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Cytochrome P450 Protocols

SECOND EDITION

Edited by

Ian R. Phillips
Elizabeth A. Shephard

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Second Edition

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Preface

Cytochromes P450 (P450) comprise a large superfamily of proteins that are of central importance in the detoxification or activation of a tremendous number of foreign hydrophobic compounds, including many therapeutic drugs, chemical carcinogens, and environmental pollutants. Many of these enzymes are induced by the compounds they metabolize. In addition, genetic polymorphisms of P450 genes can lead to adverse drug reactions. Consequently, P450s are one of the most extensively studied groups of proteins, being investigated by researchers in fields as diverse as toxicology, pharmacology, genetics, environmental biology, biochemistry, and molecular biology. The wide range of techniques that have been applied to the P450s reflects the diverse backgrounds of the many researchers active in this field.

The second edition of *Cytochrome P450 Protocols* contains a collection of key "core" techniques for the investigation of P450s. Although the emphasis is on P450s of mammalian origin, many of the methods described are suitable for the investigation of P450s from any source. Also included in this edition are chapters on the flavin-containing monooxygenases (FMOs), another family of proteins that are important in the metabolism of xenobiotics, and that share several substrates in common with the cytochromes P450.

Each chapter is written by researchers who have been involved in the development and application of the particular technique to P450s or FMOs. Protocols are presented in a step-by-step manner, with extensive cross-references to notes that highlight critical steps, potential problems, and alternative methods. We hope that this format will enable researchers who have no previous knowledge of the technique to understand the basis of the method and to perform it successfully.

Cytochrome P450 Protocols begins with a chapter on P450 nomenclature and classification, which will serve both as an introduction to those new to the field and as a guide for more experienced workers wishing to name their pet P450. Although not formally divided into sections, the remaining chapters are grouped according to topic. These include methods for spectral analysis and purification of P450s; enzymatic assays of P450s and FMOs; expression of P450s and FMOs in heterologous systems; production and use of anti-peptide antibodies; transfection of hepatocytes for gene regulation studies; P450 reporter gene assays; *in situ* hybridization; analysis of genetic polymorphisms;

and P450 allele nomenclature, including a description of the P450 allele website. Because of the increasing importance of in vitro systems for pharmacotoxicology research, we have included several chapters on the preparation and culture of rodent and human hepatocytes and the production of bone marrow stem cells. The final chapters describe more specialized techniques for the generation of mice with targeted gene disruptions.

We are extremely grateful to all the authors who contributed so generously to this volume and to the *Methods in Molecular Biology* series editor, John Walker, for his guidance and forbearance.

Ian Phillips
Elizabeth Shephard

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1

Cytochrome P450 Nomenclature, 2004

David R. Nelson

Summary

Aspects of cytochrome P450 (CYP) nomenclature are addressed. The rules for naming a P450 are outlined, though individuals should not name their own genes. The nomenclature is presented as a unifying principle to enhance communication across disciplines. Because of the historical nature of gene sequencing, sometimes names have to be changed, but this is kept to a bare minimum to avoid confusion in the literature. CYP names have now reached four digits owing to proliferation of CYP families in the fungi and lower eukaryotes. For example, *CYP5034A1* is from *Ustilago maydis*. P450 sequence motifs are described that are useful in making global alignments. CYP clans are defined as clusters of CYP families. The clan names are useful in describing higher-order evolution of the gene superfamily. The nomenclature of orthologs and pseudogenes is also discussed.

Key Words: Cytochrome P450; CYP; P450 clans; nomenclature; motifs.

1. Introduction

1.1. Moving From Hundreds to Thousands of Sequences

The previous publication of this chapter recognized 753 named P450 sequences in mid-1998. In September 2004, the Cytochrome P450 (CYP) count was 3811 and rapidly moving to 4000. Eukaryotic genomes are being sequenced in months, not years, and annotation has become the rate-limiting step. The nomenclature system for cytochrome P450, first devised in 1987, has become strained, but it is not broken ***(1)***. This system relies on evolutionary relationships as depicted in phylogenetic trees. There is an arbitrary 40% amino acid sequence identity rule for membership in a family and a 55% rule for membership in a subfamily. The actual decision to include a sequence in an existing group largely depends on how it clusters on a tree and not so much on the absolute percentage of identity, which is more or less a rule of thumb. Owing to the great diversity of P450s in insects, fungi, and bacteria, there is a need for additional layers of nomenclature, above the family/subfamily level. This is similar to the multiple levels of the Linnean classification scheme for species. The concept of clans has

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been introduced *(2,3)* as a level above family rank. It is possible that subclans or superclans may be needed, but the exact details of this are only now being discussed by those concerned with P450 nomenclature. The general Web repository for P450 nomenclature and sequence data is http://drnelson.utmem.edu/cytochromeP450.html.

1.2. Nomenclature Is Philosophy

The average person who needs to use a gene name does not consider how the name was arrived at, or what its implications are. Under the 40% rule, a new sequence submitted for naming by an individual may be 38% identical to an existing family such as CYP1, with three subfamilies. On a tree, this family may be well separated from its neighbors, so the new sequence seems to belong in the CYP1 family. In this case, it would become *CYP1D1*. However, someone under pressure for grant funding and tenure decisions, would strongly like to see the new sequence in a new family. A decision must be made based on the best interests of the nomenclature; this may disappoint an individual.

1.3. Nomenclature Is a Unifying Principal

Biochemists, geneticists, molecular biologists, and others who discover genes, often name those genes in an appropriate manner based on how they work or to what pathway they belong. Often, these are very useful names and well understood by researchers in these fields. Frequently, the gene has been named for a mutant phenotype before the gene sequence was discovered. *ERG5* and *ERG11* are two P450 genes in the ergosterol pathway of fungi, but they are also *CYP61A1* and *CYP51F1*. The Halloween genes disembodied (*dib*), phantom (*phm*), shade (*shd*), and shadow (*sad*) are required for ecdysone synthesis and its further metabolism in arthropods. They are embryonic lethal mutations in *Drosophila*. These genes are also Cyp302a1, Cyp306a1, Cyp314a1, and *Cyp315a1*, respectively *(4–6)*. Spook (*spo*) is a fifth Halloween P450 gene, but the sequence has not been revealed yet *(7)*. The two nomenclatures exist side by side and will be used by different groups for different audiences. There is nothing wrong with this. The Cyp names identify these genes as P450 genes. The numbers in the 300 range identify them as animal P450s. Furthermore, *Cyp302a1*, *Cyp314a1*, and *Cyp315a1* are in the mitochondrial clan, whereas *Cyp306a1* is in the CYP2 clan (*see* **Subheading 1.4.**). This information follows from the P450 nomenclature and shows relationships to other P450 genes in these clans. The CYP nomenclature should be cross-referenced when the phenotype-specific names are used.

1.4. Nomenclature May Change

Because names are assigned in historical order, often without the benefit of knowing whole genome P450 collections and/or related genes from other species, some names will need to be revised. When 455 P450s were named from the rice genome, 3 P450s from *Arabidopsis* were found to cluster in new locations on the phylogenetic tree *(8)*. *CYP709A1* and *CYP709A2* became *CYP735A1* and *CYP735A2*, respectively, because they clearly separated from the other CYP709 sequences. *CYP721B1* became *CYP734A1* for similar reasons. From the 272 *Arabidopsis* genes and pseudogenes to the total collection now of more than 1100 plant P450s, only three names were changed

in *Arabidopsis*. In the future, name changes in existing plant sequences should be even more rare or nonexistent, because the nomenclature stabilizes as the sequence space or diversity is more completely known.

1.5. Nomenclature Growing Pains

The 3811 P450s named to date have exceeded the capacity of the original nomenclature system that was based on fewer than 100 P450 families in eukaryotes and an open-ended number in bacteria. The first 100 families were divided among animals (CYP1–49), lower eukaryotes (CYP51–69), and plants (CYP71–99). Bacteria began with CYP101 and could go upward from there. Plants were the first to break the system. The solution was to give three-digit CYP names, allowing for about 1000 new CYP families. The numbering scheme is similar to that of the first 100, except bacteria already were in the 100 and higher range. The four divisions were assigned as follows: bacteria, CYP101–299; animals, CYP301–499; lower eukaryotes, CYP501–699; and plants, CYP701–999. Bacteria, animals, and plants still fit this three-digit system, but lower eukaryotes have already broken the CYP699 limit (*CYP699A1* is from *Agaricus bisporus*, a mushroom). A four-digit system has been set up with similar ranges: bacteria, CYP1001–2999; animals, CYP3001–4999; lower eukaryotes, CYP5001–6999; and plants, CYP7001–9999. This historical naming system may induce some CYP name prejudice, with the one- and two- digit names occupying a privileged place in the eyes of some. *CYP1A1* from vertebrates may be accorded higher status than *CYP5034A1* from *Ustilago maydis*. This is not the intent of the nomenclature, but it may reflect where the early experimental efforts on P450 first uncovered these genes. For instance, there are no vertebrate P450s above *CYP51*.

2. Naming a New P450 Gene

New P450 genes should be submitted to the Committee on Standardized Cytochrome P450 Nomenclature for naming. That committee consists of David Nelson and any other expert needed in case of a tricky nomenclature problem. Usually, that means David Nelson will name the gene, and the sequence is kept confidential. Names should not be assigned by an author without help from this central committee in order to avoid inaccurate or duplicate names. One author named a rabbit gene *CYP12* because it hydroxylated its sterol substrate on the 12 position ***(9)***. However, CYP12 is a family of insect P450s found in mitochondria, so this gene was renamed *CYP8B1*. Because many confidential sequences are named, the only way to be sure one's sequence has not already been assigned a name is to consult the committee. In addition, as described in **Subheading 4.**, there are other considerations, such as orthology, that can affect a name. The 40% rule is a guideline that can be broken for good cause. It is very helpful to do BLAST searches and identify the best matches in the public database, but one should remember that there may be even better matches in the confidential set of P450s.

There is no limit to how many sequences one may submit for naming. The record as of this writing is all the P450s found in *Fusarium graminearum, Magnaporthe grisea, Aspergillus nidulans*, and *Neurospora crassa*. The *N. crassa* genes had already been