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Possible involvement of the long terminal repeat of transposable element *17.6* in regulating expression of an insecticide resistance-associated P450 gene in *Drosophila*

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ABSTRACT P450-A and P450-B are electrophoretically defined subsets of cytochrome P450 enzymes in *Drosophila melanogaster*. P450-A is present among all strains tested, whereas expression of P450-B is associated with resistance to insecticides. Monoclonal antibodies were used to obtain cDNA clones for an enzyme from each P450 subset (i.e., P450-A1 and P450-B1). The P450-B1 cDNA was sequenced and shown to code for a P450 of 507 amino acids. Its gene has been named *CYP6A2*. Comparative molecular analyses of a pair of susceptible, 91-C, and resistant, 91-R, *Drosophila* strains were made. There was 20–30 times more P450-B1 mRNA in 91-R than in 91-C, and the small amount of P450-B1 mRNA in 91-C was significantly larger in size than that in 91-R. The P450-B1 gene in 91-R was structurally different from that in 91-C but was not amplified. The P450-B1 gene in 91-C contained a solitary long terminal repeat of transposable element *17.6* in its 3' untranslated region. It was absent in the P450-B1 gene of 91-R. On the basis of features of the long terminal repeat and its location in the gene of the susceptible fly, we propose that a posttranscriptional mechanism involving mRNA stability could be involved in regulating P450-B1 gene expression.

Metabolism by cytochrome P450 monooxygenases is a major mechanism by which insects resist the toxic effects of insecticides (1, 2). The amounts of total P450 and/or specific enzymes are generally greater in resistant insects than in their susceptible counterparts. However, the molecular mechanisms by which the levels of resistance-associated P450s are regulated are not known.

Unique features of its P450s make *Drosophila melanogaster* a useful model system for studying the molecular biology of P450-dependent insecticide resistance. *Drosophila* P450s are resolved by SDS/PAGE into two subsets. P450-A (≈59 kDa) is ubiquitous and is present in approximately equal amounts among strains tested, whereas the amount of P450-B (≈56 kDa) is much higher in resistant than susceptible strains (3–5). Putative structural and regulatory genes involved in P450-B expression are located on chromosomes 2 and 3 at, or near, major insecticide resistance loci (4). Monoclonal antibodies (mAbs) specific for an enzyme from each of the two P450 subsets (the enzymes are designated P450-A1 and P450-B1) have been produced and characterized (5). These mAbs have been used to isolate cDNA clones specific for P450-A1 and P450-B1. Characterization of these clones§ and their use as molecular probes to monitor P450-B1 gene expression and organization in the control, susceptible (91-C) *Drosophila* strain and the resistant (91-R) *Drosophila* strain are the subjects of this report. A preliminary account of some of this work has been published as an extended abstract (6).

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MATERIALS AND METHODS

***Drosophila* Strains.** Strains 91-C and 91-R were obtained from D. J. Merrell (University of Minnesota) in 1983 and have been maintained on regular food since then. Continual selection of strain 91-R for resistance to dichlorodiphenyltrichloroethane (DDT) was begun in 1952. 91-R is at least 70 times more resistant to DDT (7), and about 100 times more resistant to malathion (5), than its control, susceptible pair 91-C.

P450 Quantitation. Microsomal proteins from 91-C and 91-R adults (5 μg per lane) were resolved in triplicate by SDS/PAGE (8). The gels were either stained to estimate relative amounts of total P450 or immunoblotted to estimate relative amounts of P450-A1 and P450-B1 in the two strains (5).

cDNA and Gene Cloning. A cDNA library in λgt11 (BRL) was generated from poly(A)-RNA isolated from the 91-R strain by using oligo(dT) as the primer for cDNA synthesis. mAbs 13-2e and 8-1d were used to select those clones producing P450-A1 and P450-B1, respectively. Several overlapping clones of each specificity were obtained. DNA inserts from these were cloned into the plasmid pGEM-7Z (Promega) to facilitate their restriction mapping and sequencing.

Genomic libraries were generated with DNA from both 91-C and 91-R by using the λGEM-11 cloning system (Promega). P450-B1-specific genomic clones were identified by hybridization with a ^{32}P-labeled P450-B1 cDNA. Two 91-C clones, λ C5-1 and λ C11-2, yielded an *Eco*RI fragment of about 6.3 kilobase pairs (kbp), which contained the complete coding sequence of the gene. Two 91-R clones, λ R1-2 and λ R7-1, yielded a similar fragment, which was only about 5.9 kbp. These fragments were cloned into pGEM-7Z for further analyses.

Restriction Mapping and DNA Sequencing. Restriction sites in both cDNA and genomic DNA inserts were determined by standard procedures. Sequences were determined by the dideoxy chain-termination method (9) using overlapping clones (for cDNA), subclones, and selected sequencing primers (for cDNA and genomic DNA). Translation of DNA sequences, GenBank data base searches, and sequence homology comparisons were done using the University of Wisconsin Genetics Computer Group (GCG) sequence analysis software package (10).

RNA and DNA Blot Analysis. Total RNA was isolated from adult flies by a guanidine hydrochloride method (11), and

Abbreviations: LTR, long terminal repeat; mAb, monoclonal antibody; RFLP, restriction fragment length polymorphism.
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§The sequence reported in this paper has been deposited in the GenBank data base (accession no. M88009).

20-μg samples were resolved by electrophoresis in a denaturing 1.2% agarose gel (12). The RNA was stained with ethidium bromide, photographed, and then blotted onto a nylon membrane. P450-B1 mRNA was detected by hybridization with the ^{32}P-labeled *Xho* I/*Hin*dIII fragment of the P450-B1 cDNA as described (13). The relative amounts of P450-B1 mRNA in 91-C and 91-R were estimated by densitometric scanning of the film. Subsequently the membrane was stripped and rehybridized using the ^{32}P-labeled *Xba* I/*Hin*dIII fragment of the P450-A1 cDNA to detect P450-A1 mRNA.

Genomic DNA was isolated from adult flies as described (14). Five-microgram quantities were digested to completion with *Bam*HI, *Eco*RI, *Hin*dIII, *Pst* I, or *Sst* I and resolved by electrophoresis in a 0.7% agarose gel. The DNA was blotted onto a nylon membrane and probed as described above for the RNA blots.

RESULTS

P450 in Strains 91-C and 91-R. There is about 2 times more P450 in 91-R than in 91-C (5). As shown in Fig. 1, the amounts of total P450-A in 91-C and 91-R are nearly equal, whereas the amount of total P450-B is much greater in the resistant strain 91-R. Amounts of the immunoreactive enzymes, P450-A1 and P450-B1, closely parallel that of the total amounts of P450 in the respective subsets in the two strains (ref. 5 and Fig. 1).

cDNA Characterization. All P450-A1 and P450-B1 cDNAs from the λ library were cloned into a plasmid to facilitate their analysis. Restriction maps of the longest single cDNA of each specificity are shown in Fig. 2*A*. The P450-A1-specific cDNA clone pA1-b4 is not full length but is a convenient control probe for experiments involving the P450-B1 cDNA clone.

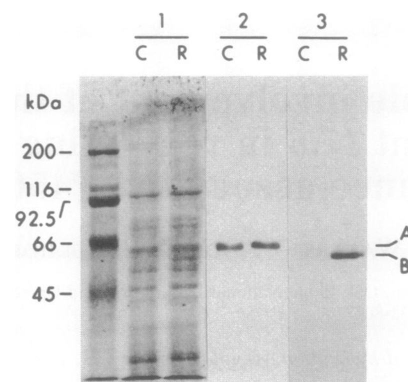

FIG. 1. Expression of total P450-A and P450-B and immunoreactive P450-A1 and P450-B1 in the susceptible 91-C and resistant 91-R strains of *Drosophila*. Total P450 in strains 91-C (lanes C) and 91-R (lanes R) was estimated by staining with Coomassie brilliant blue (panel 1). The relative amounts of P450-A1 and P450-B1 were determined by immunoblot analysis using the P450-A1-specific mAb 13-2e (panel 2) or the P450-B1-specific mAb 8-1d (panel 3). A and B indicate the location of P450-A and P450-B, respectively.

A 1689-nucleotide sequence has been determined for the P450-B1 cDNA (Fig. 2*B*). Clone pB1-b7 contained 1658 nucleotides, and the remaining 31 nucleotides, at the 3′ end, were determined from two other independent and overlapping clones, pB1-a5 and pB1-a7, which contained poly(A) stretches of 43 and 83 nucleotides, respectively. The P450-B1 cDNA has an open reading frame of 1521 nucleotides, which is capable of encoding a protein of 507 amino acids with a molecular mass of 58,835 Da. Other features of the P450-B1 cDNA sequence include a 5′ untranslated region of at least 40

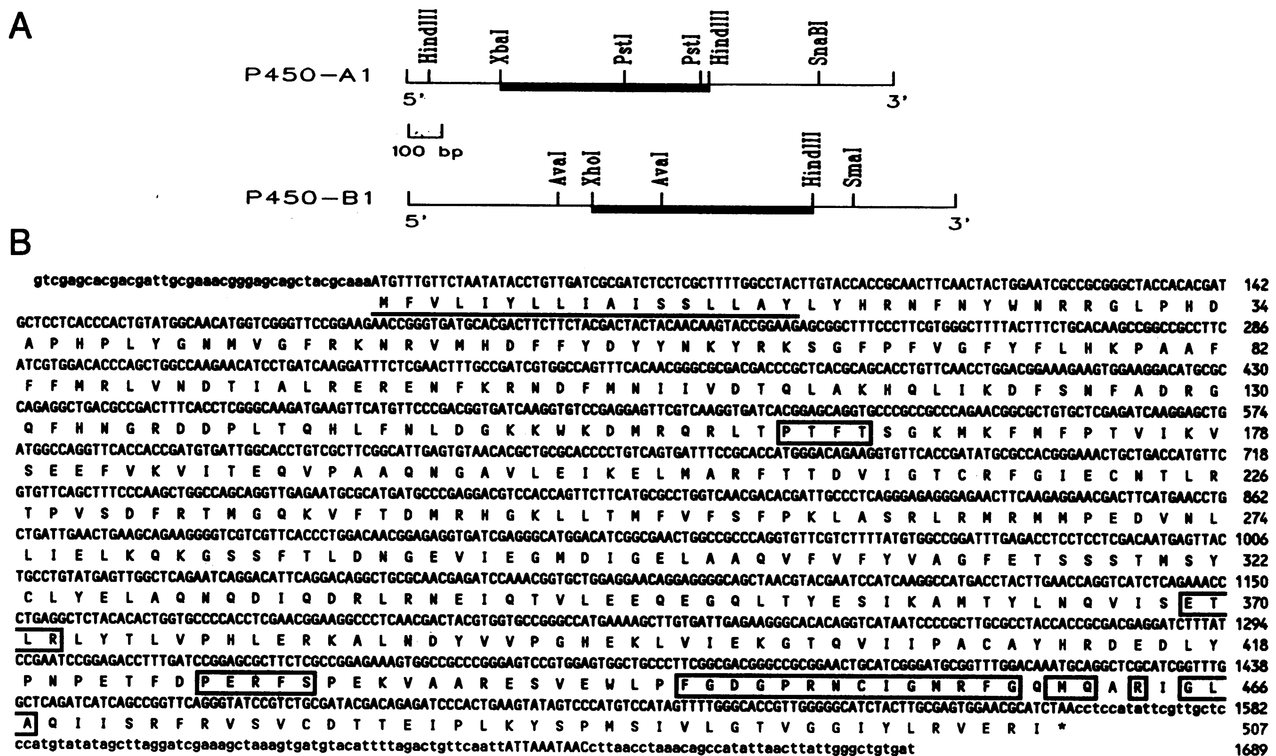

FIG. 2. Restriction maps of P450-A1 and P450-B1 cDNAs and the nucleotide sequence of P450-B1 cDNA and the deduced amino acid sequence of P450-B1. (*A*) The restriction map of the longest single clone of each cDNA—pA1-b4 (≈1470 bp) and pB1-b7 (1658 bp) corresponding to P450-A1 and P450-B1, respectively—is shown. The sequences used as probes for the RNA and DNA blot analyses shown in Figs. 3 and 4 are indicated by black bars. (*B*) The open reading frame of the P450-B1 cDNA sequence and the potential polyadenylylation signal sequence are shown in uppercase letters. The termination codon is indicated by an asterisk. The poly(A) tail, as long as 83 adenine bases, is not shown. The putative signal sequence at the amino terminus is underlined. The amino acid sequences common to family 3 P450s, as well as the amino acids around cysteine at position 452 that share positional identity with house fly CYP6A1, are boxed.

nucleotides and a 3′ untranslated region of 125 nucleotides. The nucleotide sequence CAAA, immediately preceding the start codon, is homologous with the consensus sequence ($\frac{C}{A}AA\frac{A}{C}$) that precedes translational start sites in *Drosophila* mRNAs (15). A combination of two common polyadenylylation signals, ATTAAA or AATAAC, exists in the sequence ATTAAATAAC at positions 1641–1650 (16).

The deduced amino acid sequence of P450-B1 (Fig. 2*B*) shares 49% positional identity with the house fly CYP6A1 (17), which places it in the same gene family (18). It was named CYP6A2 (D. W. Nebert, personal communication). Accordingly P450-B1 shares several amino acid sequences with members of the P450 gene family 3 (e.g., PTFT, ETLR, and PERFS). Twenty of 23 amino acids around the putative heme-binding cysteine at position 452 are identical to those around the analogous position in CYP6A1 (17). A hydrophobic, putative signal sequence of amino acids at the amino terminus (positions 1–17) of P450-B1 is a common feature among vertebrate P450s (19).

P450-B1 Gene Transcription in 91-C and 91-R. There is 20–30 times more P450-B1 mRNA in 91-R than in 91-C (Fig. 3). Also, the P450-B1 mRNA in 91-C is significantly larger in size than that in 91-R. P450-A1 mRNA is larger than P450-B1 mRNA and is expressed at similar levels in the two strains (Fig. 3).

P450-B1 Gene Organization in 91-C and 91-R. Organization of the P450-B1 gene in *Drosophila* was first investigated by Southern blot analyses of the gene-specific fragments produced by restriction enzyme digestion of genomic DNA from strains 91-C and 91-R. Restriction fragment length polymorphisms (RFLPs) were observed in DNAs digested with *Bam*HI, *Eco*RI, and *Pst* I when the P450-B1-gene-specific probe was used (Fig. 4). The minor bands observed in 91-C DNA, which corresponded to those produced by *Bam*HI, *Eco*RI, and *Pst* I digestion of 91-R DNA, were due to a slight contamination of the 91-C fly culture with 91-R flies. Subsequent experiments, using earlier passage stocks of 91-C, failed to show these bands. No RFLPs were found in the P450-A1 gene of the two strains (data not shown). Similar hybridization intensities with 91-C and 91-R DNAs indicated that the resistance-associated P450-B1 gene is not amplified in the resistant strain (Fig. 4).

The structural differences between the P450-B1 gene of susceptible and resistant flies were determined by restriction map and DNA sequence analyses of genomic DNA clones. The polymorphic *Eco*RI fragments containing the coding sequence were chosen to characterize the organization of the P450-B1 gene in 91-C and 91-R. Alignment of cDNA with the cloned genomic fragments by characteristic restriction sites indicated that the coding region of the P450-B1 gene is intronless. Further comparison of the restriction maps of the genomic clones showed a sequence of ≈500 base pairs (bp) at the 3′ end of the P450-B1 gene of 91-C that is not present in the gene of 91-R (Fig. 5*A*). This sequence was marked by the presence of a second *Nsi* I site, which is not present in the gene of 91-R. Sequence determination of this additional DNA segment identified it as the long terminal repeat (LTR) of the transposable element *17.6* (21). The LTR contains 513 bp and is flanked by the 4-bp repeated sequences ATAT; one of the two ATAT sequences and the LTR are absent from the gene in 91-R (Fig. 5).

Several additional features of the P450-B1 gene were determined from sequence analyses upstream and downstream from the cDNA (Fig. 5*B*). The sequence of at least 224 bp upstream from the cDNA was identical in the genes from 91-C and 91-R. A TATA box was present 86 bp upstream from the initiation codon. The sequences of 560 bp downstream from the cDNA poly(A) site, at positions 2430–2990, were the same for the genes in 91-C and 91-R except for single-base changes at four positions; C, T, A, and T at positions 2664, 2747, 2790, and 2970, respectively, in the gene of 91-C were changed to T, C, T, and C in the gene of 91-R (Fig. 5*B*).

The cloned *Eco*RI fragment containing the P450-B1 gene of 91-C was estimated to be only about 400 bp longer than that of the gene of 91-R. It was expected to be 517 bp longer because of the added LTR sequence and one ATAT sequence. Size analyses of fragments produced by restriction enzyme digestion of the P450-B1 genomic clones from 91-C and 91-R showed that the additional ≈100 bp in the gene of 91-R were contained in the *Nsi* I/*Xba* I fragment (Fig. 5*A*). Further DNA sequencing identified a 96-bp segment—TTCTATTAAATACCTGTCGTCAAGTCTCAACTT-TCTAGCTTTTATGGTTCCCGGATATCTCGACTTTCA-CACGAACGAACGGACATAACGGACAGA—in the clone derived from 91-R that was not present in the clone from 91-C. This segment of DNA is located 560 bp downstream of the transcribed region of the P450-B1 gene in 91-R at a site corresponding to positions 2990–2991 in the clone from 91-C (Fig. 5). A search for this sequence in GenBank (August 1991) showed it to share ≈70% identity with sequences in a *D. melanogaster* genomic clone that contains genes for Ulb small nuclear RNA variants (22). These sequences are,

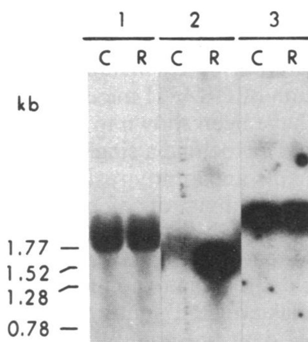

FIG. 3. Northern blot analyses of P450-A1- and P450-B1-specific mRNAs in strains 91-C (lanes C) and 91-R (lanes R). Panel 1, ethidium bromide-stained gel showing an intensely staining band near the 1.77-kb marker, which is 18S rRNA and the two fragments derived from cleavage of the 28S rRNA by heat (20). Panel 2, P450-B1-specific mRNA in 91-C and 91-R. Panel 3, P450-A1-specific mRNA in the two strains.

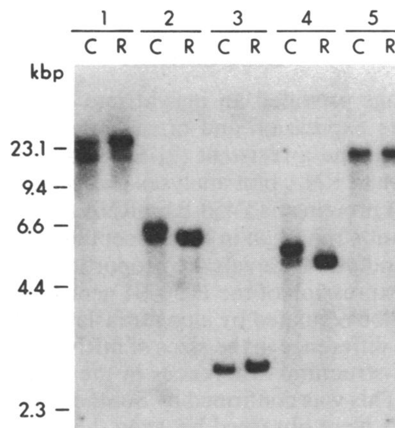

FIG. 4. Southern blot analyses of the P450-B1 gene in *Drosophila* strains 91-C (lanes C) and 91-R (lanes R). DNA was digested with either *Bam*HI (panel 1), *Eco*RI (panel 2), *Hin*dIII (panel 3), *Pst* I (panel 4), or *Sst* I (panel 5) and resolved by agarose gel electrophoresis. The DNA was blotted onto a nylon membrane and probed with the ^{32}P-labeled cDNA fragment indicated in Fig. 2*A*.

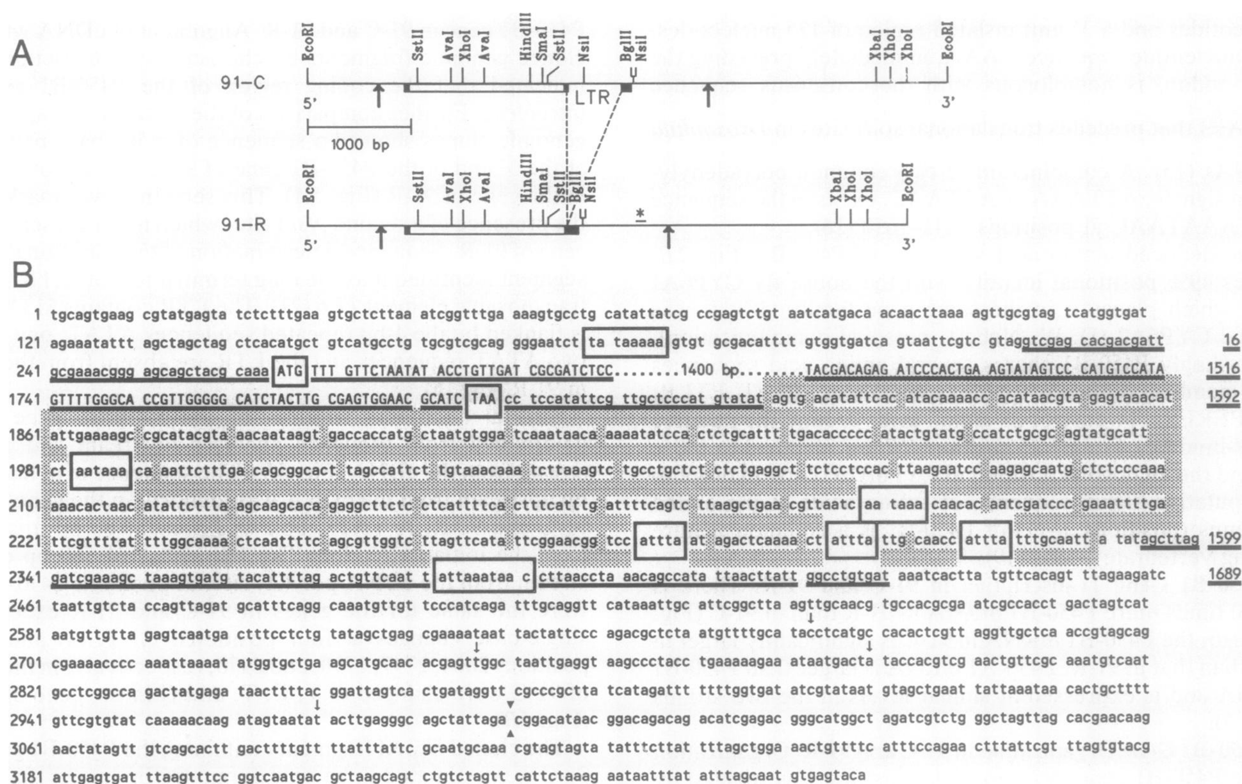

FIG. 5. Restriction maps of genomic clones containing the P450-B1 gene from 91-C and 91-R and sequence of the gene from 91-C. (A) Restriction maps. The boxes indicate the transcribed region of the gene, corresponding to the cDNA (Fig. 2B). The open part of the box is the translated region, and the closed parts are the 5′ and the 3′ untranslated regions. The DNA between the arrows has been sequenced. LTR indicates the location of the LTR of *17.6* in the gene from 91-C, and the asterisk indicates the location of the 96-bp segment unique to the gene from 91-R. (B) Sequence. The sequence that is transcribed in strain 91-R is underlined. Numbers to the right refer to the cDNA sequence shown in Fig. 2B. The TATA box upstream of the cDNA is boxed. The translated region is in uppercase letters; the start and stop codons are boxed. Polyadenylylation signals at positions 1983, 2189, and 2382 as well as ATTTA sequences at positions 2284, 2303, and 2316 are also boxed. The LTR of *17.6* is shaded. Positions downstream of the gene at which single-base changes between the genes of 91-C and 91-R occur are shown by arrows. The arrowheads at positions 2990–2991 indicate the location of a 96-bp sequence that is absent in this gene but is present in the gene from 91-R.

respectively, 308 and 175 bp downstream of the coding sequences in the Ulb genes U1-82.1 and U1-82.3. The orientation of the 96-bp sequence in the P450-B1 gene clone from 91-R is opposite to that in those sequences associated with the Ulb small nuclear RNA genes. The DNA sequence of at least another 279 bp downstream of this segment in the clone from 91-R is identical to that from positions 2991–3269 in 91-C (Fig. 5B).

DISCUSSION

This study has provided an insight into the differences in P450-B1 gene expression and organization between a susceptible (91-C) and a resistant (91-R), strain of *Drosophila*. Immunoblot and RNA blot analyses showed the amounts of both P450-B1 protein and P450-B1 mRNA to be much greater in the resistant strain than in the susceptible strain. Because the protein and mRNA levels are proportional to each other, differential expression of the P450-B1 gene in 91-C and 91-R is unlikely to be regulated by a posttranslational mechanism. A significant difference in the sizes of mRNA transcripts was indicative of structural differences in the gene between the two strains. This was confirmed by Southern blot analyses in which RFLPs were observed between the P450-B1 genes of the two strains. In all experiments, the control P450-A1 probes produced the expected results—i.e., similar amounts of P450-A1 in the two strains, similar amounts of P450-A1 mRNA (which was larger than the P450-B1 mRNA) in the two strains, and no RFLPs in the P450-A1 genes of the two strains.

It was expected that the differences in gene structure in the two strains would occur at the 5′ end of the gene and involve regulatory sequences there. DNA sequence analysis showed, however, that the difference exists at the 3′ end. Specifically, it involves the presence of the LTR of the transposable element *17.6* in the 3′ untranslated region of the gene of the susceptible fly. The LTR and one of the two ATAT sequences flanking the LTR are not present in the gene from the resistant fly. Inouye *et al.* (23) previously reported on the tendency for *17.6* to insert at ATAT sequences.

There are several examples of transposable element insertion causing gene mutation. Notable among these are the white-apricot mutation of *D. melanogaster* and the dilute coat color mutation of DBA/2J mice (24, 25). Revertants of these mutations have been shown to involve partial excision of the element, leaving behind a single LTR (26, 27). In these mutants the elements apparently reside in introns and act by causing premature termination of transcription and/or altered mRNA splicing; the solitary LTR apparently is not sufficient to produce the mutagenic effects. The case presented here appears to be an example in which the presence of a single LTR has effected mutation of the P450-B1 gene.

On the basis of data presented, it is not yet possible to define the molecular mechanism by which P450-B1 gene expression is regulated. However, gene amplification, which is responsible for esterase-dependent resistance to organophosphates in peach aphids (28) and mosquitos (29), is not involved. Features of the LTR and its location in the gene of the susceptible, 91-C strain encourage speculation that a posttranscriptional mechanism involving mRNA stability

may be involved in P450-B1 gene regulation. First, the location of the LTR, between the translation termination codon and the polyadenylylation signal of the gene, effectively moves the polyadenylylation signal ≈500 bp farther downstream. Second, the LTR contains two potential polyadenylylation signals of its own, at positions 1983 and 2189 (Fig. 5B). The one at position 2189 occurs at the junction of the U3 and R subdivisions of the LTR and is believed to be functional in element *17.6* (21). Regardless of which of the three polyadenylylation signals is used, any P450-B1 mRNA produced in 91-C would necessarily be chimeric, containing LTR sequence, and be significantly longer than the mRNA produced in 91-R. This expectation is consistent with the results of Northern blot analyses (Fig. 3).

At least two posttranscriptional mechanisms could explain the large decrease in the amount of P450-B1 mRNA produced in the susceptible strain. First, the chimeric transcript could be inherently unstable and be rapidly degraded. For example, if the polyadenylylation signal of the gene (at position 2382, Fig. 5B) was used, the transcript would contain three AUUUA sequences that are coded by sequences in the LTR at positions 2284, 2303, and 2316 (Fig. 5B). Shaw and Kamen (30) have proposed that such sequences cause mRNA to be highly unstable. Second, the chimeric mRNA might not be effectively polyadenylylated, which in itself is known to decrease mRNA stability (31, 32).

It is not known whether or not the apparently unique 96-bp sequence downstream of the coding sequence in the gene of 91-R is a functional part of the P450-B1 gene. Therefore, the possibility of this sequence having an enhancer effect on transcription of the gene in the resistant strain cannot be ignored. However, if the production of P450-A1 and its mRNA, in both 91-C and 91-R, is indicative of normal expression of a constitutive P450 gene in *Drosophila*, then it seems that the P450-B1 gene in 91-R is also normally expressed (compare Figs. 1 and 3). Consequently, it seems more logical to believe that the P450-B1 gene is down-regulated in 91-C (e.g., via decreased mRNA stability) rather than being up-regulated in 91-R by an enhancer- or promoter-dependent mechanism. Further experimentation will be necessary to determine the precise mechanism by which the P450-B1 gene is regulated in 91-C and 91-R.

In summary, this study shows that the gene for insecticide resistance-associated P450-B1 in the resistant, 91-R strain of *Drosophila* is structurally different from that in the susceptible, 91-C strain. The gene in 91-C contains the LTR of transposable element *17.6* in its 3′ untranslated region. The LTR is not present in the gene from the resistant strain. It is unlikely that this situation is limited to the 91-C/91-R pair of strains because another susceptible/resistant pair, Oregon-R and Hikone-R (BG) (3–5), yielded a P450-B1 gene-specific RFLP pattern that was indistinguishable from that of 91-C and 91-R. We propose that the LTR in the gene of 91-C causes chimeric mRNA transcripts to be produced and that such transcripts are unstable, resulting in a markedly reduced level of P450-B1 in the susceptible strain.

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