Cloning of two novel P450 cDNAs from German cockroaches, *Blattella germanica* (L.): CYP6K1 and CYP6J1

Z. Wen and J. G. Scott
Department of Entomology, Comstock Hall, Cornell University, Ithaca, NY, USA

Abstract
Two novel P450 cDNAs, CYP6K1 and CYP6J1, were isolated from German cockroaches, *Blattella germanica* (L). Both CYP6K1 and CYP6J1 are typical microsomal P450s and their deduced amino acid sequences share a number of common characteristics with other members of the P450 superfamily. Both CYP6K1 and CYP6J1 showed the highest per cent identity (based on the deduced amino acid sequence) to CYP6L1 from *B. germanica* and CYP6H1, a putative ecdysone 20-hydroxylase from *Locusta migratoria*. Using a CYP6K1 probe, two mRNA signals (~2.5 and ~2.1 kb) were detected in all life stages. Both signals were just detectable in the eggs and became stronger in later instars. The strongest signals were detected in the fifth and sixth instars as well as in adults. These two bands were also detected in the abdomens and in the remainder of bodies of both male and female adults. Southern blots suggest the two mRNA bands detected in the Northern blot might be a result of alternative splicing. No signal could be detected at any life stage using the CYP6J1 probe, suggesting that CYP6J1 was expressed at a low level.

Keywords: cytochrome P450 monooxygenase, cDNA cloning, *Blattella germanica*, Insecta.

Introduction
The cytochrome P450 monooxygenases are important because they metabolize structurally diverse endogenous and exogenous compounds (Hodgson, 1985; Porter & Coon, 1991). However, there are many P450s in each metazoan species. Genomic sequencing has revealed eighty putative P450s in *Caenorhabditis elegans* (Consortium, 1998) and eighty-six in *Drosophila melanogaster* (Adams et al., 2000). In insects, P450s are involved in the metabolism of plant allelochemicals and insecticides, as well as physiologically important endogenous compounds including juvenile hormones (JHs), ecdysteroids and pheromones (Hodgson, 1985). The isolation and characterization of insect P450s is a critical first step towards understanding the P450s involved in these important metabolic processes. Such studies have provided great insight into herbivore–plant interactions, insecticide resistance, insect development and physiology (Berenbaum, 1999; Feyereisen, 1999; Scott, 1999).

Cockroaches are one of the most primitive group of winged insects. Their body morphology has remained virtually unchanged for approximately 350 million years (Appel, 1995). Among the approximately 4000 known species of cockroaches, only about thirty are considered pests, with German cockroaches (*Blattella germanica*) being arguably the most important (Herrick, 1914; Ross & Cochran, 1975). German cockroaches are human pathogen-carriers and allergen-producers. Cockroaches carry a number of microorganisms potentially pathogenic to humans, including polio viruses, mycobacteria and helminthes (Brenner, 1995). The development of allergic respiratory diseases, especially bronchial asthma, is consistently correlated with cockroach infestation, and occurs world-wide wherever living conditions are suitable for cockroaches (Brenner, 1995; Chapman et al., 1997).

Studies of insecticide metabolism and resistance triggered the discovery of P450s in many insects, including German cockroaches. The earliest indication of P450s in German cockroaches was the discovery of a microsomal enzyme system capable of converting DDT to dicofol (Agosin et al., 1961; Agosin, 1985). Involvement of monooxygenases in the metabolism of various insecticides was later supported by *in vitro* metabolism studies or bioassays using various insecticides coupled with P450 inhibitors or inducers (Matsumura et al., 1967; Khan & Matsumura, 1972; Siegfried et al., 1990; Siegfried & Scott, 1991; Hemingway et al., 1993a; Hemingway et al., 1993b; Mahmoud et al., 1993; Valles et al., 1994; Lee et al., 1996; Scharf et al., 1997; Scott & Wen, 1997;
Results and discussion

Characterization of CYP6K1 and CYP6J1 cDNAs

The full length cDNA sequence (based on clones FB2, FB6 as well as 3' and 5' RACEs) for MCHB (accession number: AF281326) is shown in Fig. 1. MCHB was named CYP6K1 by the P450 Nomenclature Committee. CYP6K1 has an open reading frame of 1572 nucleotides with a deduced protein of 524 amino acids (AAs) and a molecular mass of 59.6 kDa. CYP6K1 is most similar to other members of the CYP6 family (Table 1), having the highest per cent AA identities with CYP6H1 (43.3%) from Locusta migratoria (Winter et al., 1999), CYP6L1 (38.2%) from Blaberus discoidalis (Bradfield et al., 1991). In this paper, we report the cloning and expression of two new P450 cDNAs, CYP6K1 and CYP6J1, from German cockroaches.

Northern and Southern analyses

When the CYP6K1 probe was used, two bands (~2.5 kb and ~2.1 kb) were detected in all life stages of German cockroaches by Northern blot analyses (Fig. 3). Both signals were hardly detectable in the eggs, but became stronger in later instars. The strongest signals were detected in the fifth and sixth instars as well as adults. These two bands were also detected in the abdomens and in the remainder of bodies of both male and female adults (Wen, 2000).

Genomic DNA (2.5 μg) was digested with four restriction enzymes (BamH I, Nco I, Sma I and Xho I) which have no cutting site within the full length CYP6K1 cDNA. Southern blots probed with CYP6K1 consistently gave a single band for each enzyme (Fig. 4). These results suggest that the CYP6K1 probe is specific. Therefore, the two mRNA bands detected by Northern blots might be the result of alternative splicing.

Expression of CYP6J1 in German cockroaches was examined in different life stages and different body parts of male and female adults by Northern blots. When probed with CYP6J1, no mRNA signal was detected in any of these samples (10 μg of total RNA was used). Given that CYP6J1 is expressed in all life stages and in both sexes of adult German cockroaches, this is similar to the expression of several other insect P450s (refs in Scott et al., 1998)).
Cloning of two novel P450 cDNAs

Figure 1. Full length cDNA sequence of CYP6K1 (accession number: AF281328) and its deduced amino acid sequence. Amino acids (AA) are numbered on the left and nucleotides (NT) on the right. Primers (Table 1) are thickly underlined and amino acids in conserved regions thinly underlined.
Table 1. Per cent identity of CYP6K1 and CYP6J1 deduced amino acid sequences from German cockroaches with other insect P450s

<table>
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<tr>
<th></th>
<th>B. germanica</th>
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<th>L. migratoria</th>
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<td>6L1</td>
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<td>36.1</td>
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</table>

Figure 2. Full length cDNA sequence of CYP6J1 (accession number: AF281325) and its deduced amino acid sequence. Amino acids (AA) are numbered on the left and nucleotides (NT) on the right. Primers (Table 1) are thickly underlined and amino acids in conserved regions thinly underlined. The putative polyadenylation signal is indicated by bold font.
not limited to the cloning of abundant P450s. Future studies employing either a suitable expression system and/or the use of isospecific specific antisera will be necessary to determine the substrates for CYP6K1 and CYP6J1.

Experimental procedures

Cockroaches, RNA isolation and cDNA synthesis

The Kenly strain of German cockroach (Scott et al., 1990) was selected with propoxur by residue exposure in 1988 and 1995 and was renamed Baygon-R. This strain has been subsequently reared (on Purina dog chow and water at 28 °C) without insecticide exposure. Total RNA was isolated from German cockroaches (Baygon-R strain) using 5 M guanidine thiocyanate solution (Chirgwin et al., 1979). mRNA was isolated from total RNA using Oligotex suspension (QIAGEN) or directly from tissues using a QuickPrep mRNA purification kit (Pharmacia) as described by the manufacturers. Superscript II (Gibco/BRL) was used to synthesize the first strand cDNA following the manufacturer's instructions. For the first strand cDNA synthesis, C3PT (Danielson et al., 1997) was used as the primer and ~500 ng of mRNA was used as template. After RNase H (2 units) treatment at 37 °C for 30 min, the first strand cDNA was isolated using a QiAquick PCR purification kit (QIAGEN) to remove the primers and short cDNA (eluted with 100 μl H2O). The purified cDNA was used as template for 3’ and 5’ RACEs (Frohman et al., 1988) as described below.

cDNA cloning

3’ cDNA cloning using a primer based on the heme binding region of P450s led to the isolation of six clones, MCH2, MCH4, MCH5, MCH9, MCH18 and MCH29, which encoded the C-terminus of two putative P450s, MCHB (MCH 4, 5, 18, and 29) and MCHC (MCH9), respectively. Based on the 3’ sequence information of MCHB, a gene specific antisense primer MCHBA1 (Table 3 and Fig. 1) was designed. Similarly, MCHCA1 (Table 3 and Fig. 2) was designed based on the 3’ sequence information of MCHC. The two gene specific primers were used to clone the 5’ cDNA sequences of MCHB and MCHC after two rounds of 5’ RACEs. All the adjacent sequences obtained by 3’ and 5’ RACEs had at least 150 bp overlap with each other. By merging the overlapping sequences from the 3’ and 5’ RACEs, two putative full-length cDNA sequences MCHB-MER (for MCHB) and MCHCMER (for MCHC) were generated. More details about the procedures used for cDNA isolation using 3’ and 5’ RACE techniques were given previously (Wen & Scott, 2001).

To ensure that the fragments used to generate MCHB-MER were from the same gene, a gene specific primer set (MCHB52/ MCHA7, Table 3 and Fig. 1) flanking the open reading frame of
MCHB2 MER was used to amplify the full length cDNA of MCHB using first strand cDNA as template. Following denaturation at 95 °C for 3 min, the PCR conditions were set for the first ten cycles in the order of 94 °C for 1 min, 45 °C for 1.5 min and 72 °C for 5 min. The next twenty-five cycles followed immediately using the same conditions except that the annealing temperature was increased to 55 °C. The PCR reaction ended with a final extension for 15 min at 72 °C. A single PCR product (1.7 kb) was observed following agarose gel electrophoresis. The band was cut and DNA purified and cloned into pCR821 vector (Invitrogen). Two positive clones (FB2 and FB6) were identified by colony PCR (Gussow & Clackson, 1989) using the MCHBS2/MCHBA7 primer set. The inserts of FB2 and FB6 were entirely sequenced.

Nearly the whole open reading frame of MCHC was cloned after two rounds of PCR amplifications. The products of the first round PCR using MCHCA1/AAP (Wen & Scott, 2000) as the primer set and first strand cDNA as template served as templates for the second round PCR using MCHCA1/MCHCS3 (Table 1 and Fig. 2) as the primer set. The PCR conditions were the same as described for cloning CYP4C21 (Wen, 2000). Four positive clones (FOS, FC12, FC13 and FC15) with inserts of 1.6 kb were identified by colony PCR using the MCHCA1/MCHCS3 primer set. The inserts of these positive clones were entirely sequenced.

Northern and Southern blot analyses

Using MCHBS2/MCHBA7 as the primer set and plasmids from FB2 clone as template a 1.7 kb cDNA fragment of CYP6K1 was PCR amplified. Similarly, a 1.6 kb cDNA fragment of CYP6J1 was PCR amplified using MCHCS3/MCHCA1 as the primer set with plasmids from FC6 clone as template. These fragments were used as templates to prepare probes for Northern and Southern hybridizations (Wen & Scott, 2000) as described below.

For Northern blots, 10 μg of total RNA was fractionated on 1% denaturing formaldehyde agarose gel containing ethidium bromide. After washing in distilled water for about 3–4 h with several changes, the RNA was transferred to a GeneScreen Plus® hybridization transfer membrane (NEN™ Life Science Products, Inc.). Equal loading was monitored by comparing the density of the 18S ribosomal RNA (rRNA) band (Savonet et al., 1997; Spiess & Ivel, 1998) on the agarose gel before transfer and/or on the membrane after transfer under UV. The above mentioned PCR fragments were labelled with 32P dCTP using the RadPrime labelling system (Amersham), having no cutting site within the cDNA sequence were used to digest 2.5 μg of genomic DNA overnight as described by the manufacturer (Gibco/BRL). Fifty units of each enzyme were used for each reaction in a volume of 100 μl. The digested DNA was then ethanol precipitated and fractionated by electrophoresis on 1% agarose gel containing ethidium bromide. After electrophoresis, the gel was placed in 0.25 μl HCl with gentle shaking until 10 min after the dyes had changed colour. After being rinsed in H2O, the gel was denatured in a solution containing 1.5 μl NaCl and 0.5 μl NaOH with gentle shaking for 30 min. The gel was then rinsed with H2O and neutralized in a solution containing 1.5 μl NaCl, 0.5 μl Tris-HCl (pH 7.2) and 0.001 μl EDTA. After rinsing in H2O, the DNA was transferred to a GeneScreen Plus® membrane (NEN™ Life Science Products, Inc.). Probe preparation, membrane hybridization and film exposure were performed exactly as described above. Southern analyses were repeated three times.

Sequence analyses

Sequences analyses were carried out using the Clustal method of MEGAALIGN program (DNASTAR Inc., Madison, Wisconsin).

Acknowledgements

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References


