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Cloning and sequencing of the cyclodiene insecticide resistance gene from the yellow fever mosquito *Aedes aegypti*

Conservation of the gene and resistance associated mutation with *Drosophila*

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In order to examine the conservation of the mechanism of cyclodiene insecticide resistance between species we cloned a cDNA from the yellow fever mosquito *Aedes aegypti* homologous to the resistance gene *Rdl* in *Drosophila*. In *D melanogaster*, resistance to cyclodienes and picrotoxinin is caused by a single amino acid substitution (alanine to serine) in the putative channel lining of a γ-aminobutyic acid gated chloride ion channel. We report that the mosquito gene not only shows high homology to that of *Drosophila* but also that resistant strains display substitution of the same amino acid. The significance of this result in relation to the evolution of pesticide resistance, the use of *Drosophila* as a model insect for resistance studies and the potential use of this gene as a selectable marker in the genetic transformation of non-Drosophilids is discussed

Cyclodienes; Insecticide resistance; Mutation; *Aedes aegypti*, *Drosophila melanogaster*

1. INTRODUCTION

There has been considerable recent debate over the number of genes involved in insecticide resistance. This discussion has focused on the relative importance of single or multiple genes (monogenic or polygenic control) in resistance mechanisms associated with control failures [1]. Resistance to cyclodiene insecticides has accounted for over 60% of reported cases of insecticide resistance [2] and is also found in vertebrates [3]. The nature and conservation of this resistance mechanism in different insect species is therefore not only highly relevant to this debate but is also of broad evolutionary importance. We have previously cloned the single gene responsible for cyclodiene resistance from *Drosophila melanogaster* [4] and identified the resistance associated mutation [5]. This species has been proposed as a genetic model for the cloning of insecticide resistance genes [6,7] but is not itself a major pest. Therefore we were further interested in comparing the molecular basis of cyclodiene resistance found in a major pest species with that in *Drosophila*, in order to validate the approach of using the latter insect as a genetic model for cloning resistance genes.

Cyclodiene resistance in *Drosophila* is caused by a single amino acid substitution in a γ-aminobutyric acid (GABA) receptor gene and substitutions at the same amino acid position are found in resistant *D. melanogaster* and *D. simulans* strains worldwide [5]. Site directed mutagenesis of this amino acid (Ala^{302}→Ser) and functional expression of the resulting GABA gated chloride ion channels in *Xenopus* oocytes has confirmed the functionality of the resistance associated mutation in *D. melanogaster* [8].

Here we report the cloning of an *Rdl* homolog from the yellow fever mosquito *Aedes aegypti*, via low stringency screening with the *Drosophila* probe. The mosquito homolog not only shows high identity to the *Drosophila* gene but resistant strains also show the same single base pair substitution as found in *D. melanogaster*.

2. MATERIALS AND METHODS

The mosquito cDNA clone (2.1.3) was isolated from a library made from RNA extracted from adult *Aedes aegypti*. RNA was polyA selected and size selected for messages greater than 2 kb. The cDNA library was constructed in the vector λGT10 by J. Williams, University of Wisconsin-Madison. The library was screened at low stringency (16 h hybridization at 50°C in 10% dextran sulfate, 0.5 M NaPO4, 5% SDS, 0.001 M EDTA and 36 μg/ml single stranded salmon sperm DNA; two washes at room temperature in 2 × SSC and 0.5% SDS for 5 min; two washes at 50°C in 2 × SSC and 1% SDS for 30 min) with a gel purified restriction fragment (0.76 kb *Eco*R1 fragment isolated in low melting temperature agarose (SeaPlaque) and radio-labelled with ^{32}P by primer extension) from the *Drosophila Rdl* clone which contains the first three of the highly conserved membrane spanning regions M1–3. DNA sequencing was carried out by the dideoxy chain termination method [9] using the Sequenase kit (United States Biochemical) and 18-mer oligodeoxyribonucleotides synthesized in an

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Applied Biosystem DNA synthesizer as primers. The predicted amino acid sequence of the mosquito cDNA was aligned and compared with that of *Drosophila* using the GAP program in the UW-GCG package [10] Northern blotting was performed with polyA selected RNA from adult mosquitoes according to standard procedures [11]. The resistance associated mutation was sequenced from the cyclodiene resistant strain Isla Verde (a kind gift of G. Craig, Notre-Dame), the Liverpool susceptible strain (a kind gift of B. Christensen, University of Wisconsin-Madison) was used for comparison. Products from the polymerase chain reaction (PCR) were cloned into the pCRII vector (Invitrogen) using the manufacturers instructions and sequenced [5]. The PCR primers used (ACF1 and ACR5) were predicted to be internal to exon 7 of the mosquito gene (Fig 1) by analogy with the genomic organization of the *Drosophila* gene [12].

3. RESULTS

3.1. *Sequence and message size of the mosquito* Rdl *homolog*

A restriction map of the mosquito clone 2.1.3 and the strategy used to sequence it are shown in Fig. 1. The predicted amino acid sequence of the mosquito cDNA is compared to that of *D. melanogaster Rdl* in Fig. 2. The two sequences show 87% identity. The two locations in the extracellular domain which show alternative splicing of two exons of equal size in *Drosophila* (termed 'a' or 'b', and 'c' or 'd'), carry sequence nearly identical to *Drosophila* exons a and c [13]. The sequence of the membrane spanning regions (M1–4) is almost identical with only one conservative amino acid substitution in M1. The sequence of the presumed extracellular domain is also nearly identical with only four substitutions. In contrast the presumed intracellular domain is much shorter in the mosquito. Although this domain still possesses repetitive strings of glycines and prolines there are fewer than in *Drosophila*. However, some repetitive motifs involving glycine/histidine and glycine/proline (*Aedes* amino acids 389–403 and 446–461 respectively) are conserved between *Drosophila* and *Aedes* suggesting they may be functional. A number of roles have been proposed for such glycine repeats including 'hinge regions' connecting two domains of a protein and regions involved in protein–protein interactions, as in the *Drosophila* genes for *Ultrabithorax* [14] and the pupal cuticilar protein EDG 91 [15], respectively. Interestingly of the three potential methionine start sites in *Drosophila* the only methionine at the start of the mosquito sequence occupies approximately the same position as the second in *Drosophila*, indicating that this may be the functional initiator of translation in the latter.

Northern analysis of polyA selected RNA from adult mosquitoes showed a transcript size of ~ 10 kb. This is consistent with the large transcript size we have observed for *Drosophila Rdl* of ~ 8 kb (our unpublished observations).

3.2. *Resistance associated mutation*

PCR based sequencing of the second membrane spanning region of the *Rdl* homolog from the resistant mosquito strain showed a single base pair substitution of a G to a T at nucleotide position 885, thus replacing an alanine (GCA) with a serine (TCA) (Fig. 3). This is exactly the same mutation as found in resistant *D. melanogaster* which was proved to be functionally involved in conferring insensitivity of expressed chloride ion channels to picrotoxinin and the cyclodiene, dieldrin [8].

4. DISCUSSION

Recent mapping of the *Aedes aegypti* genome via restriction fragment length polymorphisms has shown that the gene described here is located 23 units away from *spot* on linkage group 2 [16]. This is consistent with the previous estimation of 25–31 map units derived by measuring recombination between visible markers [17]. Together with the high amino acid identity to the *Drosophila* gene this confirms that the mosquito gene described here is an *Rdl* homolog, mutants of which confer resistance to cyclodiene insecticides.

The finding that the mutation conferring cyclodiene resistance is conserved between *Drosophila* and mosquitoes has broad implications concerning (1) ion channel receptor structure/function, (2) the genetic basis of insecticide resistance, (3) the use of *Drosophila* as a model insect and (4) the potential use of the resistance gene as

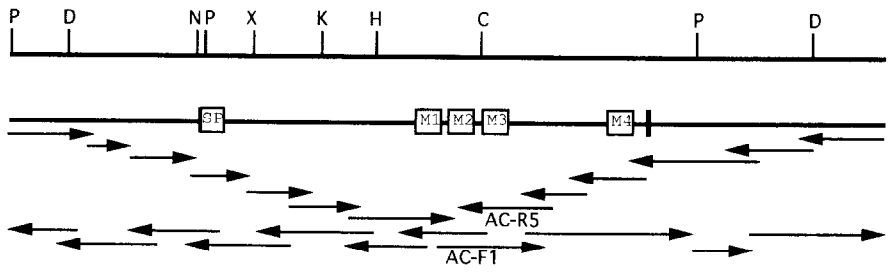

Fig. 1. Analysis of the cDNA clone 2.1.3 A schematic diagram of the predicted amino acid sequence is given showing the position of the signal peptide (SP) and the four predicted membrane spanning hydrophobic sequences (M1–4). Above is shown a restriction map P, *Pst*I; D, *Dra*I; N, *Nar*I; X, *Xba*I; K, *Kpn*I; H, *Hin*dIII; C, *Cla*I. Below the positions of the individual sequencing runs on both strands of the DNA are shown by arrows. The location of sequences derived from the PCR primers (AC-F1 and AC-R5) used in amplification of the region containing the resistance associated mutation are also given.

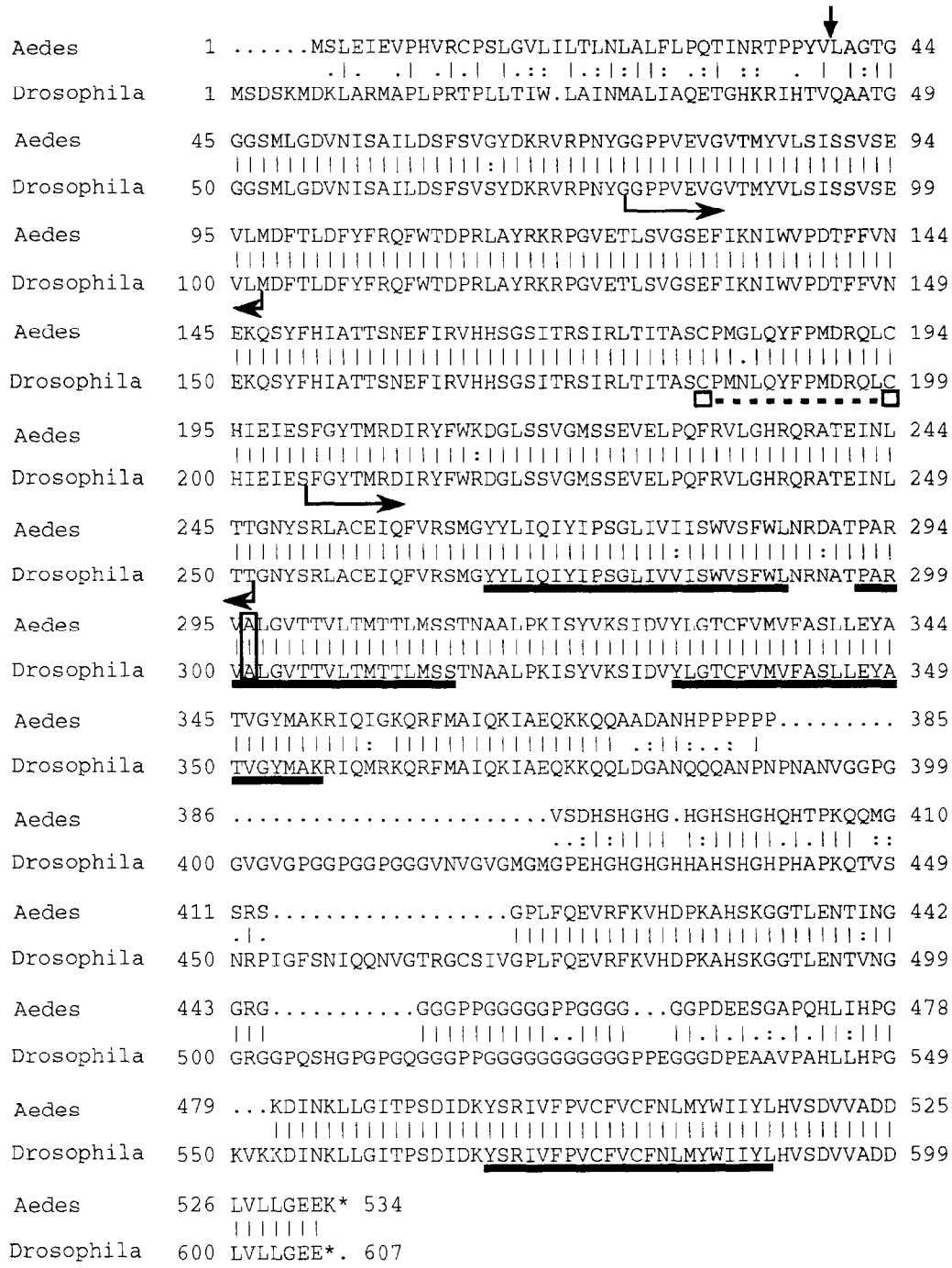

Fig. 2. Comparison of the predicted amino acid sequence of the *Drosophila* cyclodiene insecticide resistance gene *Rdl* with its homolog from the *Aedes aegypti* clone 2.1.3. The alanine residue substituted by a serine in the resistant strain is boxed. The signal cleavage site predicted from alignment of *Rdl* with vertebrate GABA receptors is shown by an arrow above the sequence. The two locations showing alternative splicing of two exons of the same size (a or b, and c or d) are indicated by arrows below the sequence. The proposed membrane-spanning hydrophobic sequences are indicated by solid bars and the β structural loop flanked by cysteines is indicated by a broken line. Vertical lines indicate amino acid identity, single and double dots between residues indicate conservative changes and dots within the sequence indicate gaps.

a selectable marker in genetic transformation. The resulting amino acid substitution of an alanine for serine in the second membrane spanning region, the region thought to line the ion channel pore of the closely related nicotinic acetylcholine receptor [18], does not introduce any change in net charge but alters polarity via the addition of a hydroxyl group. This may result in steric or electrostatic hinderance of cyclodienes with their binding site in the channel pore. The conservation of this mutation between widely separated species of

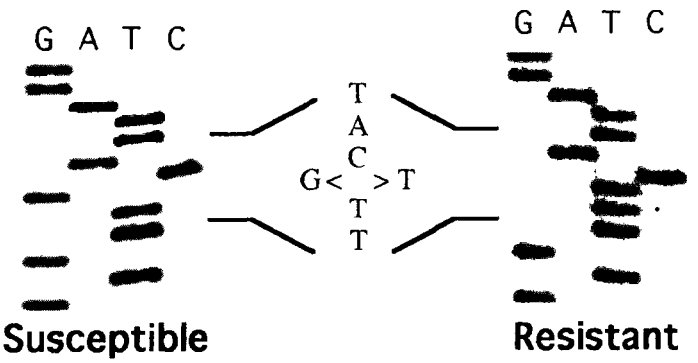

Fig. 3. Sequencing of PCR derived clones from cyclodiene susceptible and resistant *Aedes aegypti* A single nucleotide exchange (G to T) at position 908 in exon 7 is associated with resistance and causes the substitution of an alanine (GCA) with a serine (TCA) in the predicted lining of the ion channel

diptera suggests that the number of viable resistance mutations in this important functional region of the protein may therefore be extremely limited. We will investigate the effect of other mutations in this region via site directed mutagenesis and expression in *Xenopus* oocytes.

In relation to the evolution of pesticide resistance, the conservation of not only the resistance gene but also of the precise mutation, in this widespread form of insecticide resistance, strongly argues for the importance of single major genes. We will therefore proceed to examine this region of *Rdl* in other cyclodiene resistant invertebrates, and the vertebrate mosquitofish *Gambusia affinis*, in order to confirm our prediction that conservation of this resistance mechanism is widespread. Further, this similarity vindicates the applicability of using *Drosophila* as a model insect [6] for the cloning of insecticide resistance genes despite its lack of pest status.

Finally, the conservation of this receptor between insects may facilitate its use as a selectable marker in the genetic transformation of non-Drosophilid insects as previously suggested [4]. The semi-dominant nature of cyclodiene resistance allows for ready selection of an inserted susceptible or resistant copy of the gene and thus makes it an ideal marker for genetic transformation. Therefore we are building a mini-gene construct, containing the mosquito *Rdl* cDNA and the 5'-flanking genomic DNA containing the putative promoter, for use in attempts to genetically transform mosquitoes.

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REFERENCES

[1] Roush, R.T and Tabashnik, B.E (1990) in: Pesticide Resistance in Arthropods, Chapman and Hall, New York.
[2] Georghiou, G P. (1986) in: Pesticide Resistance Strategies and Tactics for Management (National Academy of Sciences, Eds.) pp. 14–43. National Academy Press. Washington DC.
[3] Yarbrough, J D., Roush, R.T., Bonner, J.C and Wise, D.A. (1986) Experientia 42, 851–853.
[4] ffrench-Constant, R.R., Mortlock, D.P., Shaffer, C.D., MacIntyre, R.J and Roush, R T. (1991) Proc Natl Acad. Sci. USA 88, 7209-7213.
[5] ffrench-Constant, R R., Steichen, J., Rocheleau, T.A., Aronstein, K and Roush, R.T. (1993) Proc Natl. Acad. Sci USA 90, 1957-1961.
[6] Wilson, T G. (1988) J Econ Entomol. 81, 22–27.
[7] ffrench-Constant, R R., Roush, R.T and Carino, F. (1992) in Molecular Approaches to Pure and Applied Entomology (M.J Whitten and J G. Oakeshott, Eds.) pp. 1–37, Springer, Berlin.
[8] ffrench-Constant, R.R., Rocheleau, T.A., Steichen, J C. and Chalmers, A.E (1993) Nature 363, in press.
[9] Sanger, F., Nicklen, S. and Coulson, A R. (1977) Proc Natl. Acad Sci USA 74, 5463–5467.
[10] Devereux, J R., Haeberli, P and Smithies, O. (1984) Nucleic Acids Res 12, 387–395
[11] Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) in: Molecular Cloning. A Laboratory Manual., Cold Spring Harbor Lab., Cold Spring Harbor, NY
[12] ffrench-Constant, R R. and Rocheleau, T (1992) J. Neurochem 59, 1562–1565.
[13] ffrench-Constant, R.H and Rocheleau, T (1993) J. Neurochem 60, in press.
[14] Beachy, P.A., Helfand, S.L and Hogness, D.S. (1985) Nature 313, 545–551
[15] Apple, R.T and Fristrom, J W. (1991) Dev Biol. 146, 569–582.
[16] Severson, D.W., Mori, A., Zhang, Y and Christensen, B.M (1993) J. Heredity, in press
[17] Lockhart, W.L., Klassen, W. and Brown, A W.A. (1970) Can. J. Genet. Cytol. 12, 407 414.
[18] Leonard, R J., Labarca, C.G., Charnet, P., Davidson, N. and Lester, H.A. (1988) Science 242, 1578–1581.