformation polymorphism analysis to identify and orient linkage groups with respect to chromosomes *I–III*. Genotypes were also scored at 57 RAPD-SSCP loci, 5 $(TAG)_n$ microsatellite loci, and 6 sequence-tagged RAPD loci. Dengue infection phenotypes were scored in 86 F_2 females. Two QTL for a midgut infection barrier were detected with standard and composite interval mapping on chromosomes *II* and *III* that accounted for ~30% of the phenotypic variance (σ_P^2) in dengue infection and these accounted for 44 and 56%, respectively, of the overall genetic variance (σ_g^2). QTL of minor effect were detected on chromosomes *I* and *III*, but these were not detected with composite interval mapping. Evidence for a QTL for midgut escape barrier was detected with standard interval mapping but not with composite interval mapping on chromosome *III*.

THE yellow fever mosquito *Aedes aegypti* is the most common vector of yellow fever and dengue fever flaviviruses. Despite the widespread availability of an effective and safe vaccine, yellow fever remains an important public health problem in much of Africa and South America (Monath 1991). Since 1986, major epidemics have occurred annually in West Africa (Miller *et al.* 1989) and mortality rates have ranged from 25 to 50%. *A. aegypti* is also the most prevalent vector of dengue viruses (serotypes 1–4) in a human-mosquito cycle in tropical and subtropical regions. Dengue fever is one of the most rapidly expanding diseases in the tropics, with more than 2 billion people at risk. An estimated 100 million human infections occur annually and several hundred thousand cases of the severe form of the disease, dengue hemorrhagic fever-shock syndrome, may occur annually (Monath 1994; Gubler and Clark 1995; Gubler 1996).

Vector competence refers to the intrinsic permissiveness of an arthropod vector to infection, replication, and transmission of a virus (Hardy 1988; Woodring *et al.* 1996). When a mosquito takes a viremic bloodmeal, the virus encounters several barriers to infection. First, the virus must establish an infection in the mosquito midgut by overcoming a midgut infection barrier. After replication in the midgut epithelium, the virus must pass through a midgut escape barrier and replicate in other tissues. Finally, the virus must infect the salivary glands and be shed in the saliva to be transmitted to the next vertebrate host.

Throughout its worldwide range *A. aegypti* exhibits variation in vector competence for flaviviruses. In Africa south of the Sahara, *A. aegypti* appears as a black "sylvan" subspecies, *A. a. formosus* that oviposits primarily in tree holes and has low vector competence for flaviviruses primarily due to a midgut infection barrier. A lighter-colored "domestic" subspecies, *A. a. aegypti*, is distributed in tropical and subtropical regions outside Africa and is relatively susceptible to flavivirus infection (Gubler *et al.* 1979; Tabachnick *et al.* 1985).

The genetics of the flavivirus midgut infection and escape barriers in *A. aegypti* is not well understood. Genetic studies of vector competence have primarily used selection to produce susceptible and resistant lines, followed by crossing these lines to analyze the susceptibility phenotype in F_1 offspring and, in some studies, susceptibility in F_2 and backcross generations. Miller and Mitchell (1991) selected lines of *A. aegypti* that were completely refractory or highly susceptible to yellow fever virus. F_1 progeny were intermediate in susceptibility, suggesting that alleles at vector competence loci act additively, and F_2 backcrosses suggested the involvement of multiple loci. We recently completed a quantitative genetic study of dengue midgut infection and escape barriers in *A. aegypti* using a standard half-sib breeding design (Bosio *et al.* 1998). *A. a. aegypti* and *A. a. formosus* mosquitoes were infected orally and, after an extrinsic incubation period of 14 days, virus titer was determined

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in the midgut and head. The heritability for a midgut infection barrier in both subspecies was 0.41 and was 0.39 for a midgut escape barrier in *A. aegypti formosus*. In *A. aegypti aegypti* a midgut escape barrier appeared to be controlled by dominant alleles.

The level of dengue infection is a quantitative rather than a discrete variable that appears to be distributed continuously among individuals and is subject to environmental effects. Recent molecular genetic and statistical advances permit the mapping of loci affecting the expression of quantitative traits; these have been termed quantitative trait loci (QTL). Severson *et al.* (1994, 1995) mapped in *A. aegypti* the QTL that condition susceptibility to filarial worms (MacDonald 1962) and those that condition avian malaria susceptibility (Kilama and Craig 1969). The purpose of the present study is to map and characterize QTL that control midgut infection and escape barriers and thus condition the vector competence of *A. aegypti* for dengue viruses.

MATERIALS AND METHODS

Mosquito strains: *A. a. aegypti* were collected as eggs in field ovitraps in the spring of 1995 in San Juan, Puerto Rico. Eggs were reared to adulthood in the laboratory starting from a population of ~1100 individuals. The first and second generations of offspring of these adults were used in all experiments. *A. a. formosus* were from Ibo village, Nigeria (Ballinger-Crabtree *et al.* 1992) and fifth- and sixth-generation mosquitoes were used.

Dengue-2 virus: The PR-159 strain of dengue was isolated from the serum of a patient with dengue fever in Puerto Rico in 1969. A confluent 75-cm^2 tissue culture flask of *A. albopictus* C6/36 cells was infected at a multiplicity of infection of 0.01–0.10, and the flask was brought to a total volume of 10 ml with L-15 cell culture medium, 2% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. After a 7-day incubation at 28°, cells were scraped into the medium to act as a source of virus. This was diluted 100-fold with 1× phosphate-buffered saline, 5% fetal bovine serum, and ~0.34 μl of this was intrathoracically inoculated into adult *A. a. aegypti* (Rex-D colony; Miller and Mitchell 1991). After 6–7 days at 28°, mosquitoes were triturated in undiluted fetal bovine serum at a concentration of 35 mosquitoes/ml serum. The triturate was centrifuged at 14,000× *g* for 20 min at 4°. The supernatant was used as the source of virus in infectious bloodmeals. Samples of the complete bloodmeal were taken before and after bloodfeeding for titration of virus and were both 7.3 $\log_{10}$ virus/ml.

Crossing design: The offspring of individual females were collected from the Puerto Rico and Ibo strains. Members of 6 Ibo families were crossed with members of 6 Puerto Rico families to generate a total of 71 F_1 families. Each cross consisted of a male from one strain and 8–10 females from the alternate strain. Reciprocal crosses were made of both strains. After 6–7 days to allow for mating, the male from each cross was collected and frozen at −70°. Egg batches from individual females were collected and held under laboratory conditions. All P_1 females of a family were infected orally with dengue with an artificial membrane feeder (Rutledge *et al.* 1964). The bloodmeal consisted of equal parts virus suspension, washed sheep erythrocytes, and fetal bovine serum with 10% sucrose. After a 14-day extrinsic incubation, all orally infected P_1 females were tested for infection by immunofluorescence assay of head squashes (Schoepp and Beaty 1984). Crosses that resulted from the pairing of a P_1 individual from a family with a high rate of infection with an individual from a family with a low rate of infection were retained for QTL mapping.

Five F_1 full sibling females were intercrossed to a brother. The resulting F_2 families were reared to adulthood and males were frozen at −70° for DNA isolation and used in linkage mapping. Females were infected orally as described above. Females that did not feed were frozen at −70° for use in mapping. After 14 days, females were frozen at −70°, awaiting the virus assay.

Bloodfed females were removed from the freezer and placed individually in tubes on dry ice. Care was taken not to transfer unattached legs, palps, or wings that might contaminate a sample with DNA from other mosquitoes. The entire midgut was dissected from the abdomen in 1× phosphate-buffered saline, rinsed twice in a drop of 1× phosphate-buffered saline, and triturated in 200 μl of L-15 medium with 2% fetal bovine serum. The head was removed and triturated in 200 μl L-15, 2% fetal bovine serum. Triturated samples were centrifuged at 12,000 × *g* at 4° for 20 min.

Reanalysis of data generated in our half-sib experiments (Bosio *et al.* 1998) demonstrated that virus titer in the midgut and head was not correlated with the rates of infection and that the heritabilities of virus titer in tissues were almost identical to heritabilities based upon the presence or absence of virus (analyses available from the authors). Therefore, samples were tested only for the presence of virus in the midgut and head. Head and midgut triturates were assayed in 96-well plates. A 10-μl aliquot of each homogenized head and midgut was plated alongside a single 10-fold dilution such that 24 individual mosquitoes were assayed on a plate. Wells were overlaid with C6/36 cells and, after 7 days, fixed, stained, and dengue was detected with an immunofluorescence assay (Schoepp and Beaty 1984).

The remnants of the abdomen and the thorax were returned immediately to a tube on dry ice and then frozen at −70°, awaiting DNA isolation. DNA was extracted from individual mosquitoes (Black and Munstermann 1996) and resuspended in 500 μl TE (50 mM Tris-HCl, 5 mM EDTA, pH 8.0) buffer. A 50-μl aliquot of DNA was overlaid with sterile mineral oil and stored at 4° for daily use in the polymerase chain reaction (PCR). The remainder was stored in plastic screw-top vials at −70°.

PCR amplification and single-strand conformation polymorphism (SSCP) gel electrophoresis: PCR was completed in thin-walled polycarbonate 96-well plates (Fisher Scientific, Pittsburgh, PA). Three plates were required to analyze all 207 individuals. Each 96-well PCR plate contained a negative control (no template DNA) and an amplification of the grandparents (P_1) and parents (F_1) of the family as a test of repeatability. PCR buffer for 100 individual reactions was prepared in one large 5-ml batch containing 4350 μl of ddH_2O, 500 μl of 10× buffer [500 mM KCl, 100 mM Tris-HCl (pH 9.0), 15 mM $MgCl_2$, 0.1% gelatin (w/v), and 1% Triton X-100; Promega Biotech, Madison, WI], 50 μl of 20 mM dNTP's, 5000 pM of each primer (1 μM final concentration), and 100 units of *Taq* polymerase. This was dispensed into each of the 96 wells in 48-μl aliquots. Into each well was added 2 μl of template DNA (~20 ng), and the wells were overlaid with two drops (~25 μl) of sterile mineral oil.

SSCP analysis of PCR products followed Black and Duteau (1996), except that 2 μl PCR products were mixed with 4 μl loading buffer (10 mM NaOH, 95% formamide, 0.05% bromophenol blue, and 0.05% xylene cyanol), and the concentration of silver nitrate in the stain was reduced to 0.075–0.100% (w/v). These modifications greatly reduced background and

made scoring of gels much easier. Each gel had 48 slots; those on the ends were loaded with size marker (1-kb ladder; GIBCO-BRL, Gaithersburg, MD), the next 4 contained PCR products from the four parents (two P_1 and two F_1), and the next five lanes (on all but the first gel) were loaded with PCR products from the last five F_2 individuals on the previous gel. Parent and overlapping F_2 DNA was loaded on each gel to test the reproducibility of SSCP patterns among gels and to ensure proper loading of samples. Loci that could not be reliably scored on all six gels or that did not show reproducibility in all cases were not considered further.

Random amplified polymorphic DNA (RAPD)-PCR: A total of nine 10-oligonucleotide primers were used in RAPD-PCR (Table 1). The amplification program consisted of 45 cycles of the following: (1) 95° for 1 min (denaturation), (2) 35° for 1 min (annealing), (3) ramp to 72° at a rate of 1°/8 sec, and (4) 72° for 2 min (extension). A final 72° extension was carried out for 7 min and the temperature was held overnight at 4°.

We initially believed that we would be able to orient the map derived in the present study with respect to our earlier RAPD-SSCP map (Antolin *et al.* 1996), the earlier cDNA maps of Severson *et al.* (1993), and the morphological/allozyme marker maps of Munstermann and Craig (1979). However, of the 57 RAPD loci markers mapped in this study, only one marker, B18.359 (chromosome *III*), was mapped in our earlier RAPD map (Antolin *et al.* 1996). Either bands of equivalent mobility could not be amplified in both families or loci mapped in the earlier study were not polymorphic in the present study. We developed three types of alternative markers to overcome this problem: sequence-tagged RAPDs (STARs), SSCP analysis of cDNA markers, and microsatellites. GenBank accession numbers, PCR primer sequences, and optimal annealing temperatures for each class of markers used in this study are listed in Table 1.

Sequence-tagged RAPDs: STAR markers were developed by flooding a polymorphic RAPD band that had been resolved on a SSCP gel with 20 μl TE. After ~30 sec, the gel and TE were scraped from the glass plate into a sterile microcentrifuge tube containing 80 μl TE. This was briefly vortexed, centrifuged at 17,000 × *g* for 10 sec, and 2 μl was used as template DNA in a RAPD-PCR reaction with the original oligonucleotide primer. The amplified product was analyzed with agarose gel electrophoresis to confirm that it was approximately the same size as the scraped fragment. If so, PCR products were purified with Qiaquick (QIAGEN, Valencia, CA), eluted into 50 μl ddH_2O, and 1 μl (~25 ng) was ligated into the pCR2.1 plasmid (50 ng) (Invitrogen Inc., San Diego) overnight at 14°. This was transformed into TOP10F′ cells (Invitrogen Inc.) and plated onto Luria-Bertani agar plates containing ampicillin (50 mg/liter; LBA) and covered with 1.6 mg 5-bromo-4-chloro-3-indolyl-β-D-galactoside (in dimethylformamide) and 4 μM isopropyl-β-D-thiogalactoside (in ddH_2O). Recombinant colonies were replated on LBA plates and insert sizes were determined directly from colonies using the T7 (5′ taa tac gac tca cta tag ggc 3′) and the M13 reverse primers (5′ cag gaa aca gct atg acc 3′). Colonies with inserts of the anticipated size (original size + 172 bp) were grown overnight in 5 ml of LBA broth. The plasmid was purified using QIAprep Spin Miniprep (QIAGEN) and both strands of the insert were sequenced using the M13 reverse and T7 primers on an Applied Biosystems sequencer (Davis Sequencing, Davis, CA). Both strands were aligned and corrected. Forward and reverse primers were designed that contained the original 10-oligonucleotide primer and the next 10 nucleotides on the sequence (Operon Technologies, Inc., Alameda, CA). Optimal annealing temperatures (T_a) were identified using a Mastercycler gradient thermal cycler (Eppendorf Scientific, Inc., Westbury, NY). Gradient cycling conditions were 98° for 5 min followed by 30 cycles of the following: (1) 95° for 1 min, (2) T_a gradient from 40°–60° for 1 min, (3) 72° for 2 min followed by a final 72° extension for 7 min, and the temperature was held at 4°. Once T_a was determined, PCR was performed on 2 μl DNA from each P_1 and F_1 parent and the first 10 F_2 offspring to determine if the STAR locus was polymorphic. The amplification program consisted of 30 cycles of the following: (1) 95° for 1 min, (2) optimal T_a for 1 min, and (3) 72° for 2 min followed by a final 72° extension for 7 min, and the temperature was held at 4°.

SSCP analysis of cDNA markers: The nucleotide sequences for the 14 genes listed in Table 1 were obtained from GenBank. Primers were designed using Primer Premier v4.11 (Premier Biosoft International, Palo Alto, CA) using a primer length of 20 nucleotides, a 100-pM DNA concentration, a 50-mM monovalent ion concentration, a 1.5-mM free Mg^{2+} concentration, a 250-mM total Na^+ equivalent, and 25° for free energy calculations. The optimal T_a was determined as outlined above, and PCR procedures followed those described above for STARs. Each of the cDNA markers were mapped in the present family, in family RA34-3 (Antolin *et al.* 1996), and in a reciprocal (Ibo × Puerto Rico) F_1 intercross (R. E. Fulton, M. L. Salasek, N. M. Duteau and W. C. Black IV, unpublished results). Also, many of the markers have been mapped previously (Severson *et al.*, 1993). Procedures for isolation and analysis of microsatellites are described in A. J. Fagerberg, R. E. Fulton and W. C. Black IV (unpublished results).

Linkage mapping: Genotypes at each putative locus were scored and entered into JoinMap 2.0 data file format for a "cross pollination" cross (Stam and Van Ooijen 1995). These were tested for conformity to Mendelian ratios with a χ^2 goodness-of-fit analysis using the JMSLA procedure in JoinMap. Loci at which Mendelian genotype ratios were observed were separated into individual linkage groups using the JMGRP and JMSPL procedures with a starting LOD threshold of 0.0 and increased to 8.0 in increments of 0.1. Pairwise Kosambi (1944) distances were estimated among loci in each of the three linkage groups using the JMREC procedure and the maximum likelihood map was estimated using the JMMAP procedure. The linkage map (Figure 1) was drawn with DrawMap1.1 (Van Ooijen 1994).

QTL mapping: Associations between genotypes at each locus and midgut infection or escape barriers were initially assessed by a contingency χ^2 analysis. The null hypothesis was that midgut infection or escape barrier rates were equal in each genotype class. Thus marginal probabilities were the frequencies of each genotype at a locus in females and the overall midgut infection or escape barrier rates. When a significant χ^2 was detected, we examined the inheritance of the alleles at that locus. Our *a priori* hypothesis was that an excess of F_2 individuals with an allele inherited from the Puerto Rico P_1 parent will become infected while an excess of F_2 individuals with an allele inherited from the refractory P_1 parent will not become infected.

The JoinMap linkage map and genotype/phenotype datasets were translated into the format used for interval mapping (Lander and Botstein 1986). BINARYQTL (Xu *et al.* 1998) is a FORTRAN program for interval mapping of QTL associated with binary traits. This algorithm assumes the presence of an unobservable continuous variable (often referred to as "liability") that underlies the binary expression of the phenotype. Most quantitative genetic models can be applied to liability even though the liability is distributed as an unobservable continuous quantitative trait. A probit model is used when the liability is assumed to be normally distributed. In using this model for dengue susceptibility we assume that below a certain liability threshold, a mosquito remains uninfected but above that threshold can become infected. BINARYQTL esti-

TABLE 1

Primer sequences, GenBank accession numbers, annealing temperatures (T_a), and anticipated PCR product size for each of the loci used to map MIB and MEB QTL in *A. aegypti*

Locus name (accession no.)	PCR product size	T_a	Pos.+[a]	Forward primer	Pos.−[a]	Reverse primer
A. Sequence-tagged RAPD markers						
B18.359 (AF191675)	360	54	1	CCACAGCAGTGTCGGTAGGAG	340	CCACAGCAGTACACAAATGGG
B18.349 (AF191676)	318	54	1	CCACAGCAGTAGAGGCGGTAG	298	CCACAGCAGTCAATGGAGCTT
B18.366 (AF191677)	364	54	1	CCACAGCAGTACAGTTCGAG	344	CCACAGCAGTGATTAAGATT
B20.220 (AF191678)	218	54	1	GGACCCTTACGACCATAATGG	198	TGACCCTTACCGTGTTAT
B20.448, B20.1300 (AF191679)	376	54	1	GGCCCTTACACGATTCGCAG	356	GGACCCTTACCCTACTGCTT
B. TAG microsatellites						
TAG66 repeats (AF191668-74)	—	42	1	TTACAGCTTGGATTTGTTCT	Variable	GAAACGAGAAGAAAACTCAT
C. cDNAs						
Lf90 (T58320)	165	48	31	AGCAGAATGGCTCCCCGTAA	175	ATGGTTTCCTTGCCGGACAG
Lf198 (T58319)	204	48	52	CTGGCGTAGATTCCGTGCTG	235	TCCGTGTTGACGTAGGTGGC
White-eye (U88851)	600	59	1968	TACCTGACSGCACTGCTGATTG	2089	TGATGACMGGCGGCCCAAC
Fxa (AF050133)	220	46	214	TTAGCACCAATCCAGCCTCA	413	TGGCACAACTGTTGGGAAGA
Early trypsin (X64362)	483	60	295	CCAACGGTGGCATCATAGTGAA	753	GATCCATTGGCGAACAGTGGA
CarboxypeptidaseA (AF165923)	398	54	68	TTGAATTGTAATGGGTTGAG	445	TTATGATAGGAATCGCTTTG
ADP/ATP translocase (AI657540)	304	58	14	CTGGCGCTACTTCATGGGTA	297	ATCGAGGTGTTCTTCGGGTC
Apolipophorin2 (AF038654)	349	54	24	GCTGGAATCGGTCAAACTCG	352	CCGGCCTTAACTTGCTGGTA
Late trypsin (X64363)	349	60	44	TGGCTTTGAAGTGCCCGTTGAG	368	CAAGTTCCTTCGTTGACCGGAGTG
Allatotropin (U65314)	392	43	193	GAACGGATGCTAGAAGAAAG	481	TTAGAATGGGACTACCGAGA
Maltase (M30442)	254	48	72	GGACTGGTGGGAACATGGAA	305	CTTATCGGACAACCGCTGGA
Defensin (AF156088)	213	54	88	CATTTGTTTCCTGGCTCTGT	280	GAGCAGCACAAGCACTATC
Apyrasel (L12389)	489	54	351	GGAATGTGACGGCGGATTT	820	TGGATCATGCGGCTGTTTG
Apyrase2 (L41391)	337	54	103	TGATTGCATCGTCGTTGATT	419	CAACTTGCGCTGTTTGTTTT

D. RAPD primers	PCR product size	Primer sequence	No. of loci
A09	—	GGGTAACGCC	12
A20	—	GTTGCGATCC	11
B15	—	GGAGGGTGTT	1
B16	—	TTTGCCCGGA	2
B18	—	CCACAGCAGT	5
C03	—	GGGGGTCTTT	10
C04	—	CCGCATCTAC	2
C13	—	AAGCCTCGTC	5
C19	—	GTTGCCAGCC	8
			56

[a] Nucleotide position of the 5′ end of each primer in the GenBank sequence.

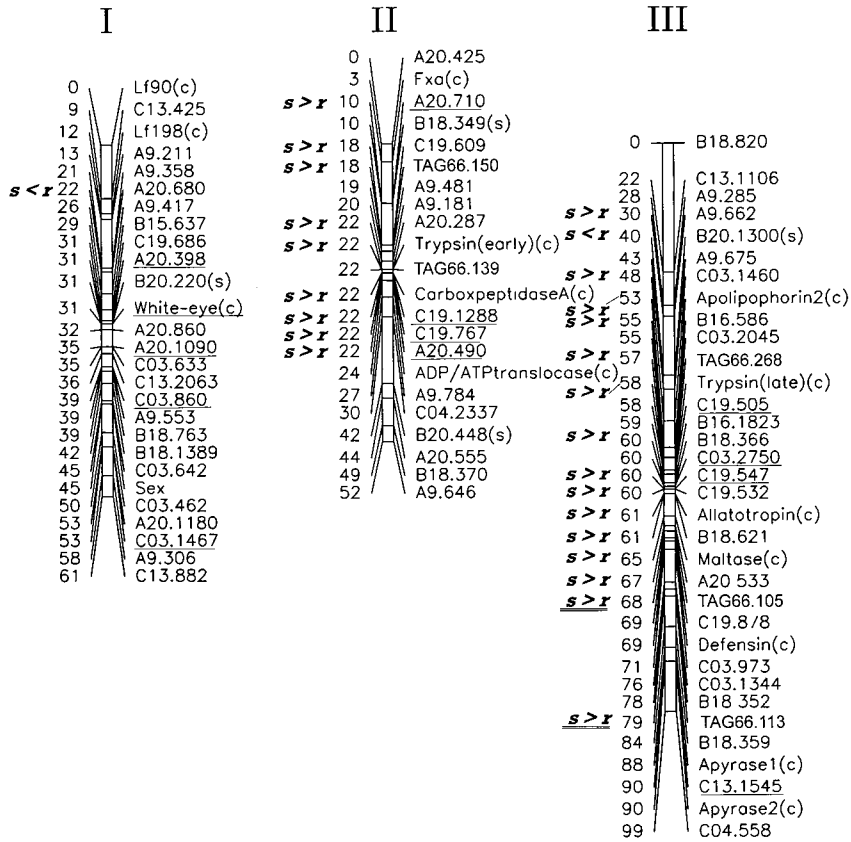

FIGURE 1.—Linkage map of the *A. aegypti* family used in the present study. STAR loci are followed by "(s)," cDNA loci are followed by "(c)," and all microsatellite loci begin with "Tag66-." Loci labeled with $s > r$ or $s < r$ were significantly associated with midgut infection barrier (MIB; $P \leq 0.05$) in the χ^2 contingency tests. Loci labeled with $\underline{s > r}$ were significantly associated with midgut escape barrier. Underlined loci were "redundant" (at equivalent map positions with adjacent loci) and were therefore removed for QTL analyses. Chromosome numbering and orientation corresponds with the map of MUNSTERMANN and CRAIG (1979) and SEVERSON *et al.* (1993).

mates liability using a single linear model with a heterogeneous residual variance and then uses standard interval mapping to determine the most probable location of QTL on the linkage map generated by JoinMap. Use of the heterogeneous residual variance model allows for calculation of the sampling variance surrounding each estimated parameter. BINARYQTL was modified to calculate 90, 95, and 99% comparisonwise thresholds at each centimorgan interval and 90, 95, and 99% experimentwise thresholds (CHURCHILL and DOERGE 1994).

Depending on their magnitude of effect and linkage relationships, LOD at different map intervals may covary with one another and this can upwardly bias LOD estimates at individual intervals. Composite interval mapping (ZENG 1994) adjusts LOD scores at individual intervals using a variable number (n_p) of markers to control for effects of other intervals in the map and a variable window size (w_s) to adjust for the effects of intervals in proximity to the interval under analysis. QTL Cartographer 1.13g (BASTEN *et al.* 1997) performs composite interval mapping using a sequential approach to characterize and rank QTL. The module SRmapqtl performs stepwise linear regression to rank the degree to which individual markers explain the phenotype variance (σ_p^2). The marker with the largest *F*-statistic is assigned a rank of one and the remaining markers are added to the model. The marker with the largest partial *F*-statistic is ranked second. This process is repeated until all the markers are ranked, regardless of the significance of their partial *F*-statistic. BINARYQTL analyses indicated two major loci with significant effects on phenotype. Thus, n_p was set to 2 and w_s = 10 cM (the recommended default value). The most probable locations for QTLs were then reanalyzed using the Zmapqtl module. Zmapqtl uses the output of SRmapqtl to identify the n_p most important markers to control for genetic background. The window size blocks out a region of the chromosome on either side of the markers flanking a test position. Any of the n_p markers that fall in the blocked area are not controlled since this would eliminate the signal from the test site itself. Zmapqtl was then rerun with 1000 permutations to estimate the 95% comparisonwise thresholds at each interval.

Estimation of variance components: Marker genotypes were numerically scored as 0, 1, or 2 according to the number of alleles inherited from the susceptible parent, and dominant genotypes from the susceptible and refractory parents were scored as 1.5 and 0.5, respectively. Pearson correlation coefficients between midgut or head infection phenotypes and marker genotypes were computed using the PROC CORR procedure in SAS 8.0 (SAS INSTITUTE 1999). Midgut, head, or overall disseminated infection phenotypes were regressed on marker genotypes. The RSQDELTA macro in SAS 8.0 (SAS INSTITUTE 1999) combines the information from PROC REG and PROC GLM to compute the change in R^2 and the associated *F*-statistics and *P* values as genotypes are added to a linear regression model. The *F*-statistics and *P* values represent a partial *F*-statistic for the general linear model.

RESULTS

Mapping family: The F_2 family selected for analysis arose from a susceptible Puerto Rico female and a refractory Ibo male. F_2 females had a midgut infection rate of 44.2% (38/86), a disseminated infection rate of 34.6% (29/84—two heads were lost in processing females with infected midguts), and a midgut escape rate of 80.5% (29/36). The ratio of females to males before bloodfeeding was 121:116. Males and unfed females

were collected 3–4 days after emergence and frozen. Thirty bloodfed females died before the 14-day extrinsic incubation period. There were 207 individuals in the mapping family: 86 bloodfed F_2 females that survived through the extrinsic incubation period, 5 unfed F_2 females, and 116 males.

Due to the large number of individual genotype determinations, we analyzed only the largest F_1 intercross family. Additional, albeit smaller, F_1 families are currently being analyzed to test the results of the current study. In principle, QTL analyses can be performed on additional, combined sets of F_2 progeny arising from the same set of F_1 siblings.

Linkage mapping: A total of 83 markers fit Mendelian ratios and were mapped among the 207 F_2 individuals. These consisted of 57 RAPD-SSCP markers amplified by each of 9 RAPD primers (Table 1), 5 $(TAG)_n$ microsatellite loci, 6 STARs, and 14 cDNA-SSCP markers, and *sex* was treated as a genetic marker. Alleles at 18 loci were codominant: 1 RAPD (B18.621), 14 cDNAs, and 3 STARs (B18.359, B18.366, and B18.220), and 9 of these were fully informative in this family. The remainder segregated as band present (dominant)/band absent (recessive) polymorphisms or were only partially informative due to equivalent genotypes between P_1 and F_1 parents. Joinmap at an LOD of 4.1 detected three linkage groups (Figure 1) and these remained intact until an LOD of 7.7.

QTL mapping: Loci that were statistically ($P \leq 0.05$) associated with a midgut infection barrier or midgut escape barrier in the contingency χ^2 tests are indicated in Figure 1. In all but two markers (A20.680 on *I* and B20.1300 on *III*), mosquitoes homozygous for an allele inherited from the susceptible P_1 parent had a significantly higher midgut infection rate than mosquitoes with one to two copies of an allele inherited from the refractory P_1 parent.

The most probable locations of QTL conditioning midgut infection (Figure 2) and escape barriers (Figure 3) were estimated with standard interval mapping using BINARYQTL (Xu *et al.* 1998) and composite interval mapping using QTL Cartographer (Basten *et al.* 1997). Comparison- and experimentwise 95% thresholds were also estimated (Churchill and Doerge 1994). Only the LOD of the chromosome *III* midgut infection barrier QTL estimated from standard interval mapping exceeded the experimentwise 95% threshold rate. However, the experimentwise 95% threshold rate tends to underestimate the number of QTL while the comparisonwise 95% threshold overestimates the number of QTL and often identifies many QTL of minor effects (Churchill and Doerge 1994; Xu *et al.* 1998). For the remainder of this article statistically significant QTL are defined relative to the comparisonwise 95% threshold.

LOD estimated for a midgut infection barrier by standard interval mapping exceeded the comparisonwise 95% threshold at 22 cM on chromosome *I*, between 20 and 22 cM on chromosome *II* and 5 LOD peaks occurred at 15, 30, 40, 52, and 63 cM on chromosome *III* (Figure 2). In general, the locations of QTL identified through standard interval mapping agree with locuswise χ^2 contingency tests (Figure 1). Genotypes at A20.680 (22 cM) on chromosome *I*, most loci between 10 and 22 cM on chromosome *II*, and most loci between 30 and 68 cM on chromosome *III* were significantly associated with a midgut infection barrier in the locuswise χ^2 contingency tests.

The most probable locations of QTL controlling midgut escape barrier were made from only 36 mosquitoes with infected midguts, of which 29 had infected heads, and of these only 7 appeared to have a midgut escape barrier. Nevertheless, genotypes at the TAG66-105 (68 cM) and TAG66-113 (79 cM) loci on chromosome *III* were statistically associated with a midgut escape barrier in the contingency χ^2 analyses. However, LOD estimated for a midgut escape barrier by standard interval mapping only exceeded the comparisonwise 95% threshold at 79 cM on chromosome *III* (Figure 3).

Composite interval mapping estimated approximately the same location and magnitude of QTL controlling a midgut infection barrier on chromosome *II* as standard interval mapping (Figure 2). However, the LOD estimated by composite interval mapping on chromosome *I* did not exceed comparisonwise 95% threshold and was not significant in the permutation test. The composite interval mapping LOD estimated on chromosome *III* exceeded the comparisonwise 95% threshold and was significant in the permutation test for the interval between 58 and 64 cM. This suggests that the magnitude and location of a midgut infection barrier QTL of minor effect on chromosomes *I* and *III* are correlated with other midgut infection barrier QTL. The chromosome *III* midgut escape barrier QTL estimated by standard interval mapping did not exceed the comparison-wise 95% threshold with composite interval mapping (Figure 3) and was not significant in the permutation test. This also suggests that the magnitude and location of midgut escape barrier QTL are correlated with other QTL.

Variance components: Pearson correlation coefficients estimated between marker genotypes and midgut infection and escape barriers were significant ($P \leq 0.05$) at the same loci that were statistically associated with midgut infection or escape barriers in the contingency χ^2 tests. For midgut infection barrier, the largest correlation coefficients were detected for *carboxypeptidase* and *early trypsin* at 22 cM on chromosome *II* and at *apolipophorin 2*, *late trypsin*, and B18.621 at 53, 58, and 61 cM on chromosome *II*, respectively. For midgut escape barrier, the largest correlation coefficients were detected for TAG66-113.

Midgut, head, or overall disseminated infection phenotypes were regressed on *early trypsin*, B18.621, or TAG66-113 genotypes (Table 2). Genotypes at the *early*

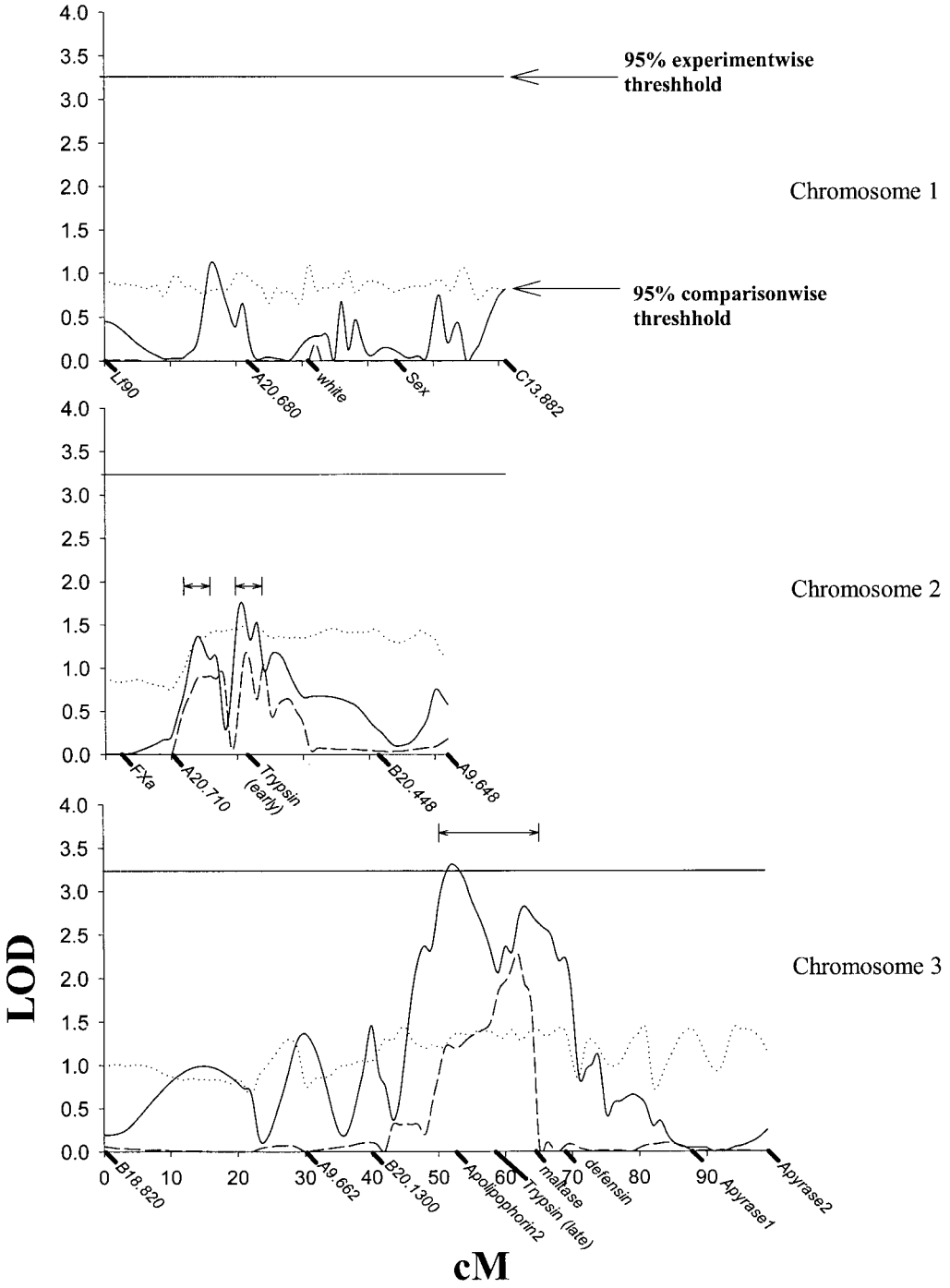

FIGURE 2.—Plot of LOD values associated with MIB along chromosomes *I–III*. Names of a few markers are listed to orient QTL positions relative to Figure 1. LOD estimated by interval mapping (IM) using a heterogeneous residual variance model for binary traits (XU *et al.* 1998) appear as a solid line. LOD estimated by composite interval mapping (CIM; ZENG 1994) appear as a dashed line. Comparisonwise 95% thresholds appear as a dotted line while 95% experimentwise thresholds are represented by the straight line along the top of each graph. Intervals at which the CIM LOD estimated in Zmapqtl (QTL Cartographer 1.13) exceeded the top 950 CIM LOD scores in 1000 permutations are bracketed by |↔|.

trypsin and B18.621 loci, respectively, accounted for 48 and 52% of σ_g^2 and cumulatively accounted for 23% of σ_p^2 in a midgut infection barrier. Genotypes at the TAG66-113 locus accounted for 13% of σ_p^2 in a midgut escape barrier. Genotypes at the *early trypsin*, B18.621, and TAG66-113 loci, respectively, accounted for 49, 54, and $<0.01\%$ of σ_g^2 and cumulatively accounted for 34% of σ_g^2 in overall disseminated infection; however, the contribution of TAG66-113 was not significant and was thus removed. Genotypes at the *early trypsin* and B18.621 loci alone, respectively, accounted for 46 and 54% of σ_g^2 and cumulatively accounted for 30% of σ_g^2 in disseminated infection. Inferences arising from analysis of genetic variance components were confirmed by plotting disseminated infection rates as a function of genotype at individual loci (Figure 4A). Alleles at the chromosome *II* QTL that cosegregated with *early trypsin* alleles appear to be additive in their effect on a midgut infection barrier. A total of 11% of individuals homozygous for the allele from the refractory parent had a disseminated infection, while 37% of heterozygous individuals had a disseminated infection and 83% of individuals homozy-

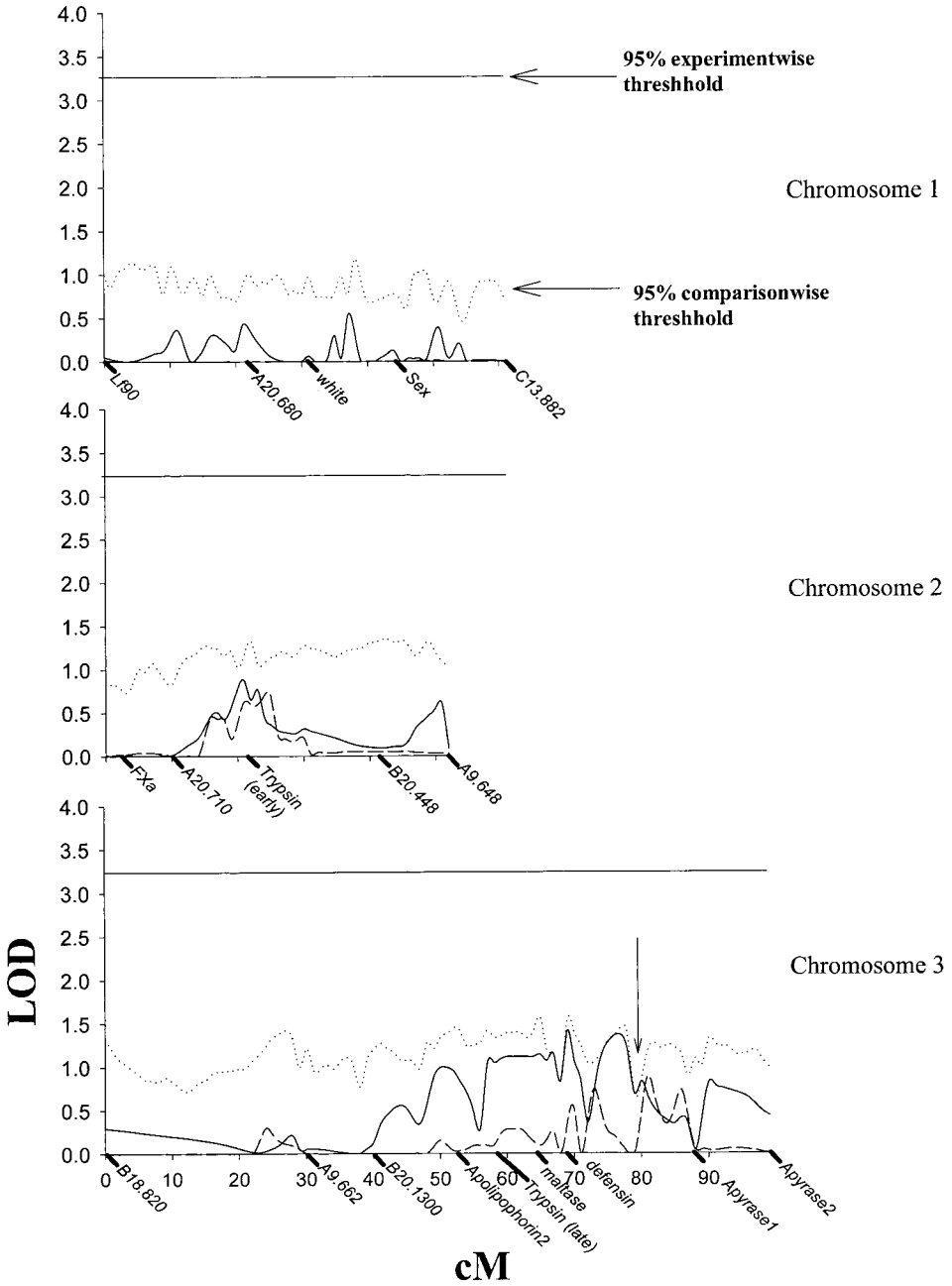

FIGURE 3.—Plot of LOD values associated with MEB along chromosomes *I–III*. Names of a few markers are listed to orient QTL positions relative to Figure 1. LOD estimated by IM (XU *et al.* 1998) appear as a solid line while LOD estimated by CIM (ZENG 1994) appear as a dashed line. Comparison-wise 95% thresholds appear as a dotted line while 95% experimentwise thresholds are represented by the straight line along the top of each graph. The only region in which the IM LOD exceeded the comparison-wise 95% threshold is indicated with a vertical arrow.

gous for the allele inherited through the susceptible parent had a disseminated infection. Linear regression analysis on the number of copies of the susceptible allele with respect to disseminated infection rate indicates that the average additive substitution rate is 32% disseminated infection/susceptible chromosome *II* QTL allele (Table 2). Similarly on chromosome *III*, alleles at the midgut infection barrier QTL that cosegregate with the B18.366 alleles appear to act additively (Figure 4A). From 7 to 13% of individuals homozygous for the allele inherited through the refractory parent had an infected midgut, while 33–41% of heterozygous individuals had disseminated infection and 69–86% of susceptible homozygous individuals had infected midguts. Linear regression analysis on the number of copies of the susceptible allele with respect to midgut infection indicates that the average additive substitution effect is 31–35% disseminated infection/susceptible chromosome *III* QTL allele.

Regression analysis of disseminated infection rate with respect to *both* chromosome *II* and *III* midgut infection barrier QTL genotypes supports a model of independent effects. Contingency χ^2 analysis showed that genotypes at the *early trypsin* and B18.621 loci were in linkage equilibrium ($\chi^2_{[4\ \text{d.f.}]} = 2.47$, $P > 0.05$). There were no mosquitoes that had no susceptibility alleles at either locus or were homozygous refractory at both loci. Individuals with three susceptible alleles at both loci

TABLE 2

Phenotypic variance accounted for by genotype markers at MIB and MEB QTL in *A. aegypti*

Phenotype	Genetic marker	ΔR^2 (% total variance)	Partial F^a
Midgut infection			
	Intercept	—	67.29***
	Early trypsin	0.109(48)	8.17**
	B18.621	0.119(52)	10.60***
	Total	0.228	
Disseminated infection			
	Intercept	—	145.00***
	TAG66-7a	0.126	4.75*
	Total	0.126	
Disseminated infection as a function of all loci			
	Intercept	—	44.32***
	Early trypsin	0.164(49)	11.52***
	B18.621	0.174(51)	18.51***
	TAG66-7a	0.001	0.12
	Total	0.339	
	Intercept	—	44.32***
	Early trypsin	0.129(46)	11.52***
	B18.621	0.167(54)	18.51***
	Total	0.295	
Disseminated infection as a function of individual loci			
Disseminated infection = 0.326***(early trypsin) + 0.076		R^2 = 12.6%	
Disseminated infection = 0.316***(B18.621) + 0.057		R^2 = 16.0%	
Disseminated infection = 0.312***(B18.366) + 0.046		R^2 = 13.9%	
Disseminated infection = 0.347***(late trypsin) + 0.107		R^2 = 17.7%	
Disseminated infection = 0.337***(early trypsin) + 0.323***(B18.621) − 0.236		R^2 = 29.6%	

*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

[a] The *F*-statistics and *P* values represent a partial *F*-statistic for the general linear model where $F = [(SSE_R - SSE_F)/(d.f._R - d.f._F)]/(SSE_F/d.f._F)$. SSE, error sum of squares; d.f., error degrees of freedom; *F*, model with all genotypes; *R*, model with one genotype. H_O is rejected when $F_c > F$ ($\alpha = 0.05$, $d.f._R - d.f._F$, $d.f._F$).

had approximately the same disseminated infection rate (82–83%; Figure 4B) as homozygous susceptible mosquitoes at either QTL (69–86%; Figure 4A). Similarly, individuals with one susceptible allele at each QTL had approximately the same disseminated infection rate (42%; Figure 4B) as heterozygous mosquitoes at either QTL (33–41%; Figure 4A) while individuals with only one susceptible allele at either had approximately the same disseminated infection rate (0–7%; Figure 4B) as homozygous refractory mosquitoes at either QTL (6–13%; Figure 4A).

DISCUSSION

The linkage map presented in the current study (Figure 1) is identical in gene order to maps generated from a reciprocal (Ibo × Puerto Rico) F_1 intercross (R. E. Fulton, M. L. Salasek, N. M. Duteau and W. C. Black IV, unpublished results), the cDNA restriction fragment length polymorphism map (Severson *et al.* 1993), and the map derived from family RA34-3 (Antolin *et al.* 1996). However, chromosome *III* linkage distances in the present family are longer than in previous maps (56–57 cM). It is possible that the families used in the two crosses vary in haploid genome size. Black and Rai (1988) reported 35% variation in the amounts of repetitive DNA among different populations of the closely related mosquito *A. albopictus*. This was chiefly due to variation in the abundance of a single highly repetitive element. Recombination can be locally reduced in heterochromatic regions (Rai and Black 1999).

This is the first study to map the locations of genes that control viral infection and escape barriers in a mosquito. Our results suggest that alleles at primarily two independently segregating loci create a midgut infection barrier in *A. aegypti* (Figure 2). Alleles at these loci act additively both within each QTL and independently among QTL (Figure 4). Other loci of minor effect may also be involved. The observed additive genetic pattern could reflect differences among genotypes in (1) the density of a virus receptor on midgut cells, (2) abundance of

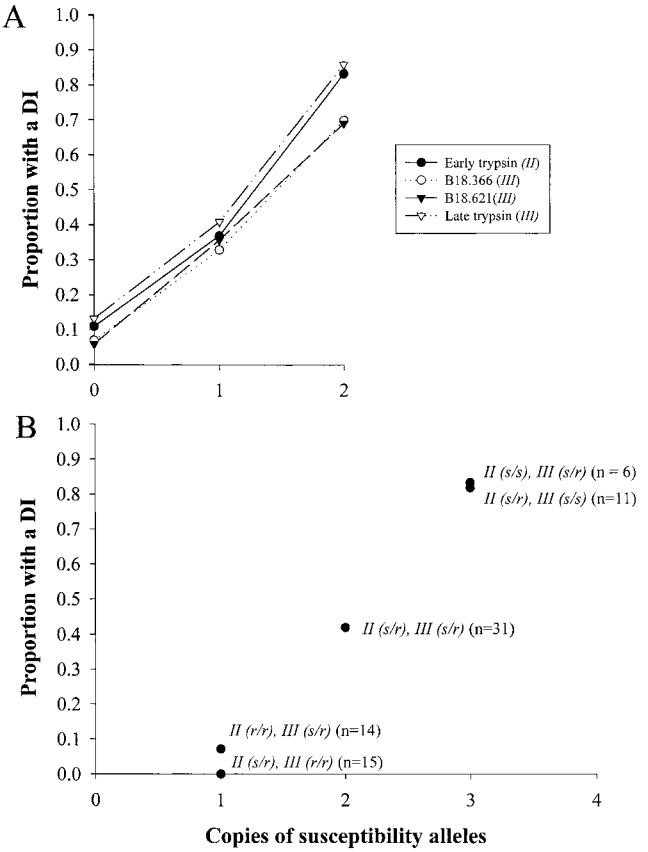

FIGURE 4.—Plot of the disseminated infection (DI) rate among *A. aegypti* with different single and dilocus genotypes at marker loci linked to chromosome *II* and *III* MIB QTL (A) A plot of DI rate as a function of the number of susceptible alleles at the *early trypsin* locus on chromosome *II* and at the *late trypsin*, B18.366, and B18.621 STAR loci on chromosome *III*. (B) Plot of midgut infection rate as a function of the number of susceptible alleles at both the *early trypsin* and B18.621 loci. The genotypes at each locus are listed next to each point.

intracellular factors needed for viral replication, or (3) abundance of intracellular inhibitors that reduce viral replication. However, very little is known about receptors or substances in mosquito midgut cells that condition arbovirus infection and replication.

The family analyzed in this study provided only weak statistical support for an association of midgut escape barrier phenotypes with genotypes (Table 2) and weak evidence for the existence of a midgut escape barrier QTL (Figure 3). This is primarily because a midgut escape barrier cannot be assayed in mosquitoes with a midgut infection barrier. This reduced the sample size for mapping midgut escape barrier QTL to 36 mosquitoes, only 7 of which appeared to have a midgut escape barrier. While small sample sizes are adequate to detect QTL with large effect, they have very limited power to detect smaller QTL (LANDER and BOTSTEIN 1986; ZENG 1993, 1994). We are attempting to resolve this problem by identifying populations in Mexico and the southeastern United States that have a high midgut infection rate, but a low disseminated infection rate.

In general, our results suggest that transmission of dengue is a quantitative genetic trait under the control of at least three loci. It is well established in laboratory studies that populations of *A. aegypti* throughout the world vary greatly in their ability to transmit dengue (GUBLER *et al.* 1979) and yellow fever (TABACHNICK *et al.* 1985) flaviviruses. A significant positive correlation was detected between head infection rates with yellow fever virus and allele frequencies at the *phosphoglucomutase* locus located at 47 cM on chromosome *II* (TABACHNICK *et al.* 1985). LORENZ *et al.* (1984) found a significant correlation between rates of head infection with yellow fever virus and allele frequencies at the *malic acid dehydrogenase* locus located at 3 cM on chromosome *II*. This correlation was found among successive generations of a single population of *A. aegypti* raised in the laboratory. These studies may have tracked cosegregation of allozymes at these two loci with the chromosome *II* QTL identified in the present study. These earlier studies found no correlation between rates of head infection and the frequencies of *hexokinase* or *phosphogluconate dehydrogenase* allozymes (both on chromosome *III*). Perhaps susceptibility alleles at the chromosome *III* QTL were fixed among all of the populations analyzed or *hexokinase* or *phosphogluconate dehydrogenase* allozymes do not cosegregate with alleles at the chromosome *III* QTL. However, isozymes were not mapped in the present study because their analysis consumes too much tissue to be useful for intensive linkage mapping of mosquito families and alleles at individual isozyme loci frequently do not segregate in individual F_1 intercross families.

Our results are consistent with a hypothesis that variation in dengue infection rates among natural populations of *A. aegypti* is due to the segregation of alleles at each of the three QTL. However, these accounted for only ~30% of σ_p^2 and the remainder of the variance is associated with environmental and random experimental effects (σ_e^2). BOSIO *et al.* (1998) showed that σ_e^2 often accounted for >50% of σ_p^2 for a midgut infection barrier and escape barriers among full siblings of *A. aegypti*. This large σ_e^2 is probably associated with factors that affect either larval or adult survivorship, or adult bloodfeeding and reproduction in the insectary. Possible factors include larval nutrition (GRIMSTAD and HARAMIS 1984; GRIMSTAD and WALKER 1991; NASCI and MITCHELL 1994) or local temperature variation (HURLBUT 1973; KRAMER *et al.* 1983) experienced by adults within the insectary.

Quantity of blood and virus ingested may also be an important component of σ_e^2 (KRAMER *et al.* 1981). During standardization of dengue infection assays for the present study, we found that a large portion of σ_e^2 may be associated with dengue virus preparation for the infectious bloodmeal. In addition to growing dengue in intrathoracically inoculated mosquitoes (as described

above), dengue was grown in insect cell culture for 7 and 14 days following SCHOEPP *et al.* (1990). The rate of head-infected mosquitoes was 54% if dengue was grown intrathoracically in mosquitoes, 24% when the virus was from 7-day cell culture, and 86% when the virus was grown in 14-day cell culture. Similar patterns were seen in Ibo where head infection was 10% when dengue was grown intrathoracically, 0% if grown in 7-day cell culture, and 65% if grown in 14-day cell culture. The 14-day incubation had the highest bloodmeal titer (8.1 $\log_{10}$ virus/ml) and the highest infection rates. The 7-day treatment, however, had a lower titer (7.7 $\log_{10}$ virus/ml) and much lower infection rates. Virus grown within intrathoracically inoculated mosquitoes had the lowest titer (7.3 $\log_{10}$ virus/ml), but yielded intermediate infection rates that were significantly higher than the 7-day cell culture virus. These results suggest that the viral titer in the bloodmeal correlates with midgut infection in mosquitoes, but also that dengue infection can vary both quantitatively and qualitatively in the laboratory when prepared by different methods. Any one or all of these factors could contribute to σ^2_ϵ in the laboratory. However, none of these three methods necessarily models the actual mechanism of dengue transmission in nature in which mosquitoes feed directly on viremic humans. Humans are infectious to mosquitoes for a short window of time during the course of illness and during the infectious period the concentration of dengue can vary a great deal among different persons. Therefore, infectious dose as well as the genotypes at the three QTL can determine if an *A. aegypti* will become infected when feeding on a dengue-infected human.

Midgut infection and escape barriers can vary among different dengue viral serotypes and among different genetic strains within a dengue serotype (GUBLER *et al.* 1979; TABACHNICK *et al.* 1985; MILLER and MITCHELL 1991). Thus, this study needs to be repeated with different genetic strains of the dengue serotype as well as different dengue serotypes. Susceptibility of *A. aegypti* to Chikungunya and Sagiyama virus (Togaviridae) was strongly statistically associated with genotypes at the *rosy eye* marker (MOURYA *et al.* 1994) on chromosome *III* (MUNSTERMANN and CRAIG 1979).

Differences in dengue susceptibility between *A. a. aegypti* and *A. a. formosus* populations may reflect differences in the frequency of alleles at the midgut infection and escape barrier loci identified in this study, but may also arise from differences in the presence of specific midgut infection and escape barrier loci between populations of the two subspecies. Therefore, we do not know whether the same loci and alleles are segregating within a single natural population of *A. aegypti*. We are currently mapping midgut infection and escape barrier among collections within Mexico, where there is active dengue transmission, to determine if alleles at the same QTL are segregating within a single population. A similar effort is underway with *A. aegypti* from Thailand, where dengue transmission is prevalent.

This research was supported in part by the MacArthur Foundation for the Network on the Biology of Parasite Vectors and by National Institutes of Health grants AI-41436 and AI-45430. Dr. Shizhong Xu kindly provided the source code for BINARYQTL modified for analysis of F_1 data. Michael Antolin, Dennis Knudson, and Boris Kondratieff served on Dr. Bosio's graduate committee. Amy Fagerberg assisted greatly in cloning and analysis of microsatellites.

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Communicating editor: Z-B. Zeng