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# Transcriptional regulation of the *Papilio polyxenes CYP6B1* gene

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## ABSTRACT

**Detoxification of host plant defensive compounds by larval Lepidoptera is mediated by cytochrome P450 monooxygenases (P450s) such as CYP6B1, which is expressed in *Papilio polyxenes* (black swallowtail) larvae in response to xanthotoxin, a linear furanocoumarin. Baculovirus-mediated expression of two cloned *CYP6B1* cDNAs in lepidopteran cell lines has demonstrated that CYP6B1 isozymes primarily metabolize the linear furanocoumarins, xanthotoxin and bergapten, and not angular furanocoumarins. To characterize the regulatory features of the *CYP6B1* transcription unit, we have isolated the first full-length *CYP6B1v3* genomic DNA clone from *P.polyxenes*. The open reading frame of this gene is interrupted by a single intron and is virtually identical to the previously characterized *CYP6B1* cDNAs. Primer extension and ribonuclease protection analyses have localized the transcription initiation site to a point 28 nucleotides upstream from the AUG initiation codon. RNase protection analyses on RNA from larvae induced by linear and angular furanocoumarins indicate that transcription of the *CYP6B1* gene is induced in insects significantly in response to xanthotoxin and only slightly in response to bergapten. Angular furanocoumarins, such as angelicin, which are not appreciably metabolized by the *CYP6B1* gene product, do not significantly induce transcription of this gene. We conclude that this P450 gene is transcriptionally regulated *in vivo* by at least one of the substrates which the encoded protein metabolizes. Transient expression of CAT fusion constructs in transfected Sf9 lepidopteran cells demonstrates that nucleotides −1 to −838 upstream from the *CYP6B1v3* transcription initiation site retain basal and xanthotoxin-inducible transcriptional activities in this heterologous cell line. These data clearly indicate that *P.polyxenes* has adapted to the presence of furanocoumarins in its host plants by evolving P450 isozymes and regulatory cascades which respond to specific toxins.**

## INTRODUCTION

The black swallowtail caterpillar *(Papilio polyxenes)* is restricted to feeding on one or two plant families, specifically the Umbelliferae and Rutaceae, which contain furanocoumarins. These plant secondary metabolites are highly toxic to a wide variety of organisms because when photoactivated they react directly and irreversibly with pyrimidine bases in DNA (1). *In vivo* analysis has demonstrated that *P.polyxenes* larvae are capable of metabolizing xanthotoxin, a linear furanocoumarin, at levels up to 10 times higher than non-adapted polyphagous lepidopteran larvae (2). In principle, this increased resistance to this plant toxin results from changes in regulatory and/or structural components of the target gene responsible for its metabolism. Although Caprio and Tabashnik (3) attributed the high levels of resistance in *P.polyxenes* solely to structural adaptations in the cytochrome P450 isozymes responsible for furanocoumarin metabolism, it is clear that xanthotoxin-metabolic activities in *P.polyxenes* are significantly induced in larvae exposed to exogenous xanthotoxin (4). This and the fact that a distantly related lepidopteran species, *Spodoptera frugiperda*, metabolizes low levels of xanthotoxin to yield metabolites identical to those produced in *P.polyxenes* (5, 6) support the notion that multiple adaptive strategies modulate resistance to plant furanocoumarins. Despite an abundance of information on the activity and inducibility of these insect isozymes, little information exists on the organization and induction patterns of insect P450 genes to verify or reject this hypothesis.

More specifically, *in vitro* analyses of the metabolic activities in *P.polyxenes* larvae have demonstrated that the linear furanocoumarin, xanthotoxin, and its isomer, bergapten, are metabolized with approximately equal efficiency and that angular furanocoumarins are metabolized at significantly reduced efficiencies (4, 7). In these and other studies, the ability to metabolize xanthotoxin is induced up to 4.5-fold by xanthotoxin and 3.6-fold by bergapten (4, 8). Angelicin, an angular furanocoumarin, also induces metabolism of xanthotoxin (8). Cloning of *P.polyxenes* cDNAs from xanthotoxin-induced larvae has allowed us to identify two P450 cDNAs, designated *CYP6B1v1* and *CYP6B1v2*, which are more than 98% identical to one another at the amino acid level (9). Baculovirus-mediated

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expression of these cloned cDNAs in two different lepidopteran cell lines, *S.frugiperda* Sf9 and *Trichoplusia ni* Tn5, has demonstrated that both *CYP6B1* gene products metabolize substantial amounts of the linear furanocoumarins, xanthotoxin and bergapten, but not angular furanocoumarins, such as angelicin and sphondin (10). These reactivities have suggested that *P.polyxenes* larvae contain at least two selective furanocoumarin-metabolic P450 loci: *CYP6B1*, which metabolizes a discrete set of linear furanocoumarins, and another P450, as yet unidentified, which metabolizes angular furanocoumarins.

Northern analysis using the cloned *CYP6B1* cDNAs at high stringency has indicated that CYP6B1-like mRNAs are induced in *P.polyxenes* larvae in response to xanthotoxin and bergapten (two linear furanocoumarins) and angelicin (an angular furanocoumarin) (8). Because of potential cross-reaction with other furanocoumarin-induced P450 transcripts, the magnitude and specificity of *CYP6B1* transcriptional regulation are difficult to establish at this level of analysis. To more specifically define transcriptional inducers and regulatory elements, we have cloned and characterized a *P.polyxenes CYP6B1v3* gene which is highly homologous to the previously cloned *CYP6B1v1* and *CYP6B1v2* cDNAs. Analysis of the transcriptional induction patterns of this *CYP6B1v3* gene in larvae has revealed that it is induced to a large extent by exposure to xanthotoxin, to a lesser extent by bergapten, and not at all by angelicin, the angular furanocoumarin. Heterologous expression of this gene indicates that xanthotoxin-responsive elements in the *CYP6B1v3* promoter are functional in transfected lepidopteran cells.

## MATERIAL AND METHODS

### Construction of a *Papilio polyxenes* genomic DNA library

Genomic DNA was extracted from a single *P.polyxenes* butterfly collected in Pennsylvania in January 1992 using a method modified from Sambrook *et al.* (11). After removal of its legs and wings, the butterfly was ground in liquid nitrogen and incubated for 4 h at 50°C in 5 ml 10 mM Tris−HCl, 100 mM EDTA (pH 8.0), and 0.5% sodium dodecyl sulfate (SDS) containing 150 μg/ml proteinase K. Potassium acetate was added to a final concentration of 0.25 M and degraded proteins were precipitated by incubating on wet ice for 30 min. After centrifuging at 10 000×*g* for 10 min, the aqueous phase was sequentially extracted with an equal volume of water-saturated phenol, phenol:chloroform:isoamyl alcohol (50:50:1), and chloroform:isoamyl alcohol (50:1). Nucleic acids were precipitated from the aqueous phase by adding 0.1 volumes 10 M ammonium acetate and 2 volumes ethanol and incubating for 15 min on dry ice and washing with 2 ml 70% ethanol. RNA was removed by digesting with 20 μg/ml RNase A (Promega) for 60 min at 37°C. The RNase-treated mixture was re-extracted with an equal volume of phenol:chloroform:isoamyl alcohol (50:50:1), an equal volume of chloroform:isoamyl alcohol (50:1) and the genomic DNA was recovered by ethanol precipitation.

The genomic DNA library was constructed by partially digesting 25 μg genomic DNA with 0.1 U *Mbo*I for 40 min at 37°C to yield approximately 15 kb restriction fragments. The *Mbo*I ends were partially filled in with dGTP and dATP as outlined in the Lambda Fix II (Stratagene) cloning protocols and ligated into *Xho*I Lambda FixII vector (Stratagene) sites which had been partially filled in with dTTP and dCTP. The recombinant DNAs were packaged into phage using GigapackII *in vitro* packaging extract (Stratagene) as described by the manufacturer.

### Identification of *CYP6B1* genomic clones

Approximately $3.2\times10^5$ phage plaques were screened at high stringency (5× SSC, 40% formamide, 5× Denhardt's solution, 50 mM sodium phosphate buffer, pH 6.5, 1% SDS at 42°C) with the $^{32}$P-labeled 1.6 kb insert of *CYP6B1v1* cDNA (9). Two positive recombinant phage were recovered and analyzed for the presence of a full-length gene by polymerase chain reaction amplication (PCR) using primers within the coding region (Fig. 1). Oligonucleotides used in this analysis are as follows: C1 (5′-GATCAAGCTTATCTGCGGACAA(T/G)GTTT-3′), C2 (5′-GGGGTGTCTGTCTTTTAC-3′), N2 (5′-TTTGCCGACA-GAGGAGTC-3′), N3 (5′-AACGTACTCGAAATGAG-3′) and N10 (5′-TTCACCATGGTGTATCTT-3′). The recombinant phage were also screened with probes containing the first 300 or last 200 nucleotides of the *CYP6B1v1* cDNA. Fragment A from the λPp6B1v3-2 phage containing the truncated 3′ end of a *CYP6B1* gene was subcloned into the pBluescript SK(−) plasmid vector (Stratagene) by PCR amplification using the C1 primer that complements sequences at the C-terminus of both *CYP6B1* cDNAs (Fig. 1), and a T3 primer, which complements flanking sequences in the Lambda FixII vector. Contiguous restriction fragments B and C from the λPp6B1v3-1 phage containing the intact *CYP6B1v3* gene were subcloned into pBluescript SK(−) as two independent *Eco*RI fragments of approximately 7.0 and 3.3 kb (Fig. 1). The coding and flanking sequences in these plasmids were dideoxy-sequenced using Sequenase 2.0 (U.S. Biochemicals) and primers 17−24 nucleotide long situated along the length of the *CYP6B1* gene. The sequences of the 5′ flanking region and the internal intron were defined by sequencing both strands of the DNA; the 3′ flanking sequences were defined by sequencing one strand of the DNA.

### Furanocoumarin treatments and RNA isolation

Final instar *P.polyxenes* larvae obtained from a laboratory colony initiated with wild *P.polyxenes* females were reared for 24 h on a host plant, parsley (*Petroselinum sativum*), treated with either acetone (control) or acetone solutions of furanocoumarins (xanthotoxin, bergapten, or angelicin) at 0.01 mmol/g fresh weight (0.2%). Individual midguts were collected by dissection and stored at −70°C.

Total RNAs for the RNase protection assays were prepared from the midguts by a method adapted from Lou *et al.* (12). In brief, a midgut from a single larvae was ground for 1 min with a Mini-Beadbeater (Biospec Products) in 1 ml buffer containing equal volumes of phenol:chloroform (1:1) and 100 mM LiCl, 100 mM Tris−HCl (pH 8.0), 10 mM EDTA, 1% SDS. After centrifugation for 2 min in an Eppendorf centrifuge, the aqueous phase was re-extracted twice with an equal volume of phenol:chloroform (1:1) and the nucleic acids were precipitated by adding 0.1 volumes 3 M sodium acetate and 2 volumes ethanol. After the precipitation, residual DNA was removed by digesting each sample with 5 U RQ1 DNase (Promega) for 60 min at 37°C, re-extracting with an equal volume of phenol:chloroform (1:1) and precipitating with 0.3 M sodium acetate and ethanol. Approximately 200 μg total RNA were recovered from each midgut.

Poly(A)$^+$ RNA for primer extension analysis was extracted directly from midgut tissue from a xanthotoxin-induced catepillar using the MicroFastTrack kit (Invitrogen).

### Ribonuclease protection assays

Ribonuclease protection assays were performed using the RPAII ribonuclease protection assay kit (Ambion). A 431 nucleotide long antisense RNA probe spanning the RNA initiation site was generated by linearizing plasmid B with *Hph*I (Fig. 1) and *in vitro* transcribing it with T7 RNA polymerase (BRL). This probe, which spans the *CYP6B1v3* gene from the *Eco*RI site (+340) to the upstream *Hph*I cleavage site at −91 (Fig. 1), was gel-purified as outlined in the Ambion RPAII protocols. For each assay, total *P.polyxenes* RNA (5, 10, or 20 μg) was denatured for 5 min at 90°C and annealed with $^{32}$P-labeled probe ($10^5$ cpm) for 18 h at 45°C. Each sample was then treated with 5 μg/ml RNase A and 10 U/ml RNase T1 for 30 min at 37°C, precipitated and resuspended in 8 μl 10 M formamide loading dye. Samples were denatured at 90°C for 5 min and analyzed on 6% acrylamide−8.3 M urea denaturing gels containing 1× TBE buffer. The protected fragments were quantified by using a PhosphorImager (Molecular Dynamics) and compared by linear regression analysis. The relative amounts of total input RNA in each assay were standardized by spectrophotometric analysis and electrophoresis on ethidium bromide stained gels.

### Primer extension assays

Fifty picomoles of a synthetic oligonucleotide (C5 primer, 5′-GTAAAAGGCCGGCCAAAACCG-3′) complementary to positions +54 to +74 relative to the transcription initiation site of the *CYP6B1v3* gene were end-labeled with [γ-$^{32}$P]ATP (Amersham) and T4 polynucleotide kinase (BRL) for 60 min at 37°C, phenol:chloroform (1:1) extracted and precipitated with one-tenth volume 7.5 M ammonium acetate and 2 volumes ethanol at −70°C. 1.5 pmoles of the kinased oligonucleotide was mixed with 3 μg poly (A)$^+$ mRNA in 1× annealing buffer (40 mM Tris−HCl, pH 8.3, 48 mM NaCl, 8 mM DTT) in a total volume of 12 μl. After heating to 90°C for 3 min, the RNA and primer were annealed for 3−6 h at 37°C. To each reaction, 6 μl 1× reverse transcriptase buffer (50 mM Tris−HCl, pH 8.), 60 mM NaCl, 10 mM DTT, 30 mM magnesium acetate), 6 μl deoxynucleotide stock solution (2 mM each dGTP, dCTP, dATP, and dTTP in 1× annealing buffer), 6 μl 1× annealing buffer and 16 U AMV reverse transcriptase (Promega) were added. After a 60 min incubation at 37°C, 57.5 μl water, 10 μl 1 M Tris−HCl (pH 8.0) and 2.5 μl 20% SDS were added and the samples were extracted with phenol:chloroform (1:1) and precipitated with ethanol. Each pellet was dissolved in 30 μl formamide loading dye and 10 μl were electrophoresed on a 7% acrylamide−8.3 M urea denaturing gel. cDNA dideoxy-sequencing products generated with the same 5′ end-labeled primer and Sequenase 2.0 (U.S. Biochemicals) were electrophoresed alongside the primer extension products.

### Chloramphenicol acetyltransferase assays

A 860 bp promoter fragment, containing nucleotides −838 to +22 relative to the RNA initiation site, was generated by PCR amplification using oligonucleotides −838CYP (5′-GGGGCT-GCAGGAATTCGATGTCTAATGGGTAACGGTCGC-3′) and +22CYP (5′-GGGGTCTAGACACGGTCACTACTCACACT-G-3′) whose positions are shown in Fig. 1. The PCR product was digested with *Pst*I and *Xba*I and cloned into the basic pCAT promoterless/enhancerless vector (Promega) that contains a chloramphenicol acetyltransferase (CAT) gene downstream of *Pst*I and *Xba*I cloning sites. The resulting 838CYP:CAT construct was purified on a Qiagen-tip 500 column (Qiagen). Twenty mg DNA were transfected into Sf9 cells grown in a 100 mm plate at a density of $5\times10^5$ cells/ml by calcium-phosphate as previously described (13). As an internal control for transfection efficiency, cells were cotransfected with an actin:β-Gal construct (kindly provided by L.Cherbas, Indiana University) containing the constitutive promoter of the *Drosophila* actin 5C gene. Sixteen hours following transfection, cells were washed with 5 ml Sf-900 serum-free media (Gibco BRL) and induced with 20, 40, 80 or 120 μg xanthotoxin (in 120 μl methanol) per 10 ml complete media [Sf-900 media containing 10% fetal bovine serum (Sigma)] or 120 μl methanol, as a control. Twenty-four hours after this treatment (40 h post-transfection), the cells were harvested and

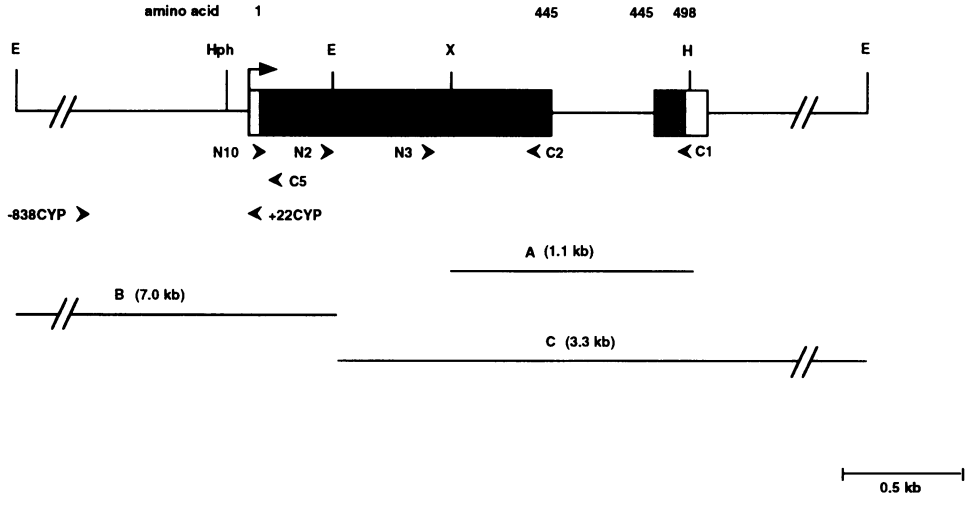


**Figure 1.** Structure of the *CYP6B1v3* gene. The structure of the cloned *CYP6B1v3* gene is drawn to scale with open boxes representing 5′ and 3′ non-translated sequences and stippled boxes representing coding sequences. Subcloned fragments (A, B and C) used in the characterization of this gene are shown below the gene. Restriction enzyme sites are as follows: E, *Eco*RI; Hph, *Hph*I; X, *Xho*I; H, *Hin*dIII. Amino acid positions are shown above the coding sequences. Primers mentioned in this report are designated with arrowheads under the gene. The transcription initiation site (+1) is indicated by a bent arrow.

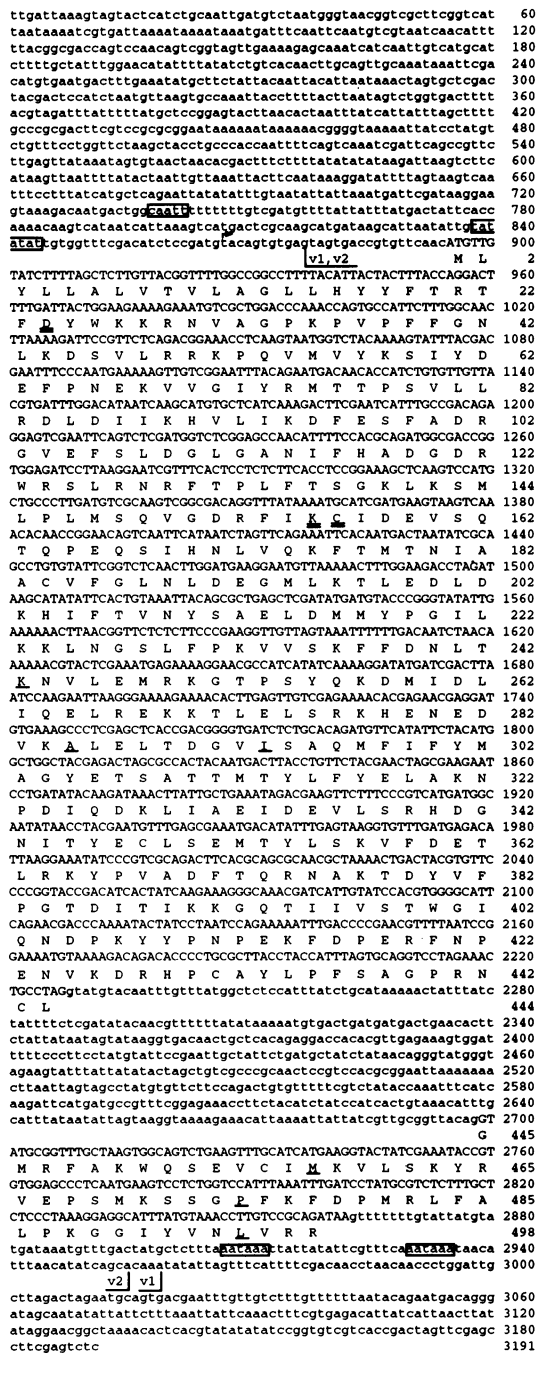

**Figure 2.** DNA and predicted amino acid sequence of the *CYP6B1v3* gene. Protein-coding nucleotides are in upper case. Non-translated nucleotides, including the single intron, are in lower case. Putative CAAT and TATA boxes are enclosed and the transcription initiation site is indicated with an arrow. The endpoints of the *CYP6B1v1* and *CYP6B1v2* cDNA clones (9) are shown below the nucleotide sequence (v1 and v2, respectively). Potential polyadenylation signals are boxed. Amino acids homologous to CYP6B1v1 are underlined, and those homologous to CYP6B1v2 are doubly underlined.

lysed in 900 $\mu$l reporter lysis buffer (Promega). Soluble fractions of cell lysates containing 2 milliunits $\beta$-galactosidase were assayed for CAT activity using [$^{14}$C]chloramphenicol and thin layer chromatography as previously described (14). The radioactive chromatograms were quantified using a PhosphorImager.

## RESULTS

### Isolation of *CYP6B1* genomic DNA clones

The *CYP6B1v1* cDNA previously identified by Cohen *et al.* (9) was used to screen a *P.polyxenes* genomic DNA library constructed from a single insect. Of the $3.2\times10^5$ phage screened, two carrying 15 kb inserts hybridized with this probe at high stringency. PCR amplification using primers within the *CYP6B1* cDNA insert (Fig. 1) on each of recombinant phage suggested that one of the phage contained a truncated 3′ portion of a *CYP6B1v3* gene while the second contained a complete coding sequence. This conclusion was verified by Southern blot analysis using probes corresponding to the first 300 nucleotides and last 200 nucleotides of the *CYP6B1v1* cDNA (not shown). The partial coding region of the truncated gene (fragment A, Fig. 1) was cloned into the pBluescript SK(−) vector for sequencing using the C1 primer, which complements sequences at the C-terminus of CYP6B1, and a T3 primer which complements the phage vector DNA. The full-length gene (fragments B and C) was directly subcloned into the pBluescript vector as two independent *Eco*RI fragments of approximately 7.0 and 3.3 kb.

Sequences obtained from these subcloned DNA fragments demonstrated that, in the region encompassed by fragment A, the full-length and truncated genes are identical and highly homologous to the *CYP6B1* cDNA. Alignment of the full-length gene sequence with the *CYP6B1v1* cDNA indicated that this *CYP6B1v3* gene contains a single intron of 471 bases interrupting the codon at position 445 just beyond the heme axial ligand present in all P450 monooxygenases (Figs 1 and 2). The allelic variants of *CYP6B1* cDNA (*CYP6B1v1, CYP6B1v2*) previously isolated from an mRNA pool obtained from several xanthotoxin-induced larvae differ in 1.81% (9/498) of their amino acids and 1.87% (28/1494) of their coding nucleotides (9). The *CYP6B1v3* gene sequence reported here is 99.53% identical in its coding nucleotides to *CYP6B1v1* cDNA and 98.59% identical to *CYP6B1v2* cDNA. The amino acid variations resulting from these few differences occur only at the positions and in the same permutations as seen in the two cDNAs. Of the nine amino acid positions in which the two cDNAs vary, six positions in the *CYP6B1v3* gene correspond to those in *CYP6B1v1* (Fig. 2, underlined) and three correspond to those in *CYP6B1v2* (Fig. 2, doubly-underlined). Variations in the 3′ non-translated region, which is not constrained by protein structure, are minimal with no differences observed between the *CYP6B1v3* genomic DNA and *CYP6B1v1* cDNA sequences. The short (18 nt) 5′ non-translated sequences present on the *CYP6B1v1* and *CYP6B1v2* cDNAs are virtually identical to those present in the *CYP6B1v3* genomic DNA clone (17/18 nt identities).

PCR amplification of genomic DNA from *P.polyxenes* using primers which span the single intron produce a single product approximately 400 nucleotides longer than that derived from *CYP6B1* cDNA (not shown). The size of this product and the absence of additional PCR products are consistent with the presence of a single genomic DNA locus in *P.polyxenes* encoding CYP6B1.

### Identification of the transcription initiation site

The 5′ end of the CYP6B1 mRNA was identified by primer extension analysis (Fig. 3) using the C5 oligonucleotide primer complementary to nucleotides +54 to +74 of the *CYP6B1v3* gene (Fig. 1). The initiation site was identified (Fig. 3, asterisked) by alignment with a *CYP6B1v3* genomic DNA sequencing ladder

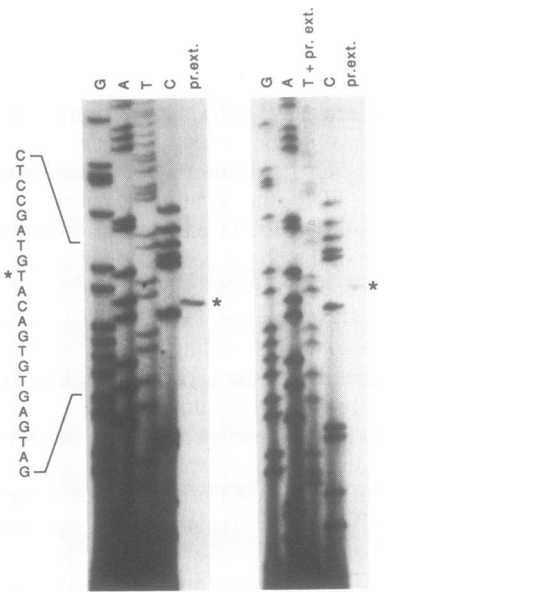


**Figure 3.** Primer extension analyses of the transcription initiation of the *CYP6B1v3* gene. Xanthotoxin-induced mRNA was primer extended using $^{32}P$ end-labeled C5 primer complementary to nucleotides +54 to +74 of the *CYP6B1v3* gene and electrophoresed on a 7% acrylamide denaturing gel. (**Left**) Lanes 1–4, dideoxy-sequencing on the *CYP6B1v3* gene using the same primer; lane 5, primer extension product obtained with 3 mg poly $(A)^+$ RNA. (**Right**) As the left panel except that in lane 3 thymidine dideoxy-sequencing products obtained with genomic *CYP6B1v3* cloned DNA were coelectrophoresed with the primer extension product. (**Bottom**) *CYP6B1v3* gene sequence surrounding the transcription initiation site (+1).

and precisely located by mixing dideoxythymidine-sequencing reactions with primer extension products prior to electrophoresis (Fig. 3, lane 3). The thymidine residue at the initiation site is situated 29 and 129 nucleotides downstream from putative TATA (TATATAT) and CAAT (CAATT) boxes, respectively (Fig. 3, bottom), and 10 nucleotides upstream from the 5′ end of the *CYP6B1v1* and *CYP6B1v2* cDNAs (9).

### Specificity of transcriptional induction

To determine which furanocoumarins induce transcription of the *CYP6B1v3* gene in *P.polyxenes* larvae, fifth instar larvae were exposed to 0.01 mmol/g fresh weight xanthotoxin, bergapten or angelicin for one day. RNase protection analyses of total RNA isolated from these larval midguts were performed using an antisense probe which spans the first 340 nucleotides of the *CYP6B1v3* gene. The relative amounts of input RNA present in each sample were estimated on ethidium bromide stained gels. For quantification, the radioactivity in the CYP6B1 protected fragment was determined by phosphorimagery and compared by linear regression analyses. These results (Fig. 4) indicate that low levels of *CYP6B1v3* transcript exist in control larvae which have ingested parsley, a host plant with extremely low levels of furanocoumarins. Transcription of the *CYP6B1v3* gene is induced 7-fold over the control level by a 24 h exposure to xanthotoxin and 2-fold by exposure to bergapten (Fig. 4, lanes 3–11). It should be noted that *in vivo* analyses suggest that bergapten fails to induce accumulation of CYP6B1 transcripts in some individuals (8). In the larvae analyzed here, angelicin fails to induce CYP6B1 transcription to any appreciable extent (Fig. 4, lanes 12–14). These results indicate that a hierarchy exists in the transcriptional regulation of the *CYP6B1* gene by furanocoumarins. Xanthotoxin, a linear furanocoumarin that is capable of being metabolized by the *CYP6B1* gene product (10), strongly induces transcription; bergapten, which is also metabolized by CYP6B1 (10), induces transcription to a far lesser degree. An angular furanocoumarin, angelicin, that is not metabolized to any significant extent by CYP6B1 (10) does not induce transcription of this gene.

### Identification of a *CYP6B1v3* promoter fragment

To determine if the 5′ flanking sequences upstream from the classic TATA box (−29) and CAAT box (−129) retain promoter activity in a heterologous system, we subcloned nucleotides −838 to +22 of the *CYP6B1v3* gene upstream from chloramphenicol acetyltransferase (CAT) coding sequences as outlined in Materials and Methods. The 838CYP:CAT construct generated represents a transcriptional fusion with the RNA initation site and first 22 nucleotides of the 5′ non-translated region derived from the *CYP6B1v3* gene and the remaining 49 nucleotides of the 5′ non-translated region derived from the basic pCAT vector (Fig. 5A). The resulting 838CYP:CAT construct was transfected into Sf9 cells derived from *S.frugiperda*, a lepidopteran distantly related to *P.polyxenes*, and assayed for CAT activity 40 h after transfection. To control for differences in transfection efficiency, Sf9 cells were cotransfected with a construct in which constitutive *Drosophila* actin 5C promoter was fused to the coding sequences of β-galactosidase (actin:β-Gal). Standardization against the β-galactosidase activities produced in these cells indicated that 838CYP:CAT transfected cells expressed a high level of basal CAT activity whereas cells transfected with the promoterless parental vector have undetectable CAT activity (Fig. 5B, lanes 1 vs. 2). To determine whether this promoter fragment retains xanthotoxin-inducible elements, varying amounts of xanthotoxin were added to 838CYP:CAT transfected cells 16 h after the initial transfection and cells were harvested 24 h later. In these assays, the level of CAT activity was at least 1.66-fold higher in cultures induced with 20 μg/10 ml xanthotoxin than in uninduced control cultures (Fig. 5C, lanes 3 vs. 4). Higher levels of xanthotoxin increase CAT activities to some extent: 40, 80, and 120 μg xanthotoxin/10 ml (Fig. 5C lanes 4–7) increased CAT activity 1.66-, 1.76-, and 2.06-fold, respectively, relative to control cultures. This indicates that this *CYP6B1v3* promoter fragment retains some, if not all, of the *cis*-elements important for xanthotoxin inducibility. More importantly, it demonstrates that the xanthotoxin signal transduction pathway is operative in transfected Sf9 cells and that, at these levels, xanthotoxin is not toxic to Sf9 cells.

## DISCUSSION

The *P.polyxenes CYP6B1v3* gene which we have described is highly homologous in its coding nucleotides to the previous cloned cDNAs, *CYP6B1v1* (99.53%) and *CYP6B1v2* (98.59%), and virtually identical in its 5′ and 3′ non-coding sequences. As such, it represents the first insect P450 gene whose corresponding

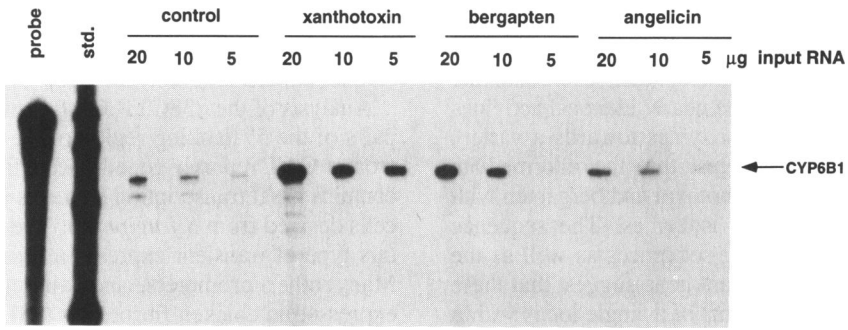


**Figure 4.** RNase protection assays. Fifth instar larvae were exposed for 1 day to 0.01 mmol/g fresh weight foilage of xanthotoxin, bergapten, or angelicin. Total RNA (5, 10, or 20 $\mu$g) from control and furanocoumarin-induced larvae was subjected to RNase protection analysis using RNases A and T1 and the $^{32}$P-labeled antisense probe spanning the first 340 nt of the *CYP6B1v3* gene. The radioactivities in each protected fragment were quantified by phosphorimagery and compared by linear regression analyses.

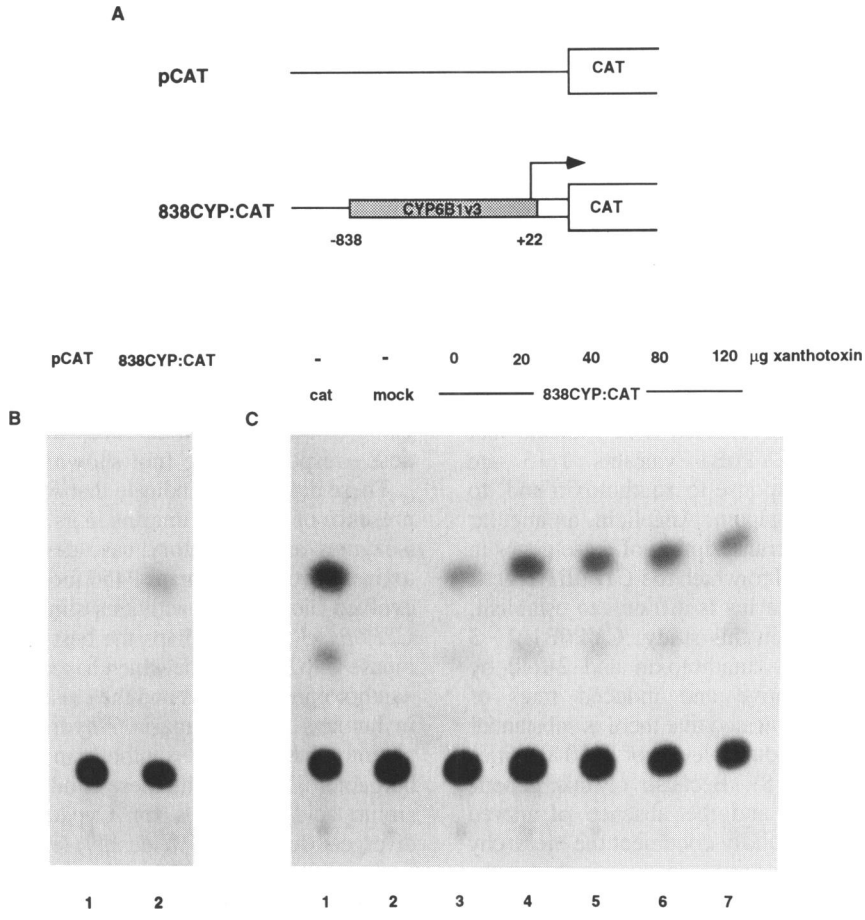


**Figure 5.** Expression of CYP6B1v3:CAT fusion constructs. (**A**) Plasmid constructs used in CAT assays. pCAT is the promoterless/enhancerless reporter vector into which the genomic subfragment (nucleotides −838 to +22) of *CYP6B1v3* was cloned to yield the 838CYP:CAT construct. (**B**) [$^{14}$C]Chloramphenicol acetyltransferase assays using protein extracted from cells transfected with actin:$\beta$-Gal DNA and pCAT (lane 1) or 838CYP:CAT (lane 2) DNA. (C) CAT assays using protein isolated from Sf9 cells 40 h after cotransfection with no DNA (mock transfection) (lane 2) or actin:$\beta$-Gal and 838CYP:CAT DNA (lane 3−7). From 16 to 40 h after transfection, the cells transfected with 838CYP:CAT DNA (lane 3−7) were treated with 0, 20, 40, 80 or 120 $\mu$g xanthotoxin/10 ml culture, as indicated above each lane. Cell lysates were normalized for $\beta$-galactosidase activity prior to CAT activity analysis. Lane 1, positive control using 2 units of chloramphenicol acetyltransferase.

cDNA has been cloned. The fact that amino acid variations occur only at the positions and in the same permutations as in the two cDNAs suggests that the *CYP6B1v3* gene represents an evolutionary intermediate between *CYP6B1v1* and *CYP6B1v2*. Closer analysis of the nucleotide and amino acid differences indicates that the *CYP6B1v3* gene may have been generated by

a cross-over between the *CYP6B1v1* and *CYP6B1v2* alleles: its first 280 amino acids (840 nucleotides) are identical to *CYP6B1v2* and its last 256 amino acids (768 nucleotides, excluding the intron) are identical to *CYP6B1v1*. The nearly identical activities of the CYP6B1v1 and CYP6B1v2 isozymes towards a variety of linear furanocoumarins (10) suggest that the intermediate CYP6B1v3 isozyme metabolizes xanthotoxin and bergapten with proficiencies similar to these other isozymes. The sequence identities in the 5′ and 3′ non-coding sequences as well as the identification of a potential recombinant gene suggest that these three isozymes represent allelic variants of a single locus within the *P.polyxenes* genome. The straightforward isolation of these allelic variants from cDNA and genomic libraries indicates that these three, and potentially others, represent common variants expressed in natural *P.polyxenes* populations and not rare alleles. Further RNase protection analysis (8) has indicated that two different alleles, corresponding to CYP6B1v3/v2 and to CYP6B1v1, are, in fact, expressed within some individuals. Together, these studies provide the first indication of the polymorphic nature of this metabolic locus and accentuate the need to correlate genotype with furanocoumarin-metabolic activities expressed in different individuals.

Other cloned P450s which have been placed in the same *CYP6* family include *CYP6A1* from an insecticide-resistant strain of house fly, *Musca domestica* (15, 16), and *CYP6A2* from an insecticide-resistant strain of *Drosophila melanogaster* (17). In locating the *CYP6B1* intron in amino acid 445, we demonstrate that the *CYP6B1v3* gene is not evolutionarily related to either *CYP6A1* which contains an intron at amino acid 364 or *CYP6A2* which is intronless. These differences, as well as the low degree of amino acid conservation (less than 32%) in these proteins, suggest that the *CYP6B1v3* gene belongs in a P450 family separate from the *CYP6A1* and *CYP6A2* genes.

The RNase protection analyses reported here indicate that transcripts corresponding to CYP6B1 variants 1−3 are specifically induced *in vivo* in response to xanthotoxin and, to a lesser extent, in response to bergapten. Angelicin, an angular furanocoumarin, does not induce transcription of these genes in *P.polyxenes* larvae. The exact level to which the *CYP6B1v3* gene is induced *in vivo* by furanocoumarins is difficult to establish. In the particular larvae analyzed in this study, CYP6B1v1−3 transcripts are induced 7-fold by xanthotoxin and 2-fold by bergapten. Analysis of constitutive and induced rates of furanocoumarin metabolism has indicated that there is substantial familial variation in the basal and induced levels of P450 activities towards different substrates (7, 8). Because of this genetic variation in natural populations and the absence of inbred *P.polyxenes* lines, our data most solidly document the hierarchy of inducers which activate transcription *in vivo* and not the absolute magnitude of the response.

The promoter sequences preceding the *CYP6B1v3* gene contain putative TATA and CAAT boxes located 30 and 129 nucleotides, respectively, upstream from the transcription initiation site. Nucleotides CGCAAGCA situated 55−48 bp upstream from the RNA initiation site are identical with those between positions −69 and −62 of the *CYP6A1* gene derived from house fly (15). Positions −717 to −703 (tTgAAAAGagcaAat) contain 7 of the 15 nucleotides in the Barbie box consensus sequence (18) which is important for barbiturate-mediated expression of P450s in *Bacillus megaterium* (19), rat (20) and *Streptomyces griseolus* (21). Though this conservation is striking, *in vitro* metabolism studies (22) suggest that phenobarbital only weakly induces xanthotoxin metabolism *in vivo*. Which of the other elements might be critical for transcriptional regulation of *CYP6B1v3* by furanocoumarins is not yet clear.

Analysis of the 838CYP:CAT constructs containing 838 base pairs of the 5′ flanking region of the *CYP6B1v3* gene upstream from a CAT reporter gene indicate that this promoter fragment contains basal transcription elements which are functional in Sf9 cells derived from *S.frugiperda*. The basal activities obtained in this type of transient expression assay are not unusually high. Many other promoters, including the SV40 early promoter expressed in chicken fibroblasts (23), the CMV promoter in B-cell line Raji and HeLa cells (24) and the *Drosophila* hsp70 promoter in *Aedes albopictus* (mosquito) (25) and Sf9 cells (26, our unpublished observations) exhibit high promoter-dependent basal activities in transient assays. Other promoters, such as the promoter of the *Drosophila copia* transposable element in *A.albopictus* cells (27) and the SV40 early promoter in Sf9 cells (our unpublished observations), exhibit negligible basal activities.

The fact that CAT activity is induced at least 2-fold above this basal activity by 2 μg/ml xanthotoxin indicates that the xanthotoxin regulatory cascade is maintained in heterologous Sf9 transfected cells and that this represents a model system for further analysis of furanocoumarin induction mechanisms. In the first of many studies along this line, we demonstrate that the 838 nucleotide promoter fragment retains some, if not all, of the elements necessary for xanthotoxin inducibility. Many explanations exist for the fact that higher levels of xanthotoxin do not increase *CYP6B1v3* gene expression significantly over those obtained with 2 μg/ml xanthotoxin but the most likely is that proteins in the signal transduction pathway exist in limiting amounts in Sf9 cells. An alternative explanation for the lack of higher induction levels, that xanthotoxin is toxic to Sf9 cells at high concentrations, can be discounted by the fact that high β-galactosidase activities are maintained throughout the dose−response curve (not shown).

These data clearly indicate that *P.polyxenes* has adapted to the presence of furanocoumarins in its host plants by evolving P450 isozymes and regulatory cascades which respond to specific toxins. Several vertebrate P450 monooxygenases appear to have evolved biochemical activities similar to those encoded by the *CYP6B1v3* gene. Perhaps the best characterized of these is the mouse Cyp2a5 isozyme which has now been shown to metabolize xanthotoxin (28). This and the closely related CYP2A6 isozyme in humans have coumarin 7-hydroxylase activities which are potently inhibited by xanthotoxin and, to a lesser extent, by bergapten (29). Despite these similar biochemical activities, the amino acid sequences for Cyp2a5 and CYP6B1 are highly divergent (less than 25% identity) (9, 30) and their gene structures are entirely different (30). This implies that the vertebrate and insect furanocoumarin-metabolic activities have evolved quite independently of one another and suggests that their transcriptional regulatory patterns may be substantially different.

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