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Identification and Characterization of CYP1 Gene family Enzymes in Xenopus laevis

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Abstract

The cytochrome p450s (CYPs) constitute a large superfamily of heme-containing monooxygenases that metabolize a wide variety of endogenous and exogenous compounds. Genes in the CYP1 family, in particular, are induced by a ligand-bound aryl hydrocarbon receptor and metabolize compounds, such as steroids, pharmaceuticals, and xenobiotics[1]. Gene duplications have resulted in many organisms possessing multiple CYP1s. For example, fish have four CYP1 subfamilies (CYP1A, CYP1B, CYP1C, and CYP1D)[2]. In *Xenopus laevis* (African clawed frog), duplicated genes are common as a result of its tetraploidy[3], which suggests that it, too, may possess multiple CYP1s. Previous studies have confirmed this notion by identifying two CYP1As in *X. laevis,* CYP1A6 and CYP1A7[4]. In this study, we present the identification of two additional CYP1s: CYP1B and CYP1C. The CYP1B and CYP1C genes were identified using homologous sequences from *Xenopus tropicalis* and extended to contain the entire open reading frame (ORF) using 3’ and 5’ rapid amplification of cDNA ends (RACE) reactions. Once the ORF was obtained, it was amplified using PCR and cloned. We ultimately seek to express all of the known CYP1s in *X. laevis* in *E. coli* using the *ompA*(+2) method[5]. The *ompA*(+2) method of heterologous expression utilizes a fusion protein of the CYP1 enzyme combined with a outer membrane protein A sequence (ompA) from *E. coli.* Attaching this sequence to the N-terminus of CYP enzymes has been shown to be an effective means of heterologously expressing CYP proteins in *E.coli*. Once these proteins are effectively expressed, we will be able to assess their activities through various alyoxyresorufin *O*-dealkylase assays.

Objective

- To obtain full length open reading frame sequences for CYP1C and CYP1B from *Xenopus laevis*.
- To generate molecular clones of CYP1A6 and CYP1A7 via RT-PCR.
- To generate a system for heterologously expressing each *X. laevis* CYP1 in bacteria. This will allow us to purify enough of each enzyme so we can conduct activity assays.

Background

- CYP1 expression is induced by the aryl hydrocarbon receptor (AhR), which becomes activated when bound to a ligand, such as the potent environmental toxicant, TCDD, which is also referred to as dioxin.

- *Xenopus laevis,* or the African clawed frog, is extremely insensitive to the toxic effects of various AhR agonists. *X. laevis* CYP1As also exhibit low inducibility in response to the aforementioned AhR agonists, which may play a role in the organism’s insensitivity.

- The CYP1s in *X. laevis* are not well characterized. CYP1A paralogs (1A6 and 1A7) were generated as a result of a gene duplication and may be either redundant or metabolically distinct.

- There are four main CYP1 subfamilies: CYP1A, CYP1B, CYP1C, CYP1D. All of these subfamilies have their own unique metabolic, and thus, physiologic, activities that are typically categorized through various alkoxyresorufin *O*-dealkylase activity assays These assays produce a fluorescent molecule called resorufin. Two examples of this activity are shown below (Fig. 5).

- Humans have three CYP1s, CYP1A1, CYP1A2, and CYP1B1. These are responsible mainly for metabolizing drugs, xenobiotics, and steroids.

- Fish have CYP1s from each of the CYP1 subfamilies, each of which exhibit different alkoxyresorufin*O*-dealkylase activities[2].

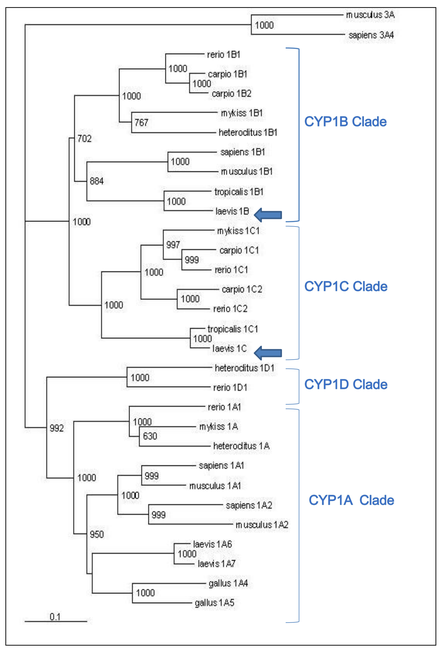
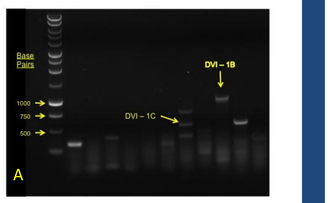


Figure 1: Phylogenetic relationship between *Xenopus laevis* CYP1B and CYP1C and other CYP family proteins. Amino acid sequences of various CYP family proteins were aligned using ClustalX software. A phylogenetic tree was generated using the Neighbor-Joining approach. The number at the nodes represent bootstrap values indicating relative confidence in assembly.

RT-PCR and RACE for X. laevis CYP1B and CYP1C



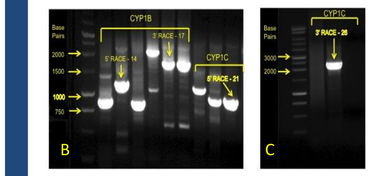


Figure 2A: RT-PCR Products showing partial CYP1B and CYP1C sequences. Total RNA was purified from XLK-WG cells after 24 hr. TCDD exposure (Final Conc. = 500 nM). Partial cDNAs were amplified via RT-PCR using degenerate primers targeting conserved regions of CYP1B and CYP1C amino acid sequences. A 612 bp product encoding 204 amino acids (DVI – 1C) was obtained that exhibited greater than 70% homology to other CYP1C sequences (BLASTx search). A second product of 963 bp encoded 620 amino acids (DVI – 1B) was also obtained that exhibited over 95% homology to various CYP1Bs (BLASTx search).

Figure 2B/C: RACE PCR Products for CYP1B and CYP1C. Using the SMART™er RACE cDNA Amplification Kit (Clontech), I generated RACE ready cDNA from RNA extracted from XLK-WG cells that had been treated with TCDD (500 nM). Gene-specific primers (Fig. 4) were designed to extend the partial CYP1B and CYP1C cDNA products (DVI – 1C and DVI – 1B) in both the 5’ and 3’ directions. In the CYP1B reaction, an 1100 bp 5’ RACE product (14) and a 1700 bp 3’ RACE product (17) were obtained. In the CYP1C reaction, a 900 bp 5’ RACE product (21) and a 2100 bp 3’ RACE product (26) were obtained.

Results: These RACE products were cloned into pCR-II-Blunt-TOPO vectors (Invitrogen), sequenced, and aligned with corresponding CYP1B or CYP1C partial sequences to ascertain complete open reading frame sequences to CYP1B and CYP1C (Fig. 3).

Assembly of CYP1B and CYP1C Open Reading Frame (ORF)

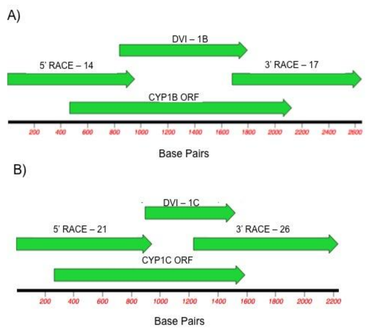


Figure 3: CYP1B and CYP1C ORF Assembly. Full length, continuous CYP1B (A) and CYP1C (B) open reading frames (ORF) were generated by analyzing contiguous segments of RT-PCR cDNA products and RACE PCR products using MacVector 10.6 Assembler and then by identifying regions flanked by start/stop codons. Green arrows indicate position and size of individual PCR products drawn to scale.

Figure 4: PCR primers used in degenerate RT-PCR and RACE reactions.

CYP1C Degenerate Primers:
213for: 5’ - AAYTCNGCRTCRTCGTG – 3’
433rev: 5’ - GGATCRTGRTTNACIGACCAYTGAT – 3’

RACE reaction primers:
5race-1C-4CMK: 5’ – GGTTTGGGAAGGTTAGAAGCCAGG – 3’
3race-1C-82CMK: 5’ – CATTCCCCAGGACACGGTGGTCT – 3’
5race-1C-17CMK: 5’ – CTGCCAGCCCCCACACTCCTTGT – 3’
3race-1C-38CMK: 5’ – GTTCCTGTCACCATTCCCCACGC – 3’

CYP1B Degenerate Primers:
78for: 5’ - TAYGGCAAYRTNTTTCAGAT – 3’
433rev: 5’ - GGATCRTGRTTNACIGACCAYTGAT – 3’

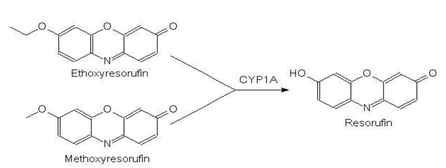
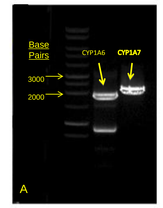


Figure 5: Example of alkoxyresorufin *O*-dealkylase activity. In humans, CYP1A1’s preferred substrate is ethoxyresorufin, while CYP1A2 interacts preferentially with methoxyresorufin.

Molecular Cloning of CYP1A6 and CYP1A7



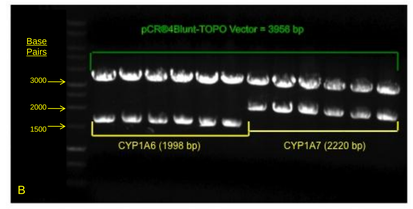


Figure 6: Molecular Cloning of CYP1A6 and CYP1A7. First, cDNA was generated from an RNA prep that was treated with TCDD (500nM). CYP1A6 and CYP1A7 complete sequences (A) were amplified using PCR and the following primers and conditions: CYP1A6: forward primer = 5’ – TCTGGCGAGTTGTTAGGTGCTG – 3’, reverse = 5’ – TTACCTGTCATCGCACCCTTCA – 3’; CYP1A7: forward = 5’ – AAGGAGAACTTTGGGACCTGGAAC – 3, reverse: 5’ – GGGGTACATGCATCCCTGTTCTTT – 3’. Cycling Conditions were: 94C/4min – [94/15s-60/15s-68/5min.] x 35 – 68/5min. PCR products were visualized using agarose gel electrophoresis and EtBr. Full-length sequences were then cloned using the Zero Blunt ® TOPO® PCR Cloning Kit (Invitrogen), cut with EcoRI, and visualized in same way to ensure proper ligation (B).

Conclusions and Future Work

Conclusions:

- In addition to CYP1A6 and CYP1A7, *X. laevis* has CYP1B and CYP1C. The activities of these enzymes are uncharacterized.

- All CYP1s appear to have emerged early in the vertebrate lineage. Some classes have lost one or more CYP1 paralogs.

- **Future Work:**

- Heterologously expressing frog CYP1A6, CYP1A7, CYP1B and 1CYP1C in bacteria can be a complicated procedure. To succeed in this endeavor we have opted to use a procedure called the *ompA*+2 modification strategy[5]. This strategy calls for the fusing one of our p450 sequences to a bacterial leader sequence (*ompA*) that contains two additional spacer amino acids. This method is useful because it avoids the issue of altering the enzyme’s native sequence. Furthermore, once expressed in the bacterium the *ompA* protein is proteolytically cleaved from the enzyme, which then releases the unaltered, native enzyme into the cell where it can easily be purified. The final technique we have employed is to optimize the codons of our sequences for *E. coli* .

- The ultimate goal of this study is to determine the activities of the individual p450s through a variety of alkoxyresorufin *O*-dealkylation activity assays, such as EROD, MROD, PROD and BROD.

- Once these activities are characterized, we will hopefully be able to correlate activity with particular amino acid sequences in the CYP binding domain.

Acknowledgements

- I would like to thank Danny Iwamoto ‘10 for conducting the RT-PCR reaction that originally identified the CYP1B and CYP1C in *X. laevis* and for his help with the phylogenetic tree.

- I would like to thank my faculty mentor, Dr. Wade H. Powell, for his guidance and support throughout my project.

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