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Genetics

The role of alternative mRNA splicing in generating heterogeneity within the *Anopheles gambiae* class I glutathione *S*-transferase family

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ABSTRACT **The class I glutathione *S*-transferases (GSTs) of *Anopheles gambiae* are encoded by a complex gene family. We describe the genomic organization of three members of this family, which are sequentially arranged on the chromosome in divergent orientations. One of these genes, *aggst1-2*, is intronless and has been described. In contrast, the two *A. gambiae* GST genes (*aggst1α* and *aggst1β*) reported within are interrupted by introns. The gene *aggst1α* contains five coding exons that are alternatively spliced to produce four mature GST transcripts, each of which contains a common 5′ exon encoding the N termini of the GST protein spliced to one of four distinct 3′ exons encoding the carboxyl termini. All four of the alternative transcripts of *aggst1α* are expressed in *A. gambiae* larvae, pupae, and adults. We report on the involvement of alternative RNA splicing in generating multiple functional GST transcripts. A cDNA from the *aggst1β* gene was detected in adult mosquitoes, demonstrating that this GST gene is actively transcribed. The percentage similarity of the six cDNAs transcribed from the three GST genes range from 49.5% to 83.1% at the nucleotide level.**

Glutathione *S*-transferases (GSTs) are a major family of detoxification enzymes found in most organisms. All eukaryotes possess multiple GSTs with different substrate specificities to accommodate the wide range of catalytic functions of this enzyme family. The mammalian GSTs have been classified into eight classes: alpha, mu, pi, theta, sigma, zeta, kappa, and a microsomal class (1–6). Nonmammalian GSTs have been assigned to the same classes if their amino acid sequences show >40% identity to other members of the class. However, the sequence of the majority of invertebrate GSTs is below the threshold for inclusion in the mammalian classes, and a separate classification system for these GSTs is more appropriate. Sequence data for nonmammalian GSTs are limited, but there is evidence for at least two classes of insect GSTs (class I and class II) and two classes of plant GSTs (7–9). The bacterial GSTs belong in a separate class because of their low homology with any other known eukaryotic enzyme (10).

The sequences of two insect class II GST genes have been published, *aggst2-1* from *Anopheles gambiae* (11) and *DmGST2* from *Drosophila melanogaster* (7), and a GenBank search identified an additional class II gene from the housefly, *Musca domestica* (accession no. U02616). Both *DmGST2* and *aggst2-1* are single-copy genes with no closely related sequences present in the genome of either species.

The insect class I GSTs, in contrast, are encoded by members of a large gene family. In *D. melanogaster,* eight divergent intronless genes are found within a 14-kb DNA segment (12), and at least five different class I GST genes are present in *M. domestica* (13). We previously have shown that *A. gambiae* also contains multiple class I GSTs, which are arranged sequentially in the genome (14).

The class I insect GSTs are of interest because of their role in insecticide resistance. Elevated GST activity has been detected in many resistant strains of insects (see, for example, refs. 15–17) and a subset of *in vitro*-expressed class I GSTs are capable of metabolizing insecticides (18, 19). GSTs are particularly important in *A. gambiae,* the major vector of malaria in Africa, as they represent one of only three major insecticide-resistance mechanisms to be found in this insect. We have cloned and expressed two class I GSTs, AGGST1–5 and AGGST1–6, from a 1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane (DDT)-resistant strain of *A. gambiae.* Both catalyze the dehydrochlorination of DDT to the noninsecticidal metabolite DDE (1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethylene) but neither belong to the subset of *A. gambiae* GSTs responsible for the majority of DDT metabolism in our laboratory-resistant strain (20).

We therefore set out to investigate the *A. gambiae* class I GST gene family further with the aim of characterizing the genetic alterations that lead to DDT resistance in this important malaria vector. We now report the identification of three *A. gambiae* class I GST cDNAs and describe the genomic organization of the genes from which these GSTs are transcribed.

METHODS

Mosquito Strains. The Suakoko 2L*a* strain originated from Liberia and is the international reference strain for *A. gambiae s.s.* mapping work (21). ZAN/U was colonized from Zanzibar, Tanzania in 1982. This strain was selected with DDT for eight generations to produce a highly resistant population.

Sequencing of *A. gambiae* Genomic DNA. The recombinant bacteriophage, Ag_B1 was isolated from a Suakoko 2L*a* genomic library by screening with a partial *A. gambiae* class I GST cDNA, *aggst1-1*, as described (14). A contiguous sequence of the entire 15.3-kb insert of Ag_B1 was obtained by manual sequencing and automated sequencing using the ALF automatic sequencer (Pharmacia). The sequences were aligned by using the LASERGENE package (DNAstar, Madison, WI). Primers designed from this sequence were used to amplify the corresponding region of the genome from the DDT-

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Abbreviations: DDT, 1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane; GST, glutathione *S*-transferase; RACE, rapid amplification of cDNA ends.
Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. AF071161, AF071162, AF071163, and AF071160).
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Table 1. Sequences of primers used in study

Primer name	Primer sequence	Size of PCR product expected from cDNA with P2 primer, nt	Size of PCR product expected from genomic DNA with P2 primer, nt
P2	ATCTGCCCCGTGCCGTGC	N/A	N/A
Pβ1.1	ATGACGCCAGTGCTGTATTAT	N/A	N/A
Pβ1.2	CAACCCACAGCACTGCATACCTACAC	N/A	N/A
P3A	CCGTTCTGCGAGGGCTGG	241	1,045
P3B	CTTCCTTGCGAATGTTTTCGT	546	2,314
P3C	AACCATTTCCTCCCGCCACAA	447	2,926
P3D	CGGGGGCGTTTGCTTTGC	553	3,700

Expected sizes of PCR products were calculated from Fig. 2. See text for further details. N/A, not applicable.

resistant ZAN/U strain. For each primer set, separate PCRs were performed by using genomic DNA extracted from two or more individual ZAN/U mosquitoes. A contig was constructed spanning 4.4 kb of ZAN/U DNA (accession no. AF071160).

PCR Amplification of GST Genes and cDNAs. mRNA was extracted from ZAN/U larvae, pupae, and adults and was used to synthesize cDNA as described (14). Genomic DNA was extracted from individual mosquitoes by using the method of Collins *et al.* (22).

PCRs were performed by using the primer sets shown in Table 1 and 1–10 ng of cDNA or 1% of total genomic DNA from a single mosquito as template. Optimal PCR conditions were determined empirically for each primer pair. *Taq* extender PCR additive (Stratagene) was used for genomic PCRs using P2/P3D and P2/P3C primer pairs.

The PCR products were separated by gel electrophoresis and transferred to a nylon membrane. A plasmid containing the *A. gambiae aggst1-6* cDNA was digested with *Sal*I, and a 170-nt fragment, encompassing the 5′ region of the gene, was isolated and used as a template for probe construction. Probes were prepared by using the Hi Prime Labeling Kit (Boehringer Mannheim). Hybridizations were performed at 42°C overnight in formamide hybridization buffer [5× SSC, 5× Denhardt's solution (0.02% polyvinylpyrrolidone/0.02% Ficoll/0.02% BSA), 1% SDS, 50% formamide, and 100 μg/ml of boiled, sheared salmon sperm DNA]. The filters were washed under conditions of high stringency (final wash 0.1× SSC, 0.1% SDS 65°C).

The 5′ untranslated region of *aggst1-5* and *aggst1-6* was amplified by PCR using DNA prepared from a cDNA library as a template. A vector primer upstream of the insert was used with antisense primers P3B or P3D.

3′ Rapid Amplification of cDNA Ends (RACE) Amplification of *aggst1β* Transcripts. A cDNA transcribed from *aggst1β* was isolated from a single female ZAN/U adult by a modified 3′ RACE procedure. RNA was extracted from single adult mosquitoes by using TRI Reagent (Sigma). First-strand cDNA was synthesized by using Superscript II reverse transcriptase (GIBCO/BRL) and an oligo(dT) adapter primer [5′-GACTCGAGTCGACATCGA$(dT)_{17}$-3′] according to the manufacturer's protocol. Primers β1.1 and β1.2 (Table 1) were designed with 100% congruence to the genomic sequence of the predicted exon 1 of *aggst1β* but with sufficient mismatches to prevent them from annealing to *aggst1α* or *aggst1-2*. An initial round of PCR amplification was carried out by using primers β1.1 and the adapter primer. One microliter of this PCR was used as the template for a second round of PCR amplification using primers β1.2 and the adapter primer. A single 700-nt product was obtained, subcloned into the TA vector (Invitrogen), and sequenced.

Sequence Analysis. Dipteran GST sequences were retrieved from the GenBank sequence database and aligned by using the CLUSTAL program (23).

RESULTS

Identification of GST–Like Sequences Within the Genomic Clone, Ag_B1. We previously have described a single, intronless class I GST gene, *aggst1-2*, from the *A. gambiae* genomic clone, Ag_B1 (14). Restriction mapping of Ag_B1 and two additional, overlapping genomic clones identified multiple fragments that hybridized to a class I GST probe. Therefore the entire insert of Ag_B1 was sequenced and compared with sequences in the database to identify putative GST genes. Six sequences, in addition to the *aggst1-2* gene previously described, had high levels of homology to insect class I GST genes. The arrangement of these GST sequences is shown in Fig. 1.

Five of these GST-like sequences are arranged sequentially on the chromosome in the same orientation. The first of these has high levels of sequence identity to the 5′ end of insect class I GSTs, while the four proceeding downstream sequences are truncated at their 5′ ends but have high sequence identities to the 3′ region of GSTs. The sixth GST-like sequence within the Ag_B1 clone is arranged in the opposite orientation and is located 4,995 bp upstream from the first of the sequences described above. This sequence has high sequence identity to the 5′ end of insect class I GSTs. The results of these homology comparisons suggest that the six sequences are exons of two divergently orientated GST genes, which we have named *aggst1α* and *aggst1β*. *aggst1α* is fully contained within the

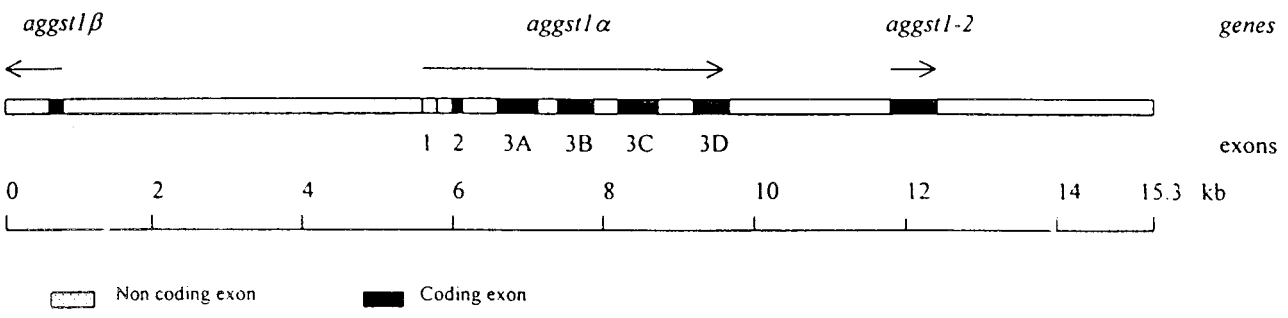


FIG. 1. Arrangement of GST genes in the recombinant bacteriophage clone, Ag_B1, isolated from an *A. gambiae* Suakoko 2L*a* genomic library. The orientations of the genes are indicated by arrows.

genomic clone Ag_B1, upstream of *aggst1-2*, whereas only the 5′ region of *aggst1β* is present in this clone.

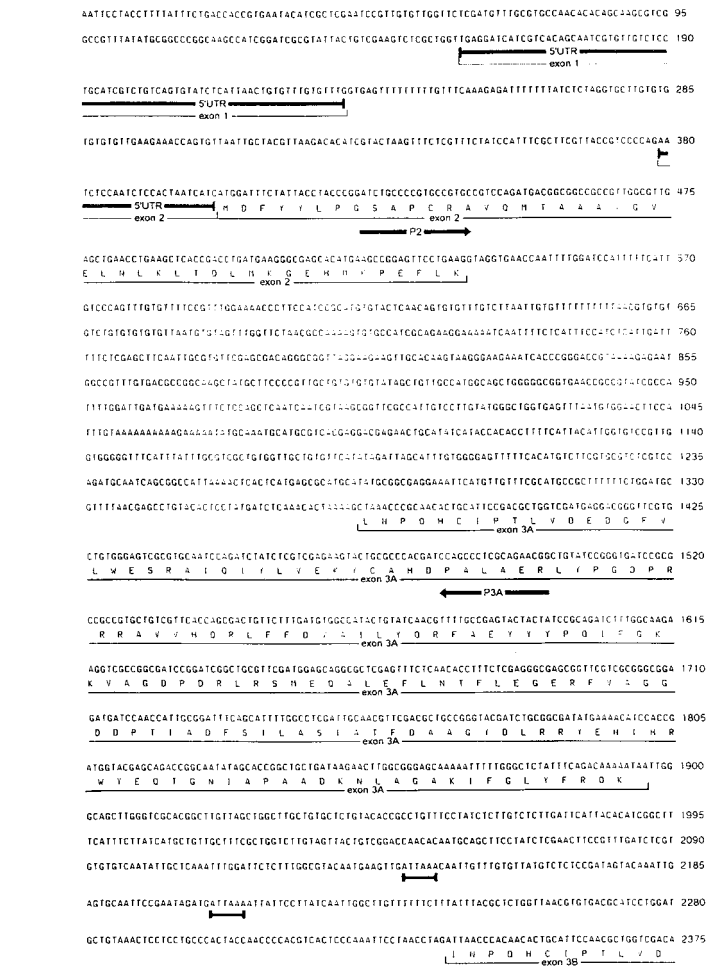
To confirm that the proposed arrangement of GST genes was not an artifact of cloning, the corresponding region of the genome from the DDT-resistant ZAN/U strain was amplified and sequenced. The sequence of this 4.4-kb region of the genome is shown in Fig. 2.

The ORF of exon 2 of *aggst1α* was aligned with the 5′ sequences of the two *A. gambiae* class I GST cDNAs, *aggst1-5* and *aggst1-6*. A single substitution was found between the first 135 nt of the ORF of *aggst1α* and the corresponding region of *aggst1-6*. An additional base pair differs between the sequence of *aggst1α* and *aggst1-5*. In addition, alignment of exons 3A, 3B, 3C, and 3D of *aggst1α* with the *A. gambiae* class I GST cDNA sequences gave a near-perfect match between the sequence of exon 3B and the 3′ region of *aggst1-5* (5/492 mismatched bases) and the sequence of exon 3D with the 3′ region of *aggst1-6* (2/492). As *aggst1-5* and *aggst1-6* are highly polymorphic (H.R., unpublished observation), these high levels of sequence identity suggest that the cDNAs *aggst1-5* and *aggst1-6* are produced by mRNA splicing within the *aggst1α* gene. If all four of the exons with homology to the 3′ region end of GST genes can be used, then alternative splicing between exon 2 and either exon 3A or exon 3C should produce additional transcripts.

To assess this possibility, a common forward primer complementary to *aggst1α* exon 2 was used with antisense primers specific to *aggst1α* exons 3A, 3B, 3C, or 3D in PCRs using cDNA extracted from ZAN/U larvae, pupae, or adults as templates. The location of these primers is shown in Fig. 2, and their sequence and expected size of product is given in Table 1. Fig. 3*A* shows that all four cDNAs were detected in all life stages of ZAN/U *A. gambiae*. The cDNAs amplified by primer sets P2/P3B and P2/P3D correspond to the previously published cDNAs *aggst1-5* and *aggst1-6,* respectively, whereas the cDNAs amplified by primer sets P2/P3A and P2/P3C, designated *aggst1–3* and *aggst1–4,* have not previously been characterized. The exon compositions of the four mature transcripts of *aggst1α* are summarized in Table 2.

Because the results shown in Fig. 3*A* do not preclude the interpretation that expression of the different transcripts is heterogeneous within the ZAN/U population, RNA was extracted from individual adult male and female mosquitoes and used as templates for reverse transcription–PCRs. All four alternatively spliced transcripts were detected in each individual, demonstrating that *agggst1-3, aggst1-4, aggst1-5,* and *aggst1-6* are simultaneously expressed (data not shown).

Confirmation of the Role of mRNA Splicing. To verify that the cDNAs described above were produced by splicing and were not transcribed from additional gene sequences elsewhere in the genome, the same primer sets were used in PCRs



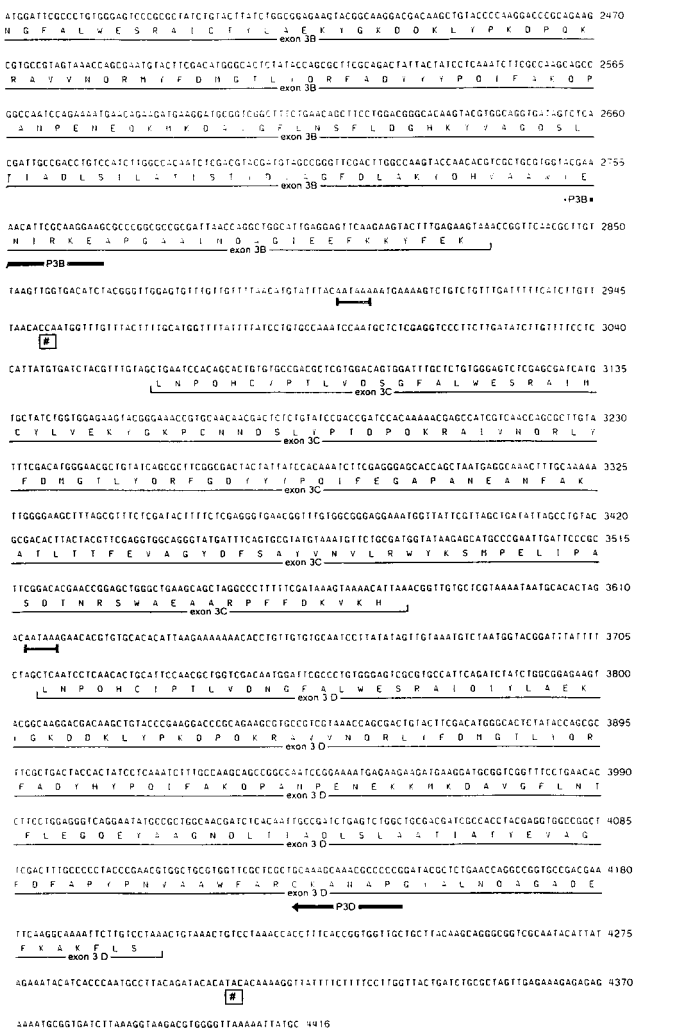


FIG. 2. Nucleotide sequence of the alternatively spliced *A. gambiae* class I GST gene, *aggst1α.* Primers designed from the sequence of the *An. gambiae* S2L*a* genomic clone, Ag_B1, were used to amplify the equivalent region of the genome from the DDT-resistant ZAN/U strain. The derived amino acid sequence of the five coding exons is shown below the nucleotide sequence. The location of the primers used to detect the splice variants are marked by arrows, putative polyadenylation sites are underscored, and the sites of addition of two of the poly(A) tails are marked with #.

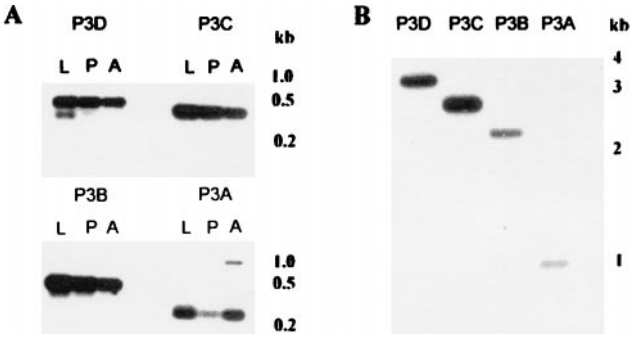


FIG. 3. PCR amplification of *aggst1α*. PCRs were performed on cDNAs from *A. gambiae* ZAN/U fourth-instar larvae (L), pupae (P), and 1-day-old adults (*A*) and *A. gambiae* genomic DNA (*B*) using the primer sets indicated. The PCR products were separated by gel electrophoresis and transferred to nylon membranes. The DNA was hybridized with a ^{32}P-labeled 5′ GST probe as described in *Methods*. The final posthybridization wash was at 65°C in 0.1× SSC and 0.1% (wt/vol) SDS.

with genomic DNA as the template. The expected size of genomic PCR product for each of the PCR sets was calculated from Fig. 2 and is shown in Table 1. With each primer set, a band of the expected size was obtained. Frequently, several additional PCR products were present, but they failed to hybridize to a 5′ class I GST probe (Fig. 3*B*).

Analysis of Nontranslated Regions and Exon Intron Boundaries. The nontranslated upstream sequences of *aggst1-5* and *aggst1-6* were amplified from DNA extracted from a cDNA library. The sequences obtained matched the published 5′ untranslated region sequence of the partial cDNA *aggst1-1,* which was obtained by 5′ RACE (14). Comparison of the cDNA sequences with the genomic sequence upstream of *aggst1α* revealed the presence of a 146-nt intron 24 nt upstream from the initiator ATG and identified an additional noncoding exon, exon 1, within *aggst1α* (Fig. 2). The exon-intron boundaries within *aggst1α* are compared with the consensus sequence in Table 2. As the distance from the 5′ splice site increases, the agreement of the 3′ splice site to the consensus sequence also increases.

Each of the four alternative 3′ exons is preceded by, and terminates in, an in-frame stop codon. The intragenic distances between them range from 130 nt (exons 3B and 3C) to 440 nt (exons 3A and 3B), and a putative polyadenylation sequence is located between each exon. The exact lengths of the 3′ nontranslated sequences of *aggst1-5* and *aggst1-6* as determined by 3′ RACE are 116 nt and 105 nt, respectively (Fig. 2).

Detection of an Additional GST cDNA by 3′ RACE PCR. A nested 3′ RACE PCR using forward primers specific to the putative 5′ GST exon of *aggst1β*, amplified a cDNA, designated *aggst1–7*, with high levels of sequence identity to the *A. gambiae* class I GST genes. No match to the 3′ sequence of this cDNA was found within the genomic clone Ag_B1, and attempts to amplify the gene from *A. gambiae* genomic DNA by using primer sets specific for the cDNA sequence *aggst1-7* were unsuccessful, suggesting that this gene is interrupted by one or more large introns.

An alignment of the predicted amino acid sequences of the six *A. gambiae* class I GSTs described in this study is shown in Fig. 4. The degree of similarity at the amino acid level ranges from 87.9% for *aggst1-5* vs. *aggst1-6* to 46.3% for *aggst1-2* vs. *aggst1-7* (Table 3).

Table 2. Exon composition and splice sites of alternative transcripts of the *A. gambiae aggst1α* gene

cDNA	Exon composition*	5′ splice site (exon/intron)	3′ splice site (intron/exon)
	Consensus	**AG/GTRAGT**	**Y_nNYAG/NN**
	exon 1 . . . 2	TG/GTGAGT	CCCCAG/AA
aggst1-3	exon 2 . . . 3A	AG/GTAGGT	AAAG/CT
aggst1-5	exon 2 . . . 3B		CCTAG/AT
aggst1-4	exon 2 . . . 3C		TTTGTAG/CT
aggst1-6	exon 2 . . . 3D		TTTTCTAG/CT

(R = A or G, Y = C or T, N = A, C, G or T).
*The presence of the noncoding exon, exon 1, has been confirmed in *aggst1-5* and *aggst1-6* only.

DISCUSSION

Genomic Organization of Class I GSTs. We have identified three cDNA clones that encode distinct *A. gambiae* GSTs. These cDNAs are in addition to the single class II GST and three class I GSTs (*aggst1-5, aggst1-6,* and *aggst1-2)* described previously (11, 14, 20). The additional sequences are classified as class I GSTs because they share 50–83% sequence identity with the previously characterized class I GSTs but are only distantly related to the class II GST (approximately 33% nucleotide identity). A serine near the N terminus is characteristic of mammalian theta GSTs and insect class I GSTs (24); this residue, which plays a vital role in the catalytic mechanism of the GSTs, is substituted by a tyrosine residue in all other GST classes (25). All of the *A. gambiae* class I GSTs sequenced to date possess this N-terminal serine (position 11 in Fig. 4).

Four of the six *A. gambiae* class I GSTs use a common 5′ exon, which is spliced to one of four alternative 3′ exons to produce mature transcripts. The translation products of these transcripts are therefore identical at the N termini (residues 1–45) but highly variable at the C termini. Crystal structures of mammalian and insect GSTs show that the majority of the active site residues involved in the binding and activation of GSH are found within the N terminal and hence this region of the protein is highly conserved between GSTs (24, 26). The divergence in the C-terminal domain confers the variation in substrate specificities of different GSTs. Therefore, the genomic organization of the *A. gambiae* GSTs provides a mechanism of increasing the diversity of GSTs produced and consequently expanding their substrate range, with a minimal increase in the length of the genome.

It is unlikely that the number of alternative 3′ exons within *aggst1α* exceeds the four reported here because no additional candidate sequences were found within the 2,285 nt of sequence separating the termination codon of the most distal 3′ exon, 3D, and the start of the intronless gene *aggst1-2*. However, the full extent of the *A. gambiae* class I GST family remains to be determined. Eight distinct fractions of GST activity were resolved by sequential column chromatography of *A. gambiae* homogenates, all of which contained multiple GSTs, which suggests that many more *A. gambiae* GST genes remain to be cloned (15). In this study we report the amplification of a single full-length GST transcript, *aggst1-7*, from an adult female mosquito, which we predict is produced by the splicing of exon 1 of *aggst1β* to one or more 3′ exons. We currently are sequencing overlapping genomic clones to investigate whether alternative splice variants of this gene also exist.

The organization of class I GSTs in *A. gambiae* contrasts with that in *M. domestica*. In this species multiple genes encode each class I GST, and additional loci code for fusion GSTs comprising the 5′ half of one GST gene joined to the 3′ half of a different GST gene (13). In addition, while the class I GST genes of *A. gambiae* and the intronless class I GST genes of *D. melanogaster* are tightly linked, the multiple loci that encode the housefly GSTs appear to be dispersed throughout the genome.

Choice of Splice Site. The factors determining the choice of a 3′ splice site remain to be determined. Different subsets of GSTs are responsible for DDT resistance in *A. gambiae* larvae and adults (27). We therefore investigated whether exon

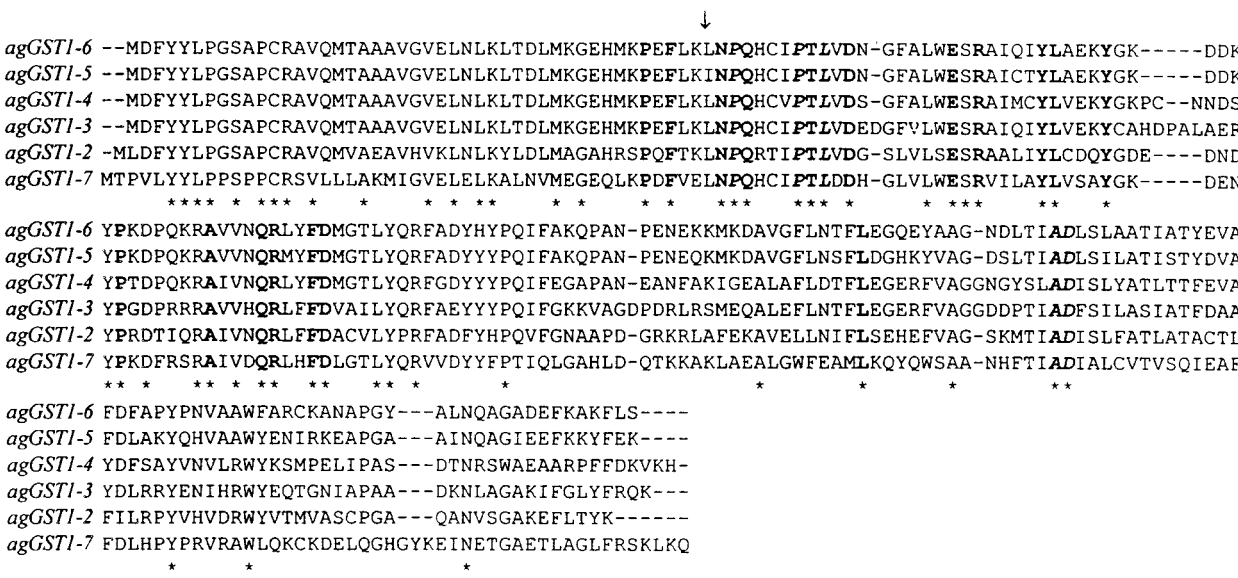


FIG. 4. Amino acid alignment of *A. gambiae* class I GST cDNAs. Gaps introduced to maximize sequence identity are shown by a horizontal dash. * indicates the presence of an identical residue in all six *A. gambiae* GSTs. Residues in bold are shared by all 27 Dipteran class I GSTs within GenBank. The five residues in bold italics are shared by all known insect GSTs. A vertical arrow marks the junction between exon 2 and exon 3 in *aggst1-3, aggst1-4, aggst1-5,* and *aggst1-6.*

choice was developmentally regulated, but the results of reverse transcription–PCR showed that all four splice variants, *aggst1-3, aggst1-4, aggst1-5,* and *aggst1-6,* are simultaneously expressed in fourth-instar larvae, pupae, and 1-day-old adults. Similarly, all exons are expressed in males and females. It seems likely that the choice of 3′ splice site is tissue dependent with specific factors regulating GST expression in each tissue.

Tissue-specific expression of GST isozymes is well documented in mammals (28), but little is known about the tissue distribution of insect GSTs. By using antisera raised against housefly GSTs, the class II enzyme, MdGST2, was shown to be expressed in the indirect flight muscles of the thorax and in the central nervous system, whereas the class I GSTs were uniformly distributed in the hemolymph cells (29). However, the anti-GST-1 antisera used in the *M. domestica* study was raised against biochemically purified class I GSTs (8), and the housefly class I GST subsequently was shown to be a complex enzyme family consisting of at least five independent subunits (13). Whether this antisera detects all members of this class has not been reported and hence the tissue distribution of insect class I GSTs remains unclear. It should now be possible to address this question for the *A. gambiae* class I GSTs, either by reverse transcription–PCR using the primer sets described in this study, or by expressing the various 3′ exons *in vitro* and raising specific antisera to the carboxyl termini of each GST isozyme that could be used in immunohistochemical studies to locate the GSTs *in vivo*.

The multiple substitutions within the C-terminal domain of the different *A. gambiae* class I GSTs are predicted to lead to variations in substrate specificities. We have shown this to be the case in an earlier report in which we expressed and characterized two members of this group (20). These GSTs, despite sharing the highest levels of sequence identity within the *A. gambiae* class I GST family, had markedly different catalytic properties. Further characterization of the substrate specificities, sites of expression, and regulatory mechanisms of the various *A. gambiae* class I GSTs will lead to a better understanding of the normal physiological role of these enzymes and their role in insecticide resistance.

Table 3. Pairwise distances between *A. gambiae* class I GST cDNAs

	aggst1-2	*aggst1-3*	*aggst1-4*	*aggst1-5*	*aggst1-6*	*aggst1-7*
aggst1-2		0.391	0.411	0.394	0.391	0.537
aggst1-3	0.432		0.284	0.267	0.269	0.503
aggst1-4	0.452	0.318		0.242	0.233	0.476
aggst1-5	0.457	0.293	0.310		0.121	0.468
aggst1-6	0.450	0.294	0.312	0.169		0.442
aggst1-7	0.505	0.464	0.471	0.452	0.430	

Mean amino acid character differences are shown above the diagonal, mean nucleotide character differences below the diagonal.

CONCLUSIONS

This study reports the involvement of alternative RNA splicing in generating multiple functional GST transcripts. Two alternatively spliced transcripts of a human mu GST have been isolated from a cDNA library, but both of these clones are incomplete and do not encode functional GSTs (30). The presence of this splicing mechanism contributes to the high level of GST class I variation reported in biochemical studies of *A. gambiae* insecticide resistance-associated GSTs. The class I GSTs of *M. domestica* and *D. melanogaster* are also highly variable and encoded by complex gene families but the genomic organization of the insect class I GST genes is dramatically different between the insect species. The variability of the insect class I GSTs is in sharp contrast to the class II GSTs, which are present as single genes in these three insect species.

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