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DNA sequence and site of mutation of the GABA receptor of cyclodiene-resistant red flour beetle, *Tribolium castaneum*

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Using polymerase chain reactions (PCR) on cDNA, the DNA sequence of a membrane spanning region of a GABA receptor of the red flour beetle, *Tribolium castaneum* was identified. The deduced amino acid sequence indicates that its basic structure is similar to the GABA receptor of *Rdl* type subunits of *Drosophila melanogaster* and of *Blattella germanica*. Particularly conserved are M_1, M_2 and M_3 segments. Within this 146 amino acid stretch, the GABA receptor from the red flour beetle differed from corresponding ones from *Drosophila* and *Rdl* subunit of *B. germanica* by 12 and eight amino acids, respectively. By using an identical approach, the corresponding DNA region was sequenced from the cDNA of a cyclodiene-resistant strain of *T. castaneum*. While two points of mutation were found only one mutation in DNA was found to result in an amino acid shift. The site of mutation was at the 5th amino acid of the M_2 cylinder where G to T conversion of the GCT codon resulted in a conversion of alanine to serine. This is qualitatively the same mutational switch of alanine to serine in resistant strains previously reported to have occurred in cyclodiene-resistant *Drosophila melanogaster*, *Aedes aegypti* and *Blattella germanica*, indicating that this amino acid change is the likely cause for evolution of the nerve insensitive type of resistance to cyclodiene insecticides.

Key words: DNA sequence; Mutation site; GABA receptor; *Tribolium castaneum*.

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Introduction

It is well known that cyclodiene resistance is a commonly occurring phenomenon among insect species particularly in those areas where any cyclodiene-type insecticides have been used for pest control in the past (Brown, 1960; Georghiou, 1986). At an early stage it has been shown that such a resistant trait depends on a major genetic factor (see Brown, 1960), and that the nervous system of the resistant strains of insects are more tolerant to the action of cyclodienes (i.e. resistance due to the 'target' insensitivities) (see Matsumura, 1987).

The idea that the GABA receptor itself is the target of these cyclodiene insecticides was initially proposed by Ghiasuddin and Matsumura (1982) and Matsumura and Ghiasuddin (1983) and later confirmed by others (see Lawrence and Casida, 1983; Eldefrawi *et al.*, 1986; Tanaka and Matsumura, 1986; Thompson *et al.*, 1993). Biochemical evidence that the resistant GABA receptors in intact neurons from the resistant insects bind less cyclodienes and are affected less than the susceptible counterparts has been obtained in several laboratories (Matsumura and Hayashi, 1966;

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Deng and Casida, 1991; ffrench-Constant *et al.*, 1993).

Recently, large portions of the sequences of the GABA receptors in three insect species have been reported including *Aedes aegypti*, (Thompson *et al.*, 1993a). The initial and complete identification of one type of GABA receptor was made by ffrench-Constant *et al.* (1991) from a DNA clone obtained from a dieldrin-resistant *Drosophila melanogaster*. This type has been designated as *Rdl*. Subsequently, Henderson *et al.* (1993) reported another type of GABA receptor from a DNA clone obtained from the same species which was named at LCCH3 belonging to a β-subunit type in comparison to well-studied mammalian subunits. Matsumura *et al.* (1993) and Kaku and Matsumura (1994) have used a polymerase chain reaction approach and sequenced the membrane spanning region of the GABA receptors from the German cockroach, *Blattella germanica*. With regard to the site of mutation in cyclodiene-resistant insects, four reports are now available indicating that the alanine to serine conversion of the 5th amino acid of the M_2 cylinder causes resistance (Ala 302 in *Rdl* terminology in *Drosophila*) (ffrench-Constant *et al.*, 1993; Thompson *et al.*, 1993a; Matsumura *et al.*, 1993). Recently, Thompson *et al.* (1993b) compared the DNA sequences of a 16 amino acid stretch of the M_2 region from five species including that of *T. castaneum* and found that in four cases the same alanine to serine conversion took place and in one case an alanine to glycine conversion was noted.

The current study was initiated to investigate whether such a mutation is the common and basic mechanism of cyclodiene resistance, and to understand comparative characteristics of the GABA receptors in a coleopteran insect.

Materials and Methods

The methods of rearing *Tribolium castaneum*, selection with dieldrin and the *in vivo* susceptibility tests have been described previously (Beeman and Stuart, 1990). These two strains were transferred to Davis California in 1992 and the resistant strain was since selected twice using dieldrin (1000 ppm mixed in feed) to maintain its level of resistance.

Isolation of Poly A$^+$ mRNA

The method used here was basically that of Bradley *et al.* (1988). Exactly 3 g of whole tissues of adult red flour beetles (susceptible and resistant strain) were ground in liquid nitrogen using a mortar with a pestle. The slurries were transferred into 50 ml cell culture tubes. A 30 ml aliquot of lysis buffer [0.2 M NaCl, 0.2 M Tris–HCl (pH 7.5), 1.5 mM $MgCl_2$, 2% SDS, 200 μg/ml Proteinase K (Boehringer Mannheim) in DEPC-treated H_2O] was added, followed by immediate homogenization using Polyton® for 30 sec. The homogenates were incubated at 45°C for 2 hr with agitation. The residue in the lysate was separated by centrifugation at 3000 rpm for 5 sec, and the supernatant was transferred into a sterile 50 ml cell culture tube. The NaCl concentration of the lysate (0.2 M) was adjusted to that of 'binding buffer' (0.5 M NaCl) with 60 μl of 5 M NaCl in the same buffer per ml lysate. Oligo (dT) cellulose (80 mg) (GIBCO BRL/Life Technologies, Gaitherburg, MD), which was equilibrated to the same concentration of binding buffer [0.5 M NaCl, 0.01 M Tris–HCl (pH 7.5)] was mixed with the lysate, followed by incubation for 1 hr at room temperature with agitation. Poly A$^+$ mRNA bound oligo (dT) cellulose was washed with the binding buffer (first with 20 ml, then four times with 10 ml). The cellulose was transferred to a sterile disposable column (BioRad, Richmond, CA), and washed three times with 10 ml of binding buffer. Poly A$^+$ mRNA was eluted with 1.3 ml DEPC-treated H_2O into polypropylene tube (# 2063 Falcon), and the eluate was kept with 2 vol. of 100% ethanol and 0.1 vol. of 3 M sodium acetate at 80°C for 30 min. The precipitated poly A$^+$ mRNA was pelleted by centrifugation at 10,000 ***g*** for 20 min, washed with ice-cold 75% ethanol, vacuum-dried for 10 min and dissolved in 50 μl of DEPC-treated H_2O. Yields of extracted poly A$^+$ mRNA were 71.3 μg (OD_{260}/OD_{280} ration = 1.60) from the susceptible strain(S) and 63.3 μg (OD_{260}/OD_{280} ration = 1.64) from the resistant strain (R).

Preparation of cDNA

The basic methods adopted here are the ones developed and used by Kaku and Matsumura (1994). Briefly, mRNAs [39.9 μg (S), 39.2 μg (R)] were reverse transcribed into first strand cDNAs in a 200 μl of reaction containing 20 mM Tris–HCl (pH 8.4 at 25°C), 50 mM KCl, 5 mM $MgCl_2$, 0.01% gelatin (wt/vol.), 1 mM deoxynucleotide triphosphates (Pharmacia LKB), 200 units RNAs in (cloned, Promega), 3000 units M-MLV Reverse transcriptase (GIBCO BRL/Life Technologies) and 1 μg oligo $(dT)_{17}$-adapter primer (5′-GACTC-GAGTCGACATCGA$(T)_{17}$-3′). The reaction mixture was incubated at room temperature for 15 min, followed by incubation at 37°C for 1 hr and 95°C for 5 min. The mixture was quickly chilled on ice and stored at −20°C until further use.

Preparation of double stranded DNA by PCR

Sequences for PCR primers were chosen using sequences from highly conserved regions of GABA gene for *Drosophila* (ffrench-Constant *et al.*, 1991), German cockroach (Kaku and Matsumura, 1994) and other species (Schofield *et al.*, 1987; Pritchett *et al.*, 1989; Ymer *et al.*, 1989; Bateson *et al.*, 1990; Harvey *et al.*, 1990). All primers were synthesized on Model 391 DNA Synthesizer (Applied Biosystems, Foster City, CA) and purified by gel filtration with Sephadex-50. The gene map and strategies used for identifying this portion of the GABA receptor are shown in Fig. 1.

Each template DNA mixture was combined in a 50 μl reaction mixture with 5 μl 10 × PCR buffer [100 mM Tris–HCl (pH 9.0 at 25°C), 500 mM KCl, 1.5 mM $MgCl_2$, 1% Triton X-100], 0.2 mM deoxynucleotide triphosphates (Pharmacia LKB Biotechnology), 1 unit tag DNA polymerase (Promega), and the same amount of both upstream and downstream primers. The mixture was overlaid with mineral oil. PCR amplification cycles used were: first round PCR: 94°C 1 min, 50°C 2 min, 72°C 3 min, 40 cycles; subsequent round PCR: 94°C 1 min, 50°C 2 min, 72°C 2 min, 40 cycles in a DNA Thermal Cycler (Precision Scientific, Chicago, IL). Each amplified product (10 μl)

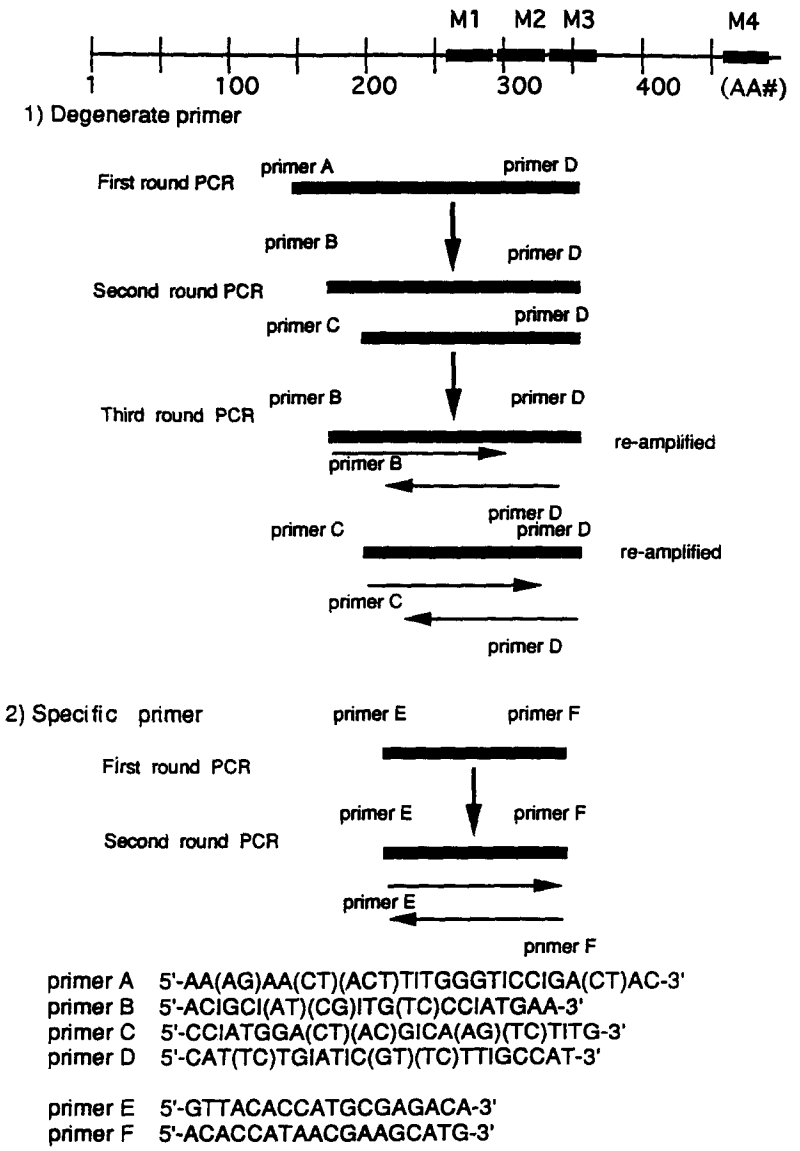

Fig. 1. The gene map of the GABA receptor of red flour beetle strategies used for its identification through polymerase chain reactions (PCR).

was run on a 2% agarose gel (FMC BioProducts, Rockland, ME) made with 1 × TBE (0.45 M Tris–HCl, 0.45 M boric acid, 10 mM EDTA) and stained with ethidium bromide. The desired PCR fragments were excised from the agarose gel and cut to pieces by using a sterile blade, rinsed by ddH_2O three times, frozen and thawed in 100 μl ddH_2O (repeated twice), and left in a refrigerator overnight. In the final round of PCR, the eluted DNA solution of six batches was reamplified in a 150 μl reaction mixture per tube to prepare the sample for DNA sequencing reaction. The mass-produced PCR solution was collected in a 1.5 ml microcentrifuge tube and mineral oil was removed with chloroform. The aqueous layer was concentrated to approximately 50 μl by Centricon-100® (Amicon) at 1000 *g* for 30 min at 4–10°C. The concentrated DNA fragment was electrophoresed on 1% agarose gel made with 1 × TBE and excised from gel, followed by purification by using QIAEX matrix (QIAEN) according to the manufacturer's protocol. The basic strategies for identification of GABA receptor gene of red flour beetle, template DNA and primer types and quality are shown in Fig. 1 and Table 1, respectively.

Sequenase® Version 2.0 system (United States Biochemical, Cleveland, OH) and DNA polymerase 1 (Klenow fragment) (Promega) were used for DNA sequencing reaction. DNA template-primer solution was made up to 10 μl volume including the purified double stranded DNA (approximately 1.4–3.4 pmol), the primer (20 times more DNA for a specific primer or 20 times each more DNA for degenerate primer) and 2 μl of 5 × sequenase buffer [200 mM Tris–HCl (pH 7.5), 100 mM $MgCl_2$, 250 mM NaCl] which was heated at 95°C for 3 min and quickly cooled in a dry ice–ethanol bath. To the denatured mixture, 3.5 μl of pre-mixed labeling solution [1.0 μl DTT (0.1 M), 2.0 μl 5 × diluted 'labeling mix' (dITP system), 0.5 μl [α-^{35}S]dATP (Du Pont NEN, Boston, MA, 5.25 μCi of 1100–1300 Ci/mmol], and 2 μl of enzyme dilution buffer [10 mM Tris–HCl (pH 7.5), 5 mM DTT, 0.5 mg/ml BSA], containing sequenase 2.0 (3.25 units) and pyrophosphatase (0.0005 units), were added, followed by incubation for 2 min at room temperature. Portions (3.5 μl) of this reaction mixture were added to 2.5 μl of each of the prewarmed (37°C 1 min) 'termination mix' (ddATP, ddCTP, ddGTP and ddTTP dITP system) and the mixture was further incubated at 37°C for 3 min, followed by the addition of 1 μl of Klenow fragment (0.25 units in enzyme dilution buffer), and incubated for 3 min at 37°C. The reaction was stopped by the addition of 4 μl of the stop solution (95% formamide, 20 mM EDTA, 0.05% Bromophenol Blue, 0.05% Xylene Cyanol FF). Prior to electrophoresis, the samples were denatured at 75°C for 3 min.

Electrophoresis using 1 × TBE system was performed for 1.75 and 4.75 hr at 60 W on 7.4 M urea/6% Long Ranger® gel (AT Biochem, Malvern, PA) (42 × 36 × 0.02 cm) with STS-45 Gel Electrophoresis Unit (IBI, New Haven, CT). The gel was dried at 80°C for 1.5 hr on Whatman 3 MM paper under vacuum and exposed to Kodak X-ray film (Rochester, NY).

Results and Discussion

The identified DNA and deduced amino acid sequence of this region of the *Tribolium* GABA receptor (Fig. 2) indicate that this receptor subunit is basically the same type found in *Drosophila melanogaster* (*Rdl* type) and the German cockroach *Blattella germanica*. It belongs to the *Rdl* subunit group which appears to be the case with other subunits isolated from several insects (ffrench-Constant *et al.*, 1991; Thompson *et al.*, 1993a; Kaku and Matsumura, 1994) and it somewhat differs from the β subunit type isolated from *Drosophila* (Henderson *et al.*, 1993) and a snail species *Lymnae stagnalis* (Harvey *et al.*, 1991).

Table 1. Description of template DNA, the types and the quantities of primers used for PCR runs

Entry	Primer	Template DNA
(Degenerate primer)		
1. First round PCR	0.5 M each (Primer A and D) 0.2 M each (Primer B and D)	2 μl of cDNA [50 μl] (Susceptible strain)
2. Second round PCR	(Primer C and D)	2 μl of eluted solution [50 μl]
3. Third round PCR	0.2 M each (Primer B and D) (Primer C and D)	4.5 μl of eluted solution [150 μl]
(Specific primer)		
4. First round PCR	0.2 M (Primer E and F)	1 μl of cDNA [50 μl] Susceptible and Resistant strain
5. Second round PCR	0.2 M (Primer E and F)	3 μl of eluted solution [150 μl]

The total amount per tube of PCR are indicated in brackets. The designation of primers and template DNA used for each PCR run are indicated in the parentheses.

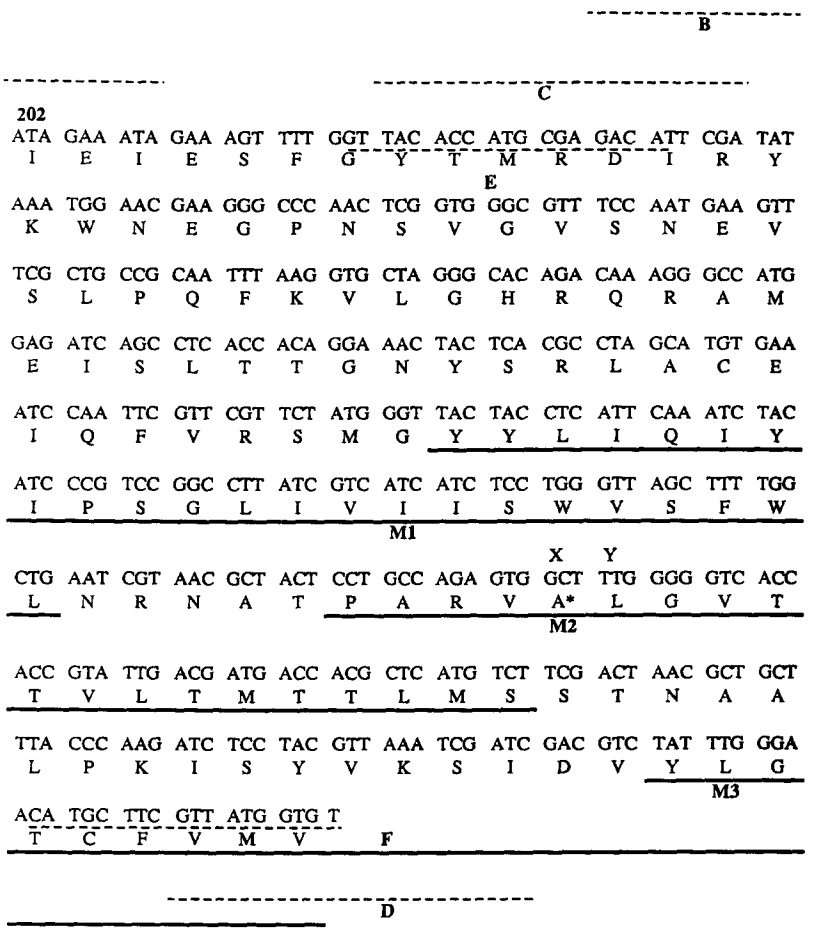

Fig. 2. The DNA and deduced amino acid sequence of the GABA receptor subunit of the red flour beetle in the susceptible strain. The numbers at the beginning indicate the corresponding amino acid number of *Drosophila Rdl* subunit (ffrench-Constant *et al.*, 1991). The site of mutation is indicated by an asterisk. The DNA regions corresponding to PCR primers are shown by dotted lines with alphabetical designations directly below the region. X and Y indicate T and C in the case of resistant DNA sequence, respectively. The putative membrane-spanning regions are indicated with horizontal lines.

Comparison of the amino acid sequence of *Tribolium* receptor to three other insect GABA receptor sequences and one rat brain beta subunit (Fig. 3) indicate that these insect subunits are relatively similar among themselves but as a group distinctly separated from the mammalian counterpart. The *Tribolium* subunit is more closely aligned to the *Rdl* subunits of the German cockroach and *Rdl* subunit of *Drosophila*. Within this range, the difference between *Tribolium* and *Blattella* was eight amino acids (out of 121) and that between *Tribolium* and *Rdl* of *Drosophila* was 12. The other β-subunit of *Drosophila*, LCCH3, appears to differ considerably from the above three.

With regard to the nature of mutation in the resistant *Tribolium*, the fact that this is qualitatively the same conversion (alanine to serine) at the same location clearly supports the view that this is the site of mutation that confers the reduced susceptibility to these insects specifically to all cyclodiene-type insecticides and picrotoxinin. Interestingly, the nature of the base substitution is somewhat different. Another difference between these two species is that despite the use of almost identical PCR approaches in the former, we did not pick up any other variation of subunits unlike the case with the latter species.

The results of comparison between *T. castaneum* of DNA sequences for the 48 base region published by Thompson *et al.* (1993b) and those of the current study indicate that the sequences of the susceptible strain were very similar except at one amino acid position (theirs NATLARVALG and ours NATPARVALG) where theirs was CTC and ours was CCT. On the other hand, the sequence of their resistant DNA strain

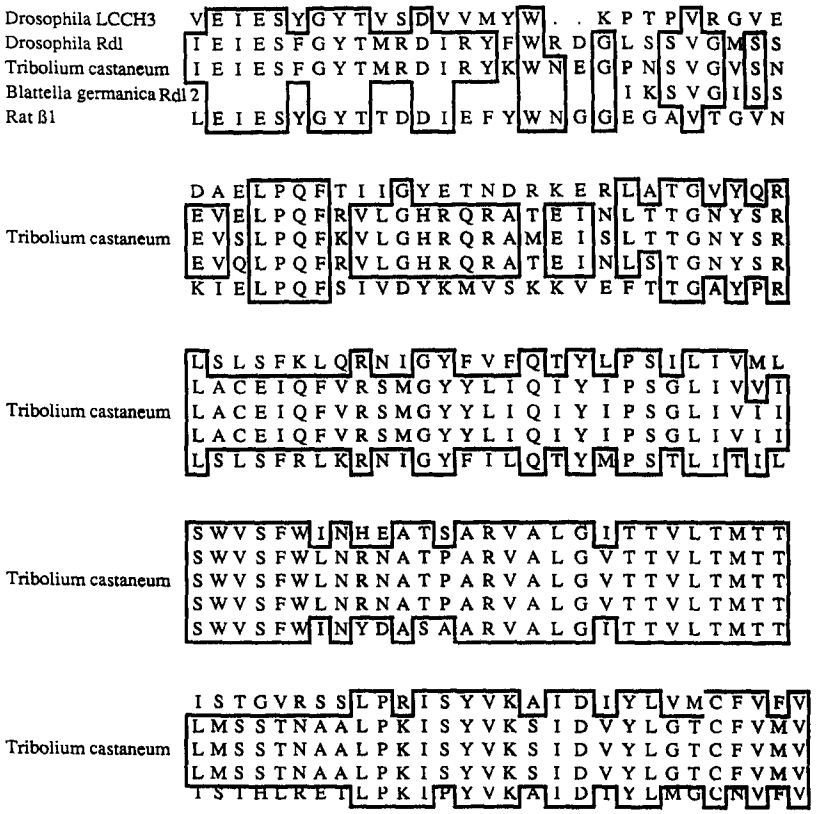

Fig. 3. Comparison of *Drosophila LCCH3* (Henderson *et al.*, 1993), *Drosophila Rdl* (ffrench-Constant *et al.*, 1991), *Blattela germanica Rdl*[2] and Rat $\beta 1$ (Ymer *et al.*, 1989) amino acid sequences with amino acid sequence from cDNA isolated from red flour beetle, *Tribolium castaneum*. Identical amino acids are enclosed by solid lines.

was quite different from ours (10–11 bases out of 48 bases). In fact their resistant strain differs from their susceptible one (by 12–13 bases). It is possible that there are more than one type of *Rdl* subunits or a large variation within populations in this species. A comparison of the DNA sequence of this same stretch among susceptible *T. castaneum* (T.), *Aedes aegypti* (A.) (Thompson *et al.*, 1993a) and *Drosophila* (D.) indicates that the number of bases different are 11 for T. vs D., 13 for T. vs A. and eight for A. vs D.

As to the functional meaning of this replacement of alanine with serine, we note the existence of a few mammalian subunits with the same serine substitution at this site (Stephenson,

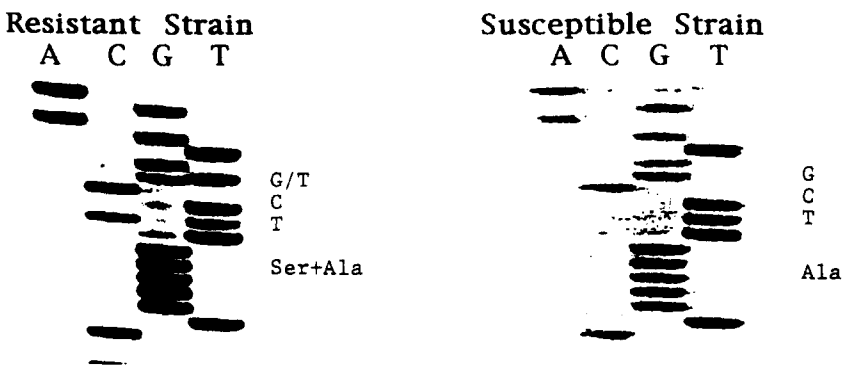

Fig. 4. Examples of DNA-sequencing gel showing the site of mutation in the resistant strain in comparison with the susceptible counterpart.

1988; Shivers *et al.*, 1989; Harrey *et al.*, 1991). Therefore, it is safe to assume that such a substitution is not likely to cause totally dysfunctional GABA receptors. It also agrees with the data provided by ffrench-Constant *et al.* (1993) who used the *Xenopus* expression system to show that the resistant *Rdl* type subunit assembly is basically functional, but differs from the susceptible counterpart in showing a reduced sensitivity to picrotoxinin and dieldrin. There are other pieces of evidence indicating that this M2 region is likely the target of picrotoxinin type chemicals in analogy to the case of inhibitory glycine receptors (Betz, 1992; Pribilla *et al.*, 1992). Furthermore, it appears that the M2 cylinders are likely to constitute the inner wall of the chloride channel proper when these subunits are assembled in a pentamer formation (Olsen and Tobin, 1990).

Regarding the issue of the reliability of PCR technologies using Taq polymerase, the basic error prone nature of this approach has also been considered by us. This is the reason why we used independent triplicates of each PCR run on each cDNA preparation. In all cases the results were consistent indicating no discrepancy within the region we sequenced. Having examined the same regions with different combinations of primers and confirming the identical sequence reading, we are confident that the sequence presented here is the correct one.

In conclusion, the current study results confirm the importance of this mutation in the phenotypic expression of insect resistance to cyclodiene type insecticides. Furthermore, the GABA receptor subunit found in *Tribolium castaneum* is most closely aligned to the *Rdl* subunits of *Blattella*.

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