INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps. Each original is also photographed in one exposure and is included in reduced form at the back of the book.

Photographs included in the original manuscript have been reproduced xerographically in this copy. Higher quality 6” x 9” black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.

UMI
A Bell & Howell Information Company
300 North Zeeb Road, Ann Arbor MI 48106-1346 USA
313/761-4700 800/521-0600
ISOLATION AND CHARACTERIZATION OF CYTOCHROME P450 3A26

by

David John Fraser

A Dissertation Submitted to the Faculty of the
COMMITTEE ON PHARMACOLOGY AND TOXICOLOGY (GRADUATE)

In Partial Fulfillment of the Requirements
For the Degree of

DOCTOR OF PHILOSOPHY

In the Graduate College

THE UNIVERSITY OF ARIZONA

1998
As members of the Final Examination Committee, we certify that we have read the dissertation prepared by David John Fraser entitled *Isolation and Characterization of Cytochrome P450 3A26* and recommend that it be accepted as fulfilling the dissertation requirement for the Degree of Doctor of Philosophy.

Dr. John Bloom  
Date  6/26/98

Dr. Paul Consroe  
Date  6/26/98

Dr. Henry Yamamura  
Date  6/14/98

Final approval and acceptance of this dissertation is contingent upon the candidate's submission of the final copy of the dissertation to the Graduate College.

I hereby certify that I have read this dissertation prepared under my direction and recommend that it be accepted as fulfilling the dissertation requirement.

Dissertation Director  
Dr. James R. Halpert  
Date  6/13/98
STATEMENT BY AUTHOR

This dissertation has been submitted in partial fulfillment of requirements for an advanced degree at The University of Arizona and is deposited in the University Library to be made available to borrowers under the rules of the Library.

Brief quotations from this dissertation are allowable without special permission, provided that accurate acknowledgement of source is made. Requests for permission for extended quotation from or reproduction of this manuscript in whole or in part may be granted by the head of the major department or the Dean of the Graduate College when, in his or her judgement, the proposed use of the material is in the interests of scholarship. In all other instances, however, permission must be obtained from the author.

SIGNED: [Signature]
ACKNOWLEDGEMENTS

There are a number of people who deserve acknowledgement for their support and guidance during the course of my studies. First and foremost, Dr. James Halpert, my advisor, who has taught me to be a scientist. He has been a great mentor and a close friend. In addition, I wish to acknowledge my committee members, Drs. Henry Yamamura, Paul Consroe, John Bloom and Rene' Feyereisen. All of these men have helped and supported me on my journey toward becoming a better person and researcher.

I also want to thank all of the members of the Halpert laboratory for their support and friendship during these last four years. These people have made my time in the lab enjoyable and have contributed to my development as a scientist in countless ways. I especially want to thank Dr. Greg Harlow for his guidance and advice which proved to be invaluable to me. A special thanks also goes to Dr. David Kroll who instilled in me a passion for science and gave me the tools to become a success.

Thanks also to my parents and brothers and sisters who taught me strength, courage, integrity, and love. Finally, and most importantly, I wish to thank my wife Lisa for her loving support. I could have never done this without you and will love you always.
DEDICATION

This dissertation is dedicated to my parents, Dennis and Darnelle Fraser, and to my wife Lisa. To my parents, who taught me the value of hard work and that the most important things in life are the ones closest to your soul. Thank you for all of your love and guidance. I also dedicate this to Lisa. You are the love of my life.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>LIST OF FIGURES</td>
<td>8</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>9</td>
</tr>
<tr>
<td>LIST OF ABBREVIATIONS</td>
<td>10</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>12</td>
</tr>
<tr>
<td>CHAPTER 1</td>
<td></td>
</tr>
<tr>
<td>INTRODUCTION</td>
<td>14</td>
</tr>
<tr>
<td>Biotransformation</td>
<td>14</td>
</tr>
<tr>
<td>Phase I Reactions</td>
<td>15</td>
</tr>
<tr>
<td>Cytochrome P450</td>
<td>16</td>
</tr>
<tr>
<td>Nomenclature</td>
<td>17</td>
</tr>
<tr>
<td>Early Studies</td>
<td>18</td>
</tr>
<tr>
<td>P450 Oxidation Cycle</td>
<td>19</td>
</tr>
<tr>
<td>Distribution</td>
<td>20</td>
</tr>
<tr>
<td>Catalytic Activity</td>
<td>22</td>
</tr>
<tr>
<td>Regulation</td>
<td>27</td>
</tr>
<tr>
<td>Structure-Function Relationships of Cytochromes P450</td>
<td>32</td>
</tr>
<tr>
<td>Family 3 Cytochromes P450</td>
<td>36</td>
</tr>
<tr>
<td>Research Problems Addressed in this Dissertation</td>
<td>41</td>
</tr>
<tr>
<td>Significance and Implications</td>
<td>42</td>
</tr>
<tr>
<td>Organization of Dissertation</td>
<td>44</td>
</tr>
</tbody>
</table>
TABLE OF CONTENTS - Continued

CHAPTER 2

ISOLATION, HETEROLOGOUS EXPRESSION AND FUNCTIONAL CHARACTERIZATION OF A NOVEL CYTOCHROME P450 3A ENZYME FROM A CANINE LIVER cDNA LIBRARY........................................45

Experimental Methods....................................................49

Results..............................................................................56

Discussion........................................................................66

CHAPTER 3

USE OF CHIMERIC ENZYMES AND SITE-DIRECTED MUTAGENESIS FOR IDENTIFICATION OF THREE KEY RESIDUES RESPONSIBLE FOR DIFFERENCES IN STEROID HYDROXYLATION BETWEEN CANINE CYTOCHROMES P450 3A12 AND 3A26........................................70

Experimental Procedures....................................................74

Results..............................................................................80

Discussion........................................................................92

CHAPTER 4

SUMMARY AND CONCLUSIONS.............................................97

REFERENCES.....................................................................104
LIST OF FIGURES

CHAPTER 1
   Figure 1.................................................................................................20

CHAPTER 2
   Figure 1.................................................................................................51
   Figure 2.................................................................................................57
   Figure 3.................................................................................................60
   Figure 4.................................................................................................61
   Figure 5.................................................................................................64

CHAPTER 3
   Figure 1.................................................................................................77
   Figure 2.................................................................................................81
   Figure 3.................................................................................................84
   Figure 4.................................................................................................89
LIST OF TABLES

CHAPTER 2
Table 1 ................................................................. 65

CHAPTER 3
Table 1 ................................................................. 87

Table 2 ................................................................. 91
**LIST OF ABBREVIATIONS**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-NF</td>
<td>α-napthoflavone</td>
</tr>
<tr>
<td>Ah</td>
<td>aryl hydrocarbon</td>
</tr>
<tr>
<td>ALA</td>
<td>δ-aminolevulinic acid</td>
</tr>
<tr>
<td>androstenedione</td>
<td>androst-4-ene-3,17-dione</td>
</tr>
<tr>
<td>BM-3</td>
<td><em>Bacillus megaterium</em></td>
</tr>
<tr>
<td>CHAPS</td>
<td>3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate</td>
</tr>
<tr>
<td>CYP</td>
<td>cytochrome P450</td>
</tr>
<tr>
<td>DLPC</td>
<td>dilauroylphosphatidylcholine</td>
</tr>
<tr>
<td>DOPC</td>
<td>dioleoylphosphatidylcholine</td>
</tr>
<tr>
<td>E. coli</td>
<td><em>Escherichia coli</em></td>
</tr>
<tr>
<td>EDTA</td>
<td>[ethylenediamine]-tetraacetic acid</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid</td>
</tr>
<tr>
<td>IPTG</td>
<td>isopropyl-β-D-thiogalactopyranoside</td>
</tr>
<tr>
<td>kb</td>
<td>kilobases</td>
</tr>
<tr>
<td>-OH</td>
<td>hydroxy</td>
</tr>
<tr>
<td>P450</td>
<td>cytochrome P450</td>
</tr>
<tr>
<td>PB</td>
<td>phenobarbital</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>sodium dodecyl sulfate-polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>SER</td>
<td>smooth endoplasmic reticulum</td>
</tr>
<tr>
<td>SRS</td>
<td>substrate recognition site</td>
</tr>
<tr>
<td>TAO</td>
<td>troleandomycin</td>
</tr>
<tr>
<td>TCDD</td>
<td>2,3,7,8-tetrachlorodibenzo-p-dioxin</td>
</tr>
<tr>
<td>TLC</td>
<td>thin layer chromatography</td>
</tr>
</tbody>
</table>
ABSTRACT

The objective of these studies was to isolate and characterize novel members of the 3A subfamily of canine cytochromes P450. This subfamily is of interest due to the wide range of endogenous and exogenous compounds metabolized. Previous studies led to the identification, isolation, and heterologous expression of 3A12, the major P450 form in canine liver. This enzyme demonstrated the ability to metabolize steroidal compounds and macrolide antibiotics in reconstituted systems. However, several lines of evidence suggested the presence of additional 3A enzymes with different substrate specificities.

Initial experiments employed the same library used to clone the 3A12 cDNA and led to the isolation of a cDNA encoding P450 3A26. This new cDNA encoded a protein of 503 amino acids with 33 nucleotide differences encoding 22 amino acid variations compared with 3A12. Immunoblots indicated that 3A26 comigrates with a previously unidentified 3A protein in PB-induced canine microsomes. The 3A26 cDNA was modified for heterologous expression and cloned into the pSE380 expression vector. Expression of 3A26 and 3A12 in E. coli was accomplished using slightly modified protocols developed in this laboratory. Steroid hydroxylase assays indicated that these two enzymes have distinct catalytic profiles, with 3A12 exhibiting a rate of 6β-hydroxysteroid product formation ranging from 4 to 50-fold higher than 3A26.

The vast differences in the activities of 3A12 and 3A26 in contrast to their similarity in structure made these enzymes an excellent model system for the identification of structure-function relationships. Studies were done to identify which of the 22 amino
acid residue differences between 3A12 and 3A26 confer differences in rates of hydroxylation of progesterone, testosterone and androstenedione. Ten different 3A12/3A26 chimeras were generated using restriction endonuclease sites. Hydroxylase assays indicated that the first four residue changes and the six differences found within an internal PstI fragment were at least partially responsible for differences in the hydroxylation rates of all three steroids tested. Site-directed mutagenesis revealed the importance of 3A26 residues Ile-187, Ser-368, and Val-369. Conversion of these positions to 3A12 residues increased the rates of 6β-hydroxysteroid formation by 10-20 fold for progesterone, testosterone, and androstenedione. These studies identified a new member of the P450 3A subfamily and are the first to use catalytically distinct cytochromes P450 3A from the same species in the elucidation of 3A structure-function relationships.
CHAPTER 1

INTRODUCTION

Biotransformation

Humans are constantly barraged with a wide variety of natural and synthetic compounds known as xenobiotics. These substances may display a wide range of toxicities in host organisms due to a number of factors including species, route of exposure, age, sex, nutritional state, sickness, and differential metabolic enzyme profiles (Sipes and Gandolfi, 1993). Generally, compounds that are more lipophilic are of pharmacological and toxicological interest as they can be easily absorbed through lipid membranes and accumulate in the organism. Successful and efficient elimination of these compounds is essential for health maintenance.

In humans, elimination of potentially toxic foreign compounds may occur through a number of different pathways including exhalation, perspiration, urination and defecation (Sipes and Gandolfi, 1993). However, lipophilic compounds will remain in tissues and fatty deposits and can accumulate if not converted to more hydrophilic structures that can be dissolved into body fluids (Wilkinson, 1983). Fortunately, systems for the removal of lipophilic compounds through conversion to hydrophilic metabolites have developed in higher organisms. The process by which hydrophobic chemical compounds are transformed by organisms to soluble products is called biotransformation.
Phase I Reactions

There exist two general classes of enzymatic reactions that make up the process of biotransformation. Phase I reactions generally convert hydrophobic chemicals to more hydrophilic metabolites through the addition or unmasking of functional groups (Sipes and Gandolfi, 1993). These groups include hydroxyl, carboxyl and amino moieties, all of which are highly polar and, thus, are water soluble (Jenner and Testa, 1981; Sipes and Gandolfi, 1993). In addition, these exposed functional groups allow for further metabolic conversion by the phase II conjugation enzymes. These enzymes add polar molecules such as glutathione, glucuronic acid, or sulfate to parent compounds as well as to the functional groups of the products of phase one metabolism (Sipes and Gandolfi, 1993). These new hydrophilic conjugates can then be moved across membranes and excreted efficiently.

The addition of functional groups to xenobiotics may lead to their direct elimination from the body and can also alter the toxicity of the compound (Anders, 1985). The reduction of the toxic potential of chemical constituents is known as detoxication, whereas an increase in toxicity of metabolites when compared with the parent compound is known as bioactivation. For example, the conjugation pathways for acetaminophen result in the sulfation or glucuronidation of the parent compound with no resultant toxicity (Sipes and Gandolfi, 1993). However, these pathways can be saturated, resulting in the activation of the parent compound by cytochrome P450 to form a benzoquinone.
intermediate. This reactive intermediate can then bind to cellular constituents, causing necrosis and organ damage.

Phase I biotransformation is accomplished essentially by two distinct classes of enzymes, the amine oxidases and members of the superfamily of cytochromes P450. Both are localized in the endoplasmic reticulum and employ molecular oxygen and the electron donor NADPH in the biotransformation of chemical compounds. It is notable that these phase I enzymes are found within membranes rather than in the cytosol in order to facilitate direct interactions between enzyme and substrate, making biotransformation and subsequent excretion of modified products more efficient. Generally, these enzymes add hydroxyl moieties to substrates via electron and oxygen transfer systems. The cytochromes P450 are the major contributors to phase I biotransformation and are the focus of the studies presented in this dissertation.

Cytochrome P450

Cytochromes P450 are found in essentially all human tissues and organs including liver (Omura and Sato, 1964), lung, intestine (Macica et al., 1993), skin (Jugert et al., 1994), and brain (Foidart, et al., 1994). This superfamily of enzymes is responsible for the vast majority of phase I biotransformation reactions and has been shown to have a major impact on the fate of xenobiotics and endogenous compounds. In addition, a large number of different cytochrome P450 enzymes have been identified, with over 300 genes encoding cytochromes P450 found to date (Nelson et al., 1996). Because of their role in
metabolism as well as the ubiquitous nature of this class of enzymes, they have been the subject of many years of intense study.

**Nomenclature**

The cytochromes P450 are the most versatile biological catalysts found in nature. The basis for the broad substrate specificity was not clear for many years because the vast number of individual members of this superfamily was unknown. Early studies identifying multiple P450 isoforms did provide some evidence that catalytic diversity may be explained in part by multiple enzymes. Due to the great number of isozymes that have been identified, a system of nomenclature has been established to replace the trivial names used previously in independent laboratories (Nelson, et al., 1996).

This system, which is updated regularly, uses a series of numbers and letters to identify P450 genes and the enzymes they encode based on their relative amino acid sequence identity. Genes encoding cytochromes P450 have been separated into different gene families designated by the root *CYP* and a family number, with members sharing greater than 40% amino acid sequence identity. Members of subfamilies share greater than 55% amino acid sequence identity and are given capital letter designations, with individual members of these subfamilies given Arabic numerals. The names for the genes are assigned according to their order of discovery. As an example, the human enzyme that is responsible for erythromycin N-demethylation is a member of family three, which is comprised of the single subfamily 3A and has been designated 3A4. This system of
nomenclature has improved the organization of this field of study and has allowed the laboratories working with members of this superfamily of enzymes to have an established system of isozyme identification, thus improving the ability of different researchers to discuss specific genes and enzymes without ambiguity.

**Early Studies**

The cytochromes P450 were first identified in liver homogenates which displayed the ability to act as mixed-function oxidases in which one atom of molecular oxygen is reduced to water and the other is incorporated into the substrate. It was also found that microsomal preparations from liver had the peculiar characteristic of a visible absorption maximum of 450 nanometers in the presence of a reducing agent and carbon monoxide (Klingenberg, 1958). The exact nature of these mixed-function oxidases remained unclear until the experiments of Sato and Omura in the early 1960s, in which they identified a protein sample that shared these spectral characteristics (Omura and Sato, 1964). The name cytochrome P450 was coined by these researchers at that time based on the spectral absorbance properties and the red pigment of the sample. These seminal studies led to the opening of the field of study of cytochromes P450 and their important role in drug oxidations.

Because the vast majority of cytochromes P450 are membrane bound, the solubilization and reconstitution of these enzymes was challenging. The sterol 11β-hydroxylase from adrenal mitochondria was the first cytochrome P450 system to be
successfully separated into its components (Omura et al., 1966; Lu and Coon, 1968). The methods used in these classic experiments have been successfully employed over the last 30 years of research in this field and have allowed for the functional characterization of hundreds of distinct cytochrome P450 enzymes. With the advent of modern molecular biology, these systems are now used in tandem, providing powerful methods for the examination of complex biochemical questions.

**P450 Oxidation Cycle**

The cytochrome P450 oxidative cycle has been examined in detail and consists of a number of different components that contribute to the oxidation of target compounds as depicted in Figure 1 below. Briefly, the P450 enzyme containing the ferric heme group binds the substrate in the active site, followed by the donation of an electron to the complex by NADPH cytochrome P450 reductase. The reduced P450-substrate complex then binds molecular oxygen. A second electron is then donated to the complex by P450 reductase or cytochrome b5. The resulting intermediate decomposes to water and an unstable oxygen-iron-substrate intermediate. The oxygen atom is then added to the substrate in the form of a hydroxyl group, allowing the oxidized iron to be reformed and reused.
Figure 1. The catalytic cycle of cytochromes P450. Each step between intermediates is numbered within the circle and described in the text. In steps one and two, substrate binds the enzyme followed by the addition of one electron to the complex from NADPH cytochrome P450 reductase. Molecular oxygen then binds to the reduced complex, followed by the addition of a second electron in step 4. Dioxygen cleavage releases a water molecule in step five. This reaction is followed by the addition of the remaining oxygen atom in the form of a hydroxyl group to the substrate in step 6, resulting in the regeneration of ferric iron, and the cycle can repeat.

**Distribution**

Cytochromes P450 have been identified in the majority of species and tissues examined. Early studies employing microsomes from subcellular liver fractions revealed high concentrations of cytochromes P450 (Whitlock and Denison, 1995). In addition, it has been observed that many tissues display the ability to oxidize foreign compounds.
suggesting the presence of cytochromes P450 and other phase I enzymes. However, cytochromes P450 have been shown to be most concentrated in liver, lung, and intestine: tissues having direct exposure to exogenous and endogenous compounds that may be toxic if accumulated in the body (Sipes and Gandolfi, 1993).

Of these organs, the liver has the greatest concentration of cytochromes P450 as a protective mechanism in filtering the blood and removing any harmful substances therein. The liver is responsible for the "first pass effect," receiving blood directly from the intestines before it passes on to the rest of the body. Any ingested harmful or toxic chemicals must pass through the liver before entering the general blood stream and can potentially be removed before serious organismic exposure occurs. This underscores the importance of the liver in xenobiotic metabolism and the significance of the cytochromes P450 found there.

In addition to the liver, the small intestine has been shown to contain substantial concentrations of cytochromes P450, including significant levels of members of the 3A subfamily of cytochromes P450 (Kolars et al., 1991; Holtbecker et al., 1996). These enzymes serve as a barrier defense system that protects the organism by converting lipophilic compounds to polar products before they can pass through lipid membranes, thus preventing xenobiotics from entering the circulatory system. These intestinal enzymes have a substantial impact on drug availability and generate marked variability in systemic concentrations of these compounds. The intestinal flora are also responsible for
the catalytic alteration of exogenous compounds, serving a significant role in protecting the organism from dangerous toxic exposure.

Finally, Clara cells in the lungs have been shown to contain cytochromes P450 from multiple subfamilies that play a role in protecting lung tissues from inhaled and ingested toxicants such as ethanol, 1-nitronapthalene and the nonselective contact herbicide paraquat. However, lung toxicity from bioactivation of these compounds is also significant and has been well documented. From the examples presented here, it is clear that the cytochromes P450 have evolved as specialized enzymes that serve to protect organisms from potentially dangerous exposures to toxic substances that are consumed, inhaled, or generated by the organism, and that bioactivation of xenobiotics can also result from P450 catalytic processes.

Catalytic Activity

The examination of cytochrome P450 activity both in vivo and in vitro are of great significance for a number of reasons. First, the use of animal models has been a foundation of research in examining the fate of xenobiotics. The catalytic profiles of mammalian model systems have been instrumental in the development of new therapeutic agents and in aiding in the determination of potential drug interactions. Also, the cellular effects of exposure to specific chemical compounds can be examined using animal model systems. Moreover, in vivo studies have been the foundation of studies examining the fate
of endogenous and exogenous compounds, as well as the cellular mechanisms employed in the detoxication and bioactivation of xenobiotics.

In addition to \textit{in vivo} systems, \textit{in vitro} systems can also be used to address questions concerning the fate of chemicals. Some of the advantages include the fact that \textit{in vitro} studies are usually faster than studies employing animals, and \textit{in vitro} systems are relatively inexpensive. In addition, studies involving specific cellular components and enzymes can be undertaken, making results less ambiguous than with animal models. However, care must be taken when extrapolating results from external systems back to animal targets. The use of multiple methods of investigation is advisable in order to be able to draw strong conclusions about scientific questions that are to be addressed.

Studies involving members of the cytochrome P450 superfamily of proteins have evolved as the techniques to examine them have developed. Each approach discussed has inherent advantages and disadvantages in the identification of catalytic profiles for cytochromes P450. Early studies focused on the purification of individual isozymes and the identification of substrates metabolized by these protein preparations (Omura and Sato, 1964; Cooper, 1973). The advantages of purification and subsequent reconstitution are that these techniques are straightforward and are very useful when the P450 involved is unknown. However, these techniques are labor intensive and time consuming and the proteins may be difficult to reconstitute successfully. Also, the activity of reconstituted enzymes may not match those identified for these isozymes \textit{in vivo}. DEAE and hydroxylapatite columns have been successfully employed to isolate individual enzyme
preparations which can be used in reconstituted systems to examine the catalytic profiles of these enzymes. However, recent studies have revealed that even small levels of contamination of column purified proteins with other P450 isozymes can result in markedly altered catalytic profiles (Strobel and Halpert). Thus, it is not possible to use these techniques alone in the determination of substrate specificity of cytochrome P450 enzymes.

Along with purification, selective induction and inhibition studies are useful in examining the catalytic profiles of specific cytochromes P450 (Ortiz de Montellano, 1988; Okey, 1990; Correia and Ortiz de Montellano, 1993). These experiments employ inducers such as ethanol or phenobarbital to induce P450 enzyme activity or compounds such as chloramphenicol that inhibit cytochromes P450. The advantages of these experimental techniques are that they are simple to employ and that they may correlate directly with known indices of toxicity. Unfortunately, the specific isozyme(s) affected by such compounds are not revealed in this type of study because both induction and inhibition may be non-selective. For example, phenobarbital induces multiple forms including many family 2 and 3 isoforms in different species (Waxman and Azaroff, 1992). Also, the level of induction or inhibition may not be directly correlated to catalytic activity.

Antibody inhibition may also be used to determine the contribution that a specific P450 may have on catalytic activity. Antibodies are highly specific and are relatively easy to use in \textit{in vitro} systems (Towbin \textit{et al.}, 1979). Antibodies are useful in the identification of the role of known isoforms in catalysis and are a valuable tool in distinguishing the roles
of different isozymes in the same preparations. Unfortunately, antibodies can have cross-reactivity between related isoforms and can also be used only in *in vitro* systems. Moreover, specific antibodies can be difficult and labor intensive to obtain.

With the development of molecular biology techniques, cloning and heterologous expression of specific cytochromes P450 can be a very useful tool in the elucidation of the catalytic activity of these isoforms. Molecular cloning has allowed for the easy and fast generation of specific cytochrome P450 enzymes and has also been pivotal in the success of structure-function analyses. These clones encoding P450 isozymes can provide unambiguous proof that a particular protein catalyzes a specific reaction or forms a specific product. Structure-function analyses have developed with the advent of DNA manipulation techniques such as PCR and site-directed mutagenesis. These tools allow for the examination of specific regions and/or amino acid residues within the enzymes and their roles in the catalytic profiles displayed in reconstituted protein assays.

Unfortunately, the catalytic rates in *in vitro* assays may not correlate directly with those observed in microsomes or *in vivo*. Also, a lack of activity may be due to a wide range of causes and can be difficult to interpret. For example, the enzyme may be inactivated in vitro or may be lacking so particular constituents that alter activity. In addition, in vitro expression may be hampered by the differences between the expression system, such as insect cells or *E. coli*, and the parent organism. For example, if the P450 being examined has been isolated from a mammalian source, it will require N-terminal modification to remove the signal anchor sequence to facilitate expression in *E. coli*. 
These modifications are necessary to insure the production of functional enzyme in a bacterial source that does not have organelles such as smooth endoplasmic reticulum (SER) in which the enzymes are inserted.

Special consideration must also be given when reconstituted systems involving 3A enzymes are used. Members of this subfamily have been resistant to reconstitution, and functional activity is directly dependent upon the reconstitution system employed. For example, it has been shown that phospholipid concentration and type are pivotal, with the use of dioleoylphosphatidylcholine (DOPC) improving activity over that found for dilauroylphosphatidylcholine (DLPC) due to increased negative charge in these systems (Imaoka et al., 1992; Ingelman-Sundberg et al. 1996). In addition, cytochrome b₅ has been found to increase the activity of these enzymes (Shet et al., 1993). Finally, differing buffer systems have been shown to affect the activity displayed by these enzymes (Shet et al., 1993; Imaoka et al., 1992). These findings limit the possibility of direct relationships between these in vitro systems and the in vivo environment.

All of the methods described here have been quite useful in the functional analysis of members of the cytochrome P450 superfamily of proteins. The information generated from studies of regulation and substrate specificity of these enzymes and their catalytic profiles has been shown to be instrumental in individual risk assessment as well as prevention and intervention strategies. Knowledge of individual, general population and sub-population metabolism profiles and tendencies is becoming increasingly helpful in
determining dosing regimens and in the prediction of potential toxicity due to drug interactions or variability in metabolic profiles.

Each of the approaches discussed here can contribute to the elucidation of the roles of individual cytochromes P450 in catalyzing specific reactions. However, it should be stressed that no single technique or methodology can provide complete information concerning the activity of a particular enzyme. Used in conjunction with one another, these approaches are powerful tools in the characterization of individual cytochromes P450 and their influence in catalyzing bioactivation and detoxification reactions within an organism.

**Regulation**

One of the hallmarks of the cytochrome P450 superfamily of enzymes is the wide variety of substrates metabolized. In some cases, the activity of members of separate subfamilies is specific to the chemical structure of target compounds. For example, members of the 1A subfamily have been shown to catalyze the metabolism of polycyclic aromatic hydrocarbons. These processes are mediated by the aryl-hydrocarbon (Ah) receptor, which is directly linked to the regulatory components controlling expression of 1A gene products. The elements responsible for induction of the family 2 and 3 cytochromes P450 have been difficult to identify and have been the focus of major research efforts in the past two decades.
To date, the majority of progress in the field of CYP gene regulation has been on the cytochrome P450 1A subfamily, focusing on P450 1A1 (Whitlock and Denison, 1995). Many factors have aided in the successful analysis of the cellular controls involved in CYP 1A gene regulation. These include the observation that induction of CYP 1A1 with 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) results in significantly higher enzyme activity than observed at basal enzyme levels (Israel and Whitlock, 1984), allowing for reasonable quantitation of and differentiation between induced and uninduced 1A1 enzyme levels in vitro.

In addition to the availability of an excellent inducer, cell culture systems responsive to TCDD mediated induction of P450 1A1 are readily available and conducive to transient transfection studies (Montisano and Hankison, 1985). This availability greatly expands the range of potential studies to include protocols examining gene promoter activity and inducer/enhancer roles in transcription, as well as facilitating the isolation and cloning of specific proteins involved in these regulatory mechanisms (Hoffinan et al., 1991). Finally, the fact that TCDD binds to the AH receptor with high affinity has led to the isolation and subsequent cloning of the receptor as well as related transcription factors (Hoffinan et al., 1991; Burbach et al., 1992). All of these factors have resulted in remarkable advances in understanding of the regulation of the TCDD-inducible CYP 1A1 gene.

Regulation of expression of members of the cytochromes P450 family 2 and 3 has proven to be more elusive than that of family 1 due to the absence of an easily identifiable
receptor that binds either substrate or inducers. It has been well established that
phenobarbital and dexamethasone are inducers of family 2 and 3 cytochromes P450, and
many studies have been done to examine the mechanisms of these cellular processes
(Nebert and Gonzalez, 1987; Okey, 1990). The CYP2 family is the most complex of the
cytochromes P450, consisting of eight subfamilies in mammals. The size of this
superfamily of proteins and the diversity of its members are reflected in the wide range of
regulatory mechanisms that control their expression and activity. Because of their
ubiquitous nature and the wide range of substrates metabolized, members of this family
have been the focus of intense and thorough scientific investigation.

Studies examining the mechanisms by which CYP genes are controlled have been
done in a variety of animal models and systems. The CYP 2 gene family has been the
subject of many of these investigations, having been done primarily in rat, mouse, and
rabbit systems (Waxman and Azaroff, 1992; Gonzalez et al., 1993). The investigation of
the mechanisms of these processes has been reasonably successful, leading to the
identification of many factors and response elements involved in P450 gene transcription
(Gonzalez and Lee, 1996).

The regulation of phenobarbital-inducible cytochromes P450 have also been the
subject of multiple scientific studies. However, the specific mechanisms by which
phenobarbital induces cytochromes P450 have been elusive. A major reason for this is the
lack of high affinity binding of the drug to cellular fractions (Tieney and Bresnick, 1981).
This finding precludes the isolation of a specific receptor mediating transport and/or
signal transduction and subsequent factors involved in these processes (Ortiz de Montellano and Costa, 1986). Moreover, it may well be that no specific receptor mediates these processes. Rather, phenobarbital induction may be the result of direct interactions between drug and transcription factors that affect the expression of specific genes.

Most of the studies done to date have employed either human or rat hepatoma cell lines (Shaw et al.; Yano et al., 1992). Unfortunately, most of these lines are not responsive to treatment with phenobarbital, thus hampering the investigation of the regulatory mechanisms involved in the induction of these genes. The recent development of cell lines and methods of culturing hepatocytes that are responsive to phenobarbital will facilitate transfection studies aimed at identifying the specific promoter regions that interact with trans-activating factors to confer enzyme induction (Lerche et al., 1996; Shaw and Fulco, 1993; Kocarek et al., 1994). The identification of specific regions of DNA that interact with cellular trans-activating factors will result in the isolation and characterization of these elements will aid in the elucidation of the specific mechanisms involved in induction of cytochromes P450 by phenobarbital.

In addition to family 2 cytochromes P450, the regulation of expression of members of the 3A subfamily have also been targeted for numerous studies. Similar to some of the family 2 cytochromes such as 2B1 and 2B2, family 3 cytochromes can be induced with phenobarbital and related compounds (Waxman and Azaroff, 1992; Gonzalez, 1989). These compounds are known as broad spectrum inducers due to the wide range of
enzymes and cellular processes that they affect. For example, PB induces proliferation of hepatic endoplasmic reticulum and increases expression of multiple phase I and phase II enzymes (Remmer and Mercker, 1963; Waxman and Azaroff, 1992). However, unlike the 1A and to some extent the 2B enzymes, the mechanisms by which 3A enzymes are regulated have been elusive and remain largely unknown.

There have been a number of factors that have contributed to the difficulties in the identification of regulatory factors for 3A enzymes. Because broad spectrum inducers such as phenobarbital affect a wide range of cellular systems, multiple mechanisms of action have been suggested for these chemicals. In addition, levels of induction may vary considerably and range from 2 to over 100 fold over basal enzyme levels (Whitlock and Denison, 1995), suggesting different genetic response components for individual genes. Also the lack of a well defined receptor with an appreciable binding constant and selectivity for PB has not been discovered despite numerous investigations. The structural diversity of 3A inducing agents may suggest that a specific receptor may not exist at all.

Some progress has been made toward the elucidation of the cellular mechanisms controlling induction of 3A enzymes. Analyses of the upstream regions of the PB-inducible CYP102 gene from Bacillus megaterium (BM-3) have revealed a 17 base pair region that is involved in the regulation of PB activity (Shaw and Fulco, 1992; Shaw and Fulco, 1993). This region, known as the Barbie Box, has also been identified in similar upstream regions of other genes including those encoding rat CYP 2B1 and 2B2 (He and
Fulco, 1991). Other studies have also provided evidence for a DNA-binding proteins that are PB-dependent (Rangarajan and Padmanaban, 1989).

Other studies have produced evidence for a more indirect mechanism for PB induction in which the PB responsiveness was inhibited by the glucocorticoid antagonist RU-486 (Shaw and Fulco, 1993). These results suggest that PB functions in an indirect fashion to regulate gene transcription. This finding may account for PB induction by a wide range of chemical compounds. In addition to these studies, transgenic mouse lines containing promoter regions from the rat 2B2 gene indicate that multiple regulatory regions exist within these regions (Ramsden et al., 1993). Clearly, the cellular processes regulating PB induction of family 2 and 3 cytochrome P450 genes are complex and elusive. Future studies involving inducible cell lines and other transgenic models will be instrumental and may provide vital insights in addressing these questions.

**Structure-Function Relationships of Cytochromes P450**

In addition to investigation of the regulation of cytochromes P450, numerous studies of the structure–function relationships that exist among related P450 enzymes have been done. Members of the family 2 enzymes have been the focus of great interest for several reasons. First, substrate specificity within this family is relatively high, with most members exhibiting distinct catalytic profiles. Also, a number of highly related members have been isolated within and across species, making functional comparison studies relatively simple. Finally, strict regio- and stereospecificity toward a number of substrates...
such as steroidal compounds has made this class of enzymes a model system for studies investigating the structural components that confer the generation of highly specific products.

Early studies of the family 2 enzymes based on comparative sequence analyses led to the identification of specific regions of these enzymes within the active site that may have direct interaction with substrate molecules (Gotoh, 1992). These regions are known as substrate recognition sites (SRSs) and can be used to predict which residue differences between two target enzymes may be responsible for any differences in catalytic activity observed between them. The SRSs have been used in multiple studies throughout the P450 field and have been shown to be quite accurately predict catalytically significant residues in members of the family 2 enzymes. Recent experiments in our laboratory have also employed the family 2 SRSs to successfully predict important regions of members of the family 3 enzymes, further demonstrating the utility of these alignments.

Other early contributions involved the use of site-directed mutagenesis to confirm the roles of specific residues in the catalytic profiles of family 2 enzymes. Studies of mouse 2A4 and 2A5, which differ by 11 amino acid residues, led to the identification of key amino acid residues including 117, 209, and 365 (Lindberg and Negishi, 1989; Juvonen et al., 1991; Iwasaki et al., 1993), as pivotal in coumarin and steroid hydroxylase activities. Members of the 2B subfamily have also been of interest, with the early identification of hydrophobic residues Leu-58 and Ile-114 found to play roles in the ability of rat 2B2 to 16β-hydroxylate testosterone and androstenedione (Aoyama et al., 1989).
These seminal studies opened the door to rigorous examination of structure-function relationships among highly related members of the cytochromes P450 superfamily of proteins.

Early studies of family 2 enzymes in the Halpert laboratory led to the identification of amino acid residues from rat, rabbit, and dog members of the 2B subfamily that are crucial in substrate specificity determination. These experiments identified residues 114 and 478 of rat 2B1 as playing important roles in the regio- and stereospecific hydroxylation of androstenedione and testosterone (He et al., 1992; Halpert and He, 1993). It was found that when Ile-114 was converted to Val, and Gly-478 was changed to either Ala or Ser, the ratio of 15α-hydroxysteroid production to 16β-hydroxysteroid could increase as much as 1000 fold. These studies were followed by further mutagenesis experiments that identified residues 363 and 206 as crucial for the alteration of rat 2B1 from a predominant 16-hydroxylase to a 2A4-like 15α-hydroxylase (Luo et al., 1994). These changes converted Phe-206 and Val-363 to Leu and Ala, respectively, as found in murine 2A4 enzymes. This interconversion of activities set the stage for a wide range of new studies in which differing catalytic profiles could be examined rigorously. Seminal studies were followed up with further mutagenesis experiments identifying additional residues 302 and 367 of 2B1 within the SRSs as significantly altering regio- and stereoselectivity of these enzymes (He et al., 1994). Importantly, the size of the residues selected was shown to have an important role in governing steroid hydroxylation from the α-face or β-face of the of the substrate.
In addition to the studies done with rat cytochrome P450 2B1, a number of different model systems from this subfamily have been examined by members of our group. The characterization of canine 2B11 led to the identification of residues Val-114, Asp-290, and Leu-363 as determinants of substrate specificity in 2B11 (Kedzie et al., 1991; Hasler et al., 1994). More recent findings have demonstrated that the replacement of the negatively charged Asp-290 in the 2B11 system results in the wholesale reduction of activity in the mutant proteins. These results underscore the impact that a single specific residue may have on the catalytic profile of a P450 enzyme.

Studies involving rabbit 2B4 and 2B5 have also been undertaken. These two enzymes are highly related, differing at only 11 amino acid residues, yet display unique steroid hydroxylase activities (Gasser et al., 1988; Szklarz et al., 1996). For example, 2B4 hydroxylates androstenedione selectively at the 16β position, whereas 2B5 produces 16α- and 15α-hydroxy products as well. In addition, benzyloxyresorufin and 7-ethoxycoumarin are oxidized readily by 2B4, but not by 2B5. The experiments undertaken in our laboratory employed a number of different techniques including the generation of hybrids through the use of restriction endonuclease sites, site-directed mutagenesis, and computer modeling to determine which of the residue differences might be responsible for the differences observed between the two enzymes. Residues 114, 294, 363, and 367 were found to be crucial in the interconversion of activity between 2B4 and 2B5, residues that align with previous findings for P450s 2B1 and 2B11. Significantly, these studies demonstrate the efficient employment of biochemical and molecular biology
techniques to successfully address scientific questions in an accurate and efficient manner. All of the previous work done on the family 2 enzymes has greatly enhanced our understanding of the structure-function relationships that exist among members of the cytochrome P450 superfamily of proteins. Moreover, these enzymes make significant contributions in human drug metabolism and these studies of their activity will aid in our ability to predict the catalytic profiles and potential drug interactions for individual patients as well as for large human populations.

**Family 3 Cytochromes P450**

The members of family three are unique among cytochromes P450 for a number of reasons. Comprised of the single subfamily, 3A, they are the most abundant of all of the cytochrome P450 enzymes in humans, accounting for as much as 55% of the P450 in human liver (Shimada et al., 1994). The substrate specificity of members of the 3A subfamily is extremely broad, metabolizing such diverse compounds as steroids (Waxman et al., 1988), macrolide antibiotics such as erythromycin and troleandomycin (Wrighton et al., 1985), the calcium channel blockers nifedipine and diltiazem (Guengerich et al., 1986), several carcinogens including benzo(a)pyrene and aflatoxin B₁ (Shimada et al., 1989; Gallagher et al., 1994), and the immunosuppressive agent cyclosporin (Kronbach et al., 1988).

These enzymes are also ubiquitous, having been identified in liver, kidney, lung, skin, intestine, and brain. Additionally, their catalytic activity is easily modified by a
number of agents including phenobarbital, glucocorticoids like dexamethasone, and macrolide antibiotics. Their distribution throughout mammalian tissues confirms the profound impact that 3A enzymes have on the metabolism of endogenous and exogenous compounds. Additionally, P450 3A effectors have also been identified, including the flavonoids such as α-napthoflavone (α-NF), that can stimulate and inhibit 3A activity (Huang et al., 1981; Schwab et al., 1988; Shou et al., 1994). Many recent studies have focused on the elucidation of the mechanisms of these processes as they relate to the catalytic profiles of these proteins. Moreover, all of these factors contribute to the importance of members of this subfamily and underscore the significance of elucidating the mechanisms that influence the expression and function of 3A enzymes.

Despite the vast array of substrates affected by 3A enzymes, the regio- and stereospecificity of these enzymes is quite rigid, generating very specific profiles for individual substrates. Unlike the cytochromes P450 of family 2, 3A enzymes within or across species exhibit no dramatic differences in substrate specificity. Additionally, heterologous expression systems for the 3A enzymes have been difficult to employ, as reconstitution of these enzymes has proven unreliable. Moreover, catalytic activities obtained in vitro have varied widely and cannot reliably be compared to one another or with in vivo findings. These characteristics have impeded rigorous scientific examination and have limited the avenues for investigation of this important class of enzymes.

Recently, significant progress has been made in the characterization of 3A cytochromes P450. Modification of the sequences encoding 3A enzymes that enable
expression in *E. coli* systems and reconstitution of these enzymes using negatively charged phospholipids such as DOPC have helped to improve and standardize *in vitro* assays employing heterologously expressed 3A proteins (John *et al.*, 1994, Born *et al.*, 1996). Additionally, the advent of molecular modeling techniques have proven effective in predicting potential structure-function relationships (Szklarz and Halpert, 1997).

Studies by our group and others have begun the process of evaluating cytochromes P450 3A at the molecular level. The focus of these studies has been on human 3A4, the most abundant and active of the human 3A enzymes. Successful expression and reconstitution of 3A4 in *E. coli* were the essential first steps in the early analyses of these proteins (Gillam *et al.*, 1993). Mutagenesis and computer modeling have since been used in the identification of important regions and residues that affect 3A4 substrate specificity. An excellent starting point for these studies was the proposition that the substrate binding sites proposed by Gotoh might also be used in alignment studies to suggest regions of interest in the 3A enzymes. Thus, alanine scanning mutagenesis in the region of 3A4 that corresponds to SRS2 was undertaken, leading to the identification of residues 210 and 211 as prominent contributors to substrate specificity (Harlow and Halpert, 1997). It was found that the conversion of leucine residues at these sites to alanines resulted in the alteration of the regioselectivity of testosterone hydroxylation. Additionally, an altered response to the 3A effector α-napthoflavone was observed, with diminished responses over a range of substrate concentrations.
Site-directed mutagenesis of residues aligning with SRS5 also led to the identification of residues 369, 370 and 373 as effectors of steroid hydroxylase activity. Catalytic activities of 12 distinct mutants were examined using progesterone and testosterone as substrates. Although the three important mutants hydroxylated the steroid substrates at the 6β position similar to that of wild-type enzyme, L369V showed suppressed progesterone 16α-hydroxylase activity. Alternatively, A370V exhibited enhanced progesterone 16α-hydroxylase activity. and L373H generated an entirely new product from both progesterone and testosterone. Other studies examining SRS4 residues 301, 304, 305, and 309 indicate that these residues are important in α-naphthoflavone stimulation of steroid hydroxylase activity and/or in the stereo- and regioselectivity of these enzymes (Domanski et al., 1998). Moreover, assessment of the kinetics of progesterone hydroxylation indicated that progesterone may act as an activator similar to α-NF at high substrate concentrations.

All of these current studies have been based on molecular models and alignment comparisons with bacterial and family 2 cytochromes P450, respectively. These systems have proven to be very useful in the identification of regions and specific residues that are important in the catalytic activity of mammalian 3A enzymes. Unfortunately, no members of the 3A subfamily from the same species yet having distinct catalytic profiles have been identified to date, hindering structure-function analyses considerably. It would be useful to identify highly related members of the 3A subfamily that do not share such closely overlapping substrate specificities in order to advance our understanding of the structure-
function relationships of this important class of catalytic enzymes. The studies presented in this dissertation address these issues, using canine cytochromes P450 3A12 and 3A26 as a model system for the examination of questions concerning the structural components that influence the catalytic activity of members of the 3A subfamily of enzymes.
Research Problems Addressed in this Dissertation

The studies described in this dissertation were undertaken in an effort to isolate novel members of the cytochrome P450 3A subfamily of enzymes and to elucidate any differences in catalytic activity between them based on differences in molecular structure and enzymology. These studies are divided into two main sections. Chapter 2 describes the isolation and characterization of a novel canine 3A enzyme, 3A26. Chapter 3 details the identification of key residues that are responsible for the differences in catalytic activity observed between the 3A26 and the previously characterized canine 3A12. The research described herein is based on the hypothesis that novel canine cytochromes P450 3A exist and that they have distinct catalytic profiles. The specific aims of these investigations and the central subject of the research described in the following chapters were to 1) isolate and clone a novel canine P450 3A cDNA from a canine liver cDNA library, 2) to heterologously express and characterize the activity of this novel enzyme, 3) to compare the activity of the novel 3A26 to that of the previously characterized canine P450 3A12 enzyme, 4) to use hybrid enzymes and site-directed mutagenesis to identify the specific amino acids responsible for differences in catalytic activity between 3A12 and 3A26.
Significance and Implications

The canine is a model drug metabolizing system used extensively in the pharmaceutical industry. However, mechanisms of regulation and substrate specificities of canine cytochromes P450 are not as well known as for rodent and human enzymes. The objective of this research has been to isolate, heterologously express, and characterize novel cytochromes P450 and to elucidate the structural differences that confer functional variations between these highly related enzymes.

Modern molecular biology techniques have facilitated many of the advances in knowledge of the cytochrome P450 systems examined here. Such experiments as the isolation of novel cDNAs encoding new cytochromes P450, cloning strategies, successful heterologous expression, generation of hybrids using convenient restriction endonuclease sites, and site-directed mutagenesis have all become routine using molecular biology tools. All of these tools have been used successfully, coupled with advanced enzymology for the evaluation of the catalytic activities of the enzymes being studied.

Previous studies using family 2 and human 3A enzymes have also been essential to the design of the project. These experiments have led us to the understanding of the fundamental mechanisms of these complex systems, allowing us to draw correlations and optimize our chances of success. Important regions and specific residues of other cytochromes P450 from these studies directly influenced our choices as to how these investigations could best be done and which residue differences should be targeted for analysis.
The current investigations have resulted in the identification of 3 residue differences that are responsible for the substantial differences in substrate specificity between canine cytochromes 3A26 and 3A12. Further evidence exists implicating one of these differences in alteration of human cytochrome P450 3A4 catalytic activity. The importance of findings that confer the ability to predict changes in the activity of cytochromes P450 across species is of great interest and importance. The ability of the cytochromes P450 to catalyze specific reactions in vitro can now be correlated somewhat to activities found in vivo. These profiles are therefore significant in the areas of drug development and metabolism, where the ability to predict specific metabolism profiles and potential drug interactions is of paramount importance. The studies described here contribute to that body of knowledge and may be useful in the determination of these parameters.
Organization of Dissertation

The specific aims of this research have been addressed in the following chapters of this dissertation. Chapter 2 describes the isolation, heterologous expression, and catalytic characterization of the novel canine cytochrome P450 3A26. Chapter 3 details the identification of three specific residues responsible for catalytic differences between 3A12 and 3A26 as determined by hybrid enzymes and site-directed mutagenesis. The research described herein has been published or will be submitted for publication to peer-reviewed scientific journals. Each chapter contains its own introductory statements regarding the research questions addressed, followed by the methods used, the results obtained, and a discussion. A final summary chapter contains a discussion of the major conclusions of my work.
CHAPTER 2

ISOLATION, HETEROLOGOUS EXPRESSION AND FUNCTIONAL CHARACTERIZATION OF A NOVEL CYTOCHROME P450 3A ENZYME FROM A CANINE LIVER CDNA LIBRARY

A cDNA encoding a new member of the cytochrome P450 3A subfamily, P450 3A26, has been isolated from phenobarbital-induced canine liver. The sequence encodes a protein of 503 amino acids with 33 nucleotide differences conferring 22 amino acid substitutions when compared with the previously identified canine CYP3A12 enzyme. Nine of the amino acid differences are within the substrate recognition sites (SRSs) identified for P450 family 2, with five residue substitutions clustered within SRS-6. To facilitate heterologous expression in *Escherichia coli*, the N-terminus of 3A26 was modified. The expressed protein comigrated with a 3A-immunoreactive protein in dog liver microsomes with a slightly greater electrophoretic mobility on SDS-PAGE than 3A12, suggesting that 3A26 corresponds to a previously noted but never characterized 3A enzyme in dog. Functional characterization of 3A26 was undertaken using progesterone, testosterone, and androstenedione as substrates. Assays of expressed 3A26 and 3A12 demonstrated that 3A26 displays low steroid hydroxylase activity. Identification of an additional canine 3A enzyme should increase our understanding of xenobiotic metabolism in this important animal model. These findings also suggest that 3A26 and 3A12 may be
an interesting model system for the investigation of structure-function relationships involved in steroid metabolism catalyzed by members of the cytochrome P450 3A subfamily.

Cytochromes P450 constitute a superfamily of hemoproteins that play a central role in the metabolism of a wide variety of xenobiotics and endogenous compounds. In the past decade, the study of these enzymes has been greatly advanced by the cloning of P450 cDNAs and expression of these proteins in heterologous systems. P450 family 3, which consists of the single subfamily 3A, is of particular importance due to its metabolism of a wide range of pharmacologically, physiologically, and toxicologically important agents. Compounds metabolized by P450 3A enzymes include macrolide antibiotics such as erythromycin and triacytyloleandomycin (Wrighton et al., 1985), the calcium channel blockers nifedipine and diltiazem (Guengerich et al., 1986), the immunosuppressive agent cyclosporine (Kronbach et al., 1988), steroidal compounds (Waxman et al., 1988), and a number of carcinogens including benzo(a)pyrene and aflatoxin B1 (Shimada et al., 1989; Gallagher et al., 1994). In addition, adverse pharmacokinetic drug interactions have been observed clinically with the concomitant use of multiple drugs that are metabolized by 3A enzymes (Periti et al., 1992). In spite of these numerous studies, relatively little is known about the structure-function relationships of previously identified 3A enzymes. Unlike cytochromes P450 of family 2, 3A enzymes within or across species exhibit few dramatic substrate specificity differences that could provide obvious leads for site-directed mutagenesis of particular residues. Identification
of substrates that can distinguish the various members of the 3A subfamily or of novel enzymes with altered substrate specificity would be very useful in the determination of the roles that specific residues play in conferring the distinctive catalytic activities of the 3A subfamily.

The rat, human, and mouse P450 3A subfamilies have been shown to consist of multiple members, which differ in their regulation (Nelson et al., 1996). Canine models have been used extensively in drug metabolism studies, but to date, only a single canine cytochrome P450 3A enzyme, 3A12, has been isolated (Ciaccio et al., 1991). Considerable evidence suggests that multiple canine 3A forms exist and that these may have different catalytic properties. Immunoblots of liver microsomes from phenobarbital-treated dogs were probed with a polyclonal antibody generated against canine hepatic 3A12. The results indicated the presence of two distinct proteins, one with an apparent molecular weight of 51 kDa, corresponding to 3A12 and the second with an apparent molecular weight of 49.5 kDa (Ciaccio et al., 1989). In addition, previous studies demonstrated differences between the 6β-hydroxylation of steroids and troleandomycin (TAO) complex formation in canine liver microsomes. In particular, although both reactions were induced by phenobarbital and inhibited by antibodies to 3A12, TAO-P450 complex formation had little effect on steroid 6β-hydroxylation. These data suggested that some PB-inducible cytochrome P450 3A other than P450 3A12 might be responsible for TAO complex formation in dog liver microsomes. Finally, complex Southern blot hybridization patterns were observed when canine genomic DNA was probed with the
3A12 cDNA (Ciaccio et al., 1989). Thus, several lines of evidence support the hypothesis that multiple canine cytochromes P450 3A exist, and that these enzymes may differ in their substrate specificity.

This study describes the isolation of a cDNA encoding a novel member of the cytochrome P450 3A subfamily, P450 3A26, and its subsequent heterologous expression and functional characterization. Degenerate oligonucleotide PCR techniques were used to probe the cDNA library, resulting in the isolation of a 1.97 kbp fragment encoding the 3A26 enzyme. The cDNA encodes a protein of 503 amino acids and differs from 3A12 at 22 amino acid positions. 3A26 was expressed in E. coli as described previously (Barnes et al., 1991; Born et al., 1996). The different catalytic activities ascribed to this enzyme will be useful in the elucidation of structure-function relationships between 3A26 and the previously identified 3A12.
EXPERIMENTAL PROCEDURES

**Materials.** Restriction endonucleases and media for bacterial growth were purchased from GIBCO-BRL (Grand Island, NY). The pKK233-2 and pSE380 expression plasmids were purchased from Pharmacia (Alameda, CA). Primers for PCR amplification were obtained from the University of Arizona Macromolecular Structures Facility (Tucson, AZ). PCR products were purified using the GeneClean II kit from Bio101 (Vista, CA). TOPP3 cells were obtained from Stratagene (La Jolla, CA). CHAPS, progesterone, testosterone, androstenedione, erythromycin, troleandomycin, NADPH, and DOPC were purchased from Sigma Chemical Co. (St. Louis, MO). (4-\(^{14}\)C) Testosterone was obtained from Amersham Life Science (Arlington Heights, IL). (4-\(^{14}\)C) Progesterone and (4-\(^{14}\)C) androstenedione were obtained from Dupont-New England Nuclear (Boston, MA). HEPES was purchased from the Calbiochem Corp. (La Jolla, CA). Thin-layer chromatography plates (silica gel, 250 μm, Si 250 PA (19C)) were purchased from Baker (Phillipsburg, NJ). All other reagents and supplies not listed were obtained from standard sources.

**Isolation and sequencing of the cDNA encoding P450 3A26.** A λgt11 cDNA library previously generated and used to isolate the canine 3A12 cDNA (Ciaccio et al., 1991) was employed for the isolation of the cDNA encoding P450 3A26. The overall cloning scheme is presented in Figure 1. PCR was used in conjunction with degenerate oligonucleotide primers to probe the canine cDNA library for 3A sequences. The N-
terminal [5'-TTTGC(GT)GG(AGCT)TATGA(AG)AC(AC)AC(AGCT)AGCAG-3'] and C-terminal [5'-CCTCAT(GT)CCAA(GT)GCA(AG)TT-3'] degenerate primers were based on highly conserved regions of mammalian P450 3A sequences, corresponding to amino acid residues 304-311 and 441-446 of 3A12 respectively. Use of these primers resulted in the amplification of a 0.4 kbp product (Figure 1, Step 1). Reaction conditions were: one cycle of 94°C for 2 minutes, 54°C for 2 minutes, and 72°C for 2 minutes, followed by 30 cycles of 94°C for 1 minute, 54°C for 1 minute, and 72°C for 1 minute. The total reaction volume was 100 μl and all reactions were done in duplicate. A 20-μl aliquot of each reaction was run on a 1.2% agarose gel, and a single band at 0.4 kbp was identified and excised from the gel. The PCR products were isolated from the gel using the GeneClean II kit (Bio101, Vista, CA) and cloned into the pCRII cloning vector using the TA Cloning Kit (Invitrogen, San Diego, CA). Two types of clones could be distinguished. One type was identical in sequence to 3A12, and the other type possessed a Sau961 site and lacked an Ear I site when compared with 3A12.
Step 1  
cDNA Library  
| PCR with 3A Degenerate Primers  

Sau96 I  
3A26 Fragment

Step 2  
PCR of Putative 3A Clones  
Using Universal Primers

Sau96 I  TGA

Step 3  
PCR of cDNA Library Using  
Selective 3A26 Primer and  
Universal Primer

ATG Sau96 I TGA

Figure 1. A representation of the cloning steps that led to the isolation of the cDNA encoding cytochrome P450 3A26. In Step 1, degenerate oligonucleotide primers based on conserved mammalian 3A sequences were used in conjunction with PCR to generate fragments of cDNAs encoding members of the canine 3A subfamily including 3A12 and 3A26. Only the 3A26 fragment is depicted in this figure. This fragment carried several sequence differences when compared to 3A12, resulting in the identification of a Sau96 I restriction endonuclease site not found in 3A12. In Step 2, previously isolated clones
from a screen of λgt11 phage carrying canine cDNA sequences which interacted with 3A oligonucleotide probes were analyzed for the presence of Sau96 I restriction sites as a means of identifying novel 3A clones. PCR of these λgt11 clones using universal primers (gray boxes) and subsequent restriction analyses resulted in the identification of a cDNA clone encoding a partial 3A26 sequence. In Step 3, primers based on differences between 3A26 and 3A12 sequences found 3’ to their respective translation stop sites and selective for 3A26 (black box) were used in conjunction with a universal primer to generate a PCR product encoding the entire coding region of 3A26. This sequence was subsequently cloned into the pCRII cloning vector and sequenced. Step 3 reactions were done in duplicate and sequences of individual clones isolated from separate reactions were compared to insure the fidelity of the PCR reactions.

These sequence variations were used to re-examine 13 clones isolated in the original homology screen of a λgt11 cDNA library that produced the 3A12 cDNA (Ciaccio et al., 1991) (Figure 1, Step 2). The cDNA inserts were amplified from the phage isolates using λgt11 forward and reverse primers. Two types of phage inserts were isolated in these experiments, the previously identified 3A12 and a variant clone lacking an Ear I site and possessing a Sau96 I site. However, because none of the variant clones were full length, it was necessary to go back to the library to isolate a cDNA encoding the complete 3A enzyme coding region (Figure 1, Step 3). A region after the translation stop site was used to design a primer specific for the novel cDNA. That primer, 5’-

AACCGGATAGGTTGAGTCTAC-3’, was used in conjunction with the forward λgt11 primer to produce a 1.8 kbp PCR product, which was cloned into the pCRII cloning vector and sequenced.
N-terminal modification and heterologous expression of 3A26. The N-termini of canine 3A26 and 3A12 are identical in sequence until the first variation is encountered at base pair 295. Modifications to the N-terminus of 3A12 have been described previously (Born et al., 1996). Restriction endonucleases and subcloning were used in the modification of 3A26 for expression in *E. coli*. The unmodified N-terminus of 3A26 was removed using *Nco I* and *Sca I* restriction endonuclease sites and replaced with the corresponding fragment from the modified N-terminus of 3A12. These alterations removed 10 amino acids in the signal anchor sequence of 3A26 and changed the second amino acid residue from aspartic acid to alanine, changes that have been shown to facilitate expression in *E. coli* (Barnes et al., 1991; Born et al., 1996). Both 3A26 and 3A12 constructs were subsequently subcloned into the pSE380 expression vector using an *NcoI* site at the 5'-end and *Bam HI* or *Hind III* sites at the 3'-end, respectively.

Heterologous expression and preparation of solubilized *E. coli* membranes was done essentially as described previously (John et al., 1994). Briefly, pSE3A26- and pSE3A12-containing TOPP3 *E. coli* cells were grown at 37°C with 240 rpm shaking in 250 ml liquid TB media (12 g Bacto tryptone, 24 g Bacto yeast extract, 4 ml glycerol / L to mid log phase. IPTG (final conc. 1.0 mM) and 80 mg / L δ-aminolevulinic acid (ALA) were added, and cells were harvested after an additional incubation at 30°C with 190 rpm shaking. Maximum expression of 3A26 was observed at 42 hours after IPTG/δ-ALA addition, and typically 5-8 nmol of P450 3A26 was recovered per liter of culture.
Maximum expression of 3A12 was observed at 72 hours after IPTG/δ-ALA addition and yields of 40-55 nmol per liter of culture were routine.

**Immunohchemical detection of canine cytochromes P450 3A from**

**heterologous expression systems and PB-induced canine liver microsomes.**

Polyclonal antibodies raised against canine cytochrome P450 3A12 were isolated and characterized previously (Ciaccio et al., 1989). Analyses of purified proteins, heterologously expressed proteins, and microsomes by SDS-PAGE (8% polyacrylamide gels) were performed essentially as described by Laemmli et al. (Laemmli, 1970) and resolved proteins were stained with Coomassie blue or transferred to nitrocellulose membranes.

**Functional characterization of *E. coli*-expressed canine P450s 3A26 and 3A12.** Steroid hydroxylase assays were performed using CHAPS-solubilized *E. coli* membrane preparations of 3A26 and 3A12 as described previously (John et al., 1994; Born et al., 1996). Briefly, 10 pmol P450 was reconstituted with 40 pmol *E. coli* expressed rat NADPH-P450 reductase, 10 pmol rat cytochrome b₅, and 0.1 mg / ml dioleoylphosphatidylcholine (DOPC) in a minimal reaction volume. Assays were performed in 100 μl of 50 mM HEPES (pH 7.6) with 15 mM MgCl₂ and 100 μM EDTA, 0.1 mg / ml DOPC, and 0.06% CHAPS. Steroid stock solutions were made in 100% methanol. Methanol concentrations in all reactions were equivalent and did not exceed 1% of the total reaction volume. Individual testosterone, progesterone and androstenedione concentrations were 250 μM for all catalytic assays performed.
Identification of metabolites was by relative mobility on TLC and by comparison to authentic standards (Waxman, 1991).
RESULTS

Isolation of a cDNA encoding canine cytochrome P450 3A26. The isolation of a cDNA clone encoding P450 3A26 is described in Materials and Methods and outlined in Figure 1. Notably, both 3A12 and 3A26 were isolated from a cDNA library created with RNA from a single phenobarbital-induced dog liver, eliminating any possibility that the differences identified are due to strain or inter-individual variations. In addition, PCR reactions were done in duplicate and one 3A26 clone from each reaction was isolated and analyzed via dideoxy-sequencing. These sequences were found to be identical, indicating that differences in DNA sequence between 3A26 and 3A12 are extremely unlikely to be a result of PCR error.

A comparison of P450 3A26 with the previously published canine P450 3A12 sequence is shown in Figure 2. Both enzymes are 503 amino acids in length and share 95.6% amino acid identity. The two enzymes exhibit 33 nucleotide and 22 amino acid differences. Most of the amino acid differences are found in the C-terminal half of the sequence. It is also interesting to note that the 5'-untranslated region of 3A26 is identical in sequence to that found in the 3A12 clone, whereas significant variations are present in the 3'-untranslated regions of 3A26 and 3A12. These differences allowed for the design of a primer selective for 3A26, thus facilitating the isolation of the clone encoding the entire sequence from the λgt11 cDNA library (Figure 1, Step 3).
Figure 2. The complete sequence of cytochrome P450 3A26 isolated from a canine cDNA library. The sequence for 3A26 was determined and amino acids were deduced. The nucleotide and amino acid residue differences identified within the coding regions of 3A12 compared to 3A26 are shown above and below those of 3A26, respectively. The 5'-and 3'-noncoding regions of 3A12 were not aligned. A total of 33 nucleotide differences conferring 22 amino acid alterations were identified within the coding regions of the two sequences.
Putative substrate recognition sites (SRSs) (Gotoh, 1992) for P450 family 2 enzymes are predicted to contain key residues involved in enzyme-substrate interactions. The limited number of amino acid differences between 3A26 and 3A12 present interesting experimental possibilities in the investigation of structure-function relationships of P450 enzymes. Figure 3 graphically represents the positions of amino acid sequence differences between 3A12 and 3A26. Putative 3A SRSs have been indicated, and the specific residue differences between 3A12 and 3A26 within these regions are noted by showing the one-letter code for the 3A12 residue, the position, and the single letter code for the 3A26 residue. Changes in residues outside of the SRSs are noted with asterisks. The majority of the changes found in the SRSs occur in sites 5 and 6, with only one difference in each of SRSs 2 and 4. No differences in amino acid sequence were observed in SRSs 1 or 3.
Figure 3. An alignment of the relative positions of variations in amino acid sequence between 3A12 and 3A26 with the proposed substrate recognition sites for P450 family 2 enzymes. Restriction sites used to generate hybrid proteins are noted above sequence lines. SRSs are denoted with thick black lines. Residue differences between 3A12 and 3A26 found within SRSs are identified below SRS lines. Residue differences found outside of SRSs are denoted with asterisks.

Comparison of differences found in P450 3A26 with analogous residues in other mammalian members of the cytochrome P450 3A subfamily. Many of the residue differences identified between cytochromes P450 3A26 and 3A12 occur at
positions that are generally well conserved in other mammalian P450 3A enzymes. Figure 4 represents a comparison of residue differences within the putative SRSs found in 3A26 with other mammalian 3A enzymes. 3A26 has significant alterations in amino acid sequence in terms of residue volume, charge, and hydrophilicity in these SRSs. For example, serine residues are found at positions 368 and 474 in 3A26, whereas proline is found at those positions in all other mammalian 3A sequences. In addition, the highly conserved lysine residue at position 476 has been replaced by a larger arginine residue in 3A26. These differences may prove to be of significance in determining the metabolic profile of this enzyme.

<table>
<thead>
<tr>
<th>209</th>
<th>301</th>
<th>368</th>
<th>369</th>
<th>474</th>
<th>476</th>
<th>477</th>
<th>479</th>
<th>480</th>
</tr>
</thead>
<tbody>
<tr>
<td>3A26</td>
<td>N</td>
<td>V</td>
<td>S</td>
<td>V</td>
<td>S</td>
<td>R</td>
<td>I</td>
<td>T</td>
</tr>
<tr>
<td>3A12</td>
<td>K</td>
<td>I</td>
<td>P</td>
<td>I</td>
<td>P</td>
<td>K</td>
<td>L</td>
<td>A</td>
</tr>
<tr>
<td>3A1</td>
<td>K</td>
<td>I</td>
<td>P</td>
<td>I</td>
<td>P</td>
<td>K</td>
<td>L</td>
<td>R</td>
</tr>
<tr>
<td>3A2</td>
<td>K</td>
<td>I</td>
<td>P</td>
<td>I</td>
<td>P</td>
<td>K</td>
<td>L</td>
<td>R</td>
</tr>
<tr>
<td>3A4</td>
<td>K</td>
<td>I</td>
<td>P</td>
<td>I</td>
<td>P</td>
<td>K</td>
<td>L</td>
<td>L</td>
</tr>
<tr>
<td>3A5</td>
<td>K</td>
<td>I</td>
<td>P</td>
<td>V</td>
<td>P</td>
<td>K</td>
<td>L</td>
<td>T</td>
</tr>
<tr>
<td>3A6</td>
<td>R</td>
<td>I</td>
<td>P</td>
<td>I</td>
<td>P</td>
<td>K</td>
<td>L</td>
<td>K</td>
</tr>
<tr>
<td>3A7</td>
<td>K</td>
<td>I</td>
<td>P</td>
<td>V</td>
<td>P</td>
<td>K</td>
<td>L</td>
<td>F</td>
</tr>
</tbody>
</table>

Figure 4. Alignment of 3A enzymes at SRS positions where 3A26 and 3A12 differ. From a total of 22 amino acid differences identified between 3A26 and 3A12, 9 occur within these SRS regions.

Heterologous expression of the modified cytochrome P450 3A26 enzyme in *E. coli*. Heterologous expression and preparation of CHAPS-solubilized membranes of the
modified 3A26 and 3A12 in *E. coli* were performed as described previously (John et al., 1994). Expression levels for 3A26 were found to be relatively low when compared with levels obtained for 2B or 3A constructs (John et al., 1994; Born et al., 1996; Harlow et al., 1997), resulting in maximal recovery of 5-8 nmol P450 per liter of culture. Maximal expression of P450 3A26 was found to occur at 42 hours after addition of IPTG and 8-ALA when cultures were grown at 30°C. Culture temperatures of 24°C and 37°C resulted in reduced levels of recovered protein. In addition, variation of IPTG and 8-ALA concentrations as well as time of induction and order of addition of compounds were all examined and resulted in no increase in expression of P450 3A26 from the parameters described in Materials and Methods.

Immunoblot analyses of heterologously expressed P450s 3A26 and 3A12 and PB-induced canine liver microsomes. Previous studies in this laboratory (Ciaccio et al., 1989; Ciaccio et al., 1991) resulted in the isolation, identification, and characterization of the canine cytochrome P450 3A12 enzyme. It was noted at that time that immunoblots of liver microsomes from phenobarbital-induced dogs using a polyclonal antibody to 3A12 produced two distinct immunoreactive bands. It was therefore of interest to examine the electrophoretic mobility of the expressed 3A26 enzyme. Immunoblot data of canine liver microsomes as well as heterologously expressed P450s 3A26 and 3A12 are shown in Figure 5. Differences in electrophoretic mobility of these two cytochromes are clearly in evidence, with 3A26 having greater mobility than 3A12. In microsomes from phenobarbital-induced dogs, two distinct bands were discernible, with the upper band
corresponding in electrophoretic mobility to 3A12 and the lower band corresponding to 3A26. The possibility existed that, because of N-terminal modifications that removed 10 residues of the signal-anchor sequence, electrophoretic mobility of the expressed cytochrome P450 3A enzymes was altered significantly from that observed for their microsomal counterparts. However, no difference was observed between heterologously expressed 3A12 and the purified hepatic enzyme. These findings demonstrate that the differences in electrophoretic mobility observed for 3A26 and 3A12 are not likely to be the result of modifications that were introduced for heterologous expression. In order to demonstrate that other constituents in the samples had no influence on electrophoretic mobility differences, samples of 3A26 and 3A12 were combined and run alongside induced microsomal samples. It is evident from these experiments that P450 3A12 has decreased electrophoretic mobility relative to P450 3A26 on SDS-PAGE, and that each enzyme corresponds to immunoreactive bands identified in dog liver microsomes.
Figure 5. Immunoblot of *Escherichia coli*-expressed 3A12 and 3A26 liver microsomal samples from phenobarbital-induced dogs, and purified hepatic canine PBD-1 (3A12). 5 pmol of each cytochrome P450 enzyme were loaded into their respective lanes. A polyclonal antibody raised against 3A12 was used in these experiments. Due to the limited number of differences in their amino acid sequences, no differences in antibody recognition of the enzymes were expected or noted. Lane 1 of immunoblot B contains 5 pmol of 3A26 and 3A12 combined.
Characterization of the catalytic activity of cytochrome P450 3A26. Steroid-hydroxylase assays were performed using solubilized E. coli membrane preparations containing 3A26 or 3A12. The major steroid metabolite formed by other mammalian 3A enzymes is the 6β-OH product (Waxman et al., 1988; Ciaccio et al., 1989; Born et al., 1996). The studies performed here (Table 1) indicate a significant reduction in steroid hydroxylase activity of P450 3A26 when compared with P450 3A12. Three different steroid substrates, androstenedione, testosterone, and progesterone, were examined to determine the relative activities of heterologously expressed P450s 3A26 and 3A12. P450 3A12 exhibited high rates of steroid hydroxylase activity for all of the steroids employed, whereas the relative rates of steroid hydroxylation for 3A26 did not exceed 22% of 3A12 activity for any individual hydroxylated steroid metabolite. Interestingly, although steroid hydroxylase rates for 3A26 are low and determination of exact metabolite ratios is therefore difficult, metabolite profiles do seem to differ between 3A12 and 3A26.

**TABLE 1**

Steroid hydroxylase activities of canine P450s 3A12 and 3A26

Solubilized E. coli membrane preparations containing 10 pmol of 3A26 or 3A12 were reconstituted with 40 pmol cytochrome P450 reductase and 10 pmol cytochrome b5 and analyzed for androstenedione, progesterone, and testosterone hydroxylase activity. Substrate concentrations of 250 mM were used throughout. Rates are given in nmol product/min/nmol P450 and represent the mean of 2-4 incubations with two separate preparations of each enzyme. Numbers in parentheses represent 3A26 activity as a percentage of 3A12 activity.

<table>
<thead>
<tr>
<th>P450</th>
<th>Androstenedione</th>
<th>Progesterone</th>
<th>Testosterone</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>6β</td>
<td>6β</td>
<td>6β</td>
</tr>
<tr>
<td>3A12</td>
<td>3.5, 3.7</td>
<td>5.6, 5.9</td>
<td>0.81, 0.99</td>
</tr>
<tr>
<td>3A26</td>
<td>0.47, 0.33 (11)</td>
<td>0.11, 0.08 (2)</td>
<td>0.02, 0.02 (2)</td>
</tr>
</tbody>
</table>
DISCUSSION

Previous studies have suggested that multiple canine 3A forms exist and have distinct metabolic profiles (Ciaccio et al., 1989). The results presented here describe the isolation and initial characterization of a new canine cytochrome P450 3A enzyme, P450 3A26, from a cDNA library generated from phenobarbital-induced canine hepatic tissue. The 1.9 kb cDNA encoding 3A26 exhibited 33 nucleotide and 22 amino acid differences when compared with canine P450 3A12. The sequence identity between CYP3A12 and CYP3A26 at the N-terminal and 5' untranslated region, and the sequence differences found mostly at the C-terminal and 3' untranslated region suggest that CYP3A26 might be the product of a recent "gene conversion" or "unequal crossing over" event involving CYP3A12. According to this hypothesis, the 5' portion of the CYP3A26 gene would be derived from CYP3A12 and the 3' portion would be derived from either an ancestral CYP3A26 gene which was subsequently lost, or from a putative third canine CYP 3A gene. Southern blot evidence does not allow us to exclude the existence of a third, related CYP3A gene (Ciaccio et al., 1989). Recombination between closely related genes of the CYP2 family has been documented previously in the rat CYP2D subfamily (Matsunaga et al., 1990).

The recovery of heterologously expressed 3A26 was relatively limited and may be a limiting factor in future assays investigating its metabolic profile. However, it may be possible to augment the recovery of expressed 3A26. Recent mutagenesis studies examining the key residues involved in P450 3A4 substrate recognition (He et al., 1997)
may give some indication of the cause of relatively low levels of expression of 3A26 in *E. coli*. These studies noted that the substitution of Pro-368 with Ser led to a 30-fold lower expression level of 3A4 than that of wild type 3A4. Because residue 368 of 3A26 is a serine and all other mammalian P450s 3A examined contain a proline at this site, it is possible that expression of 3A26 is hampered by this single difference in amino acid sequence. Another serine for highly conserved proline substitution is observed at residue 474 of 3A26, suggesting a second potential site which could have a negative impact on expression. While no direct evidence from expression studies using 3A26 would indicate that these particular residues are contributing to lower expression levels in *E. coli*, these 3A4 mutagenesis findings do suggest that Ser-368 may be responsible for the low expression levels of 3A26 in *E. coli*.

Immunoblot data presented here indicate that 3A26 has electrophoretic mobility characteristics similar to those of a canine 3A band identified in previous studies (Ciaccio et al., 1989). Specifically, the heterologously expressed 3A26 enzyme has slightly greater mobility than expressed 3A12 on SDS-PAGE. These differences are analogous to those seen in phenobarbital-induced canine liver microsomes in which two separate immunoreactive bands are discernible, suggesting that the 3A26 enzyme is analogous to the lower immunoreactive band and 3A12 corresponds to the upper band. It is interesting to note that these two enzymes are each 503 amino acids in length and share only 22 amino acid differences between them. Moreover, the molecular masses of 3A26 and 3A12, 57,689 Da and 57,684 Da respectively, differ by only 5 Daltons. As observed in
Figure 4, in which microsomal preparations and purified hepatic 3A12 were compared with heterologously expressed enzyme samples, it was found that the N-terminal modifications that deleted 10 amino acids and reduced the size of each heterologously expressed enzyme by 1.160 Da had no discernible effect on electrophoretic mobility. Taken together, these data indicate that differences in molecular weight cannot account for the differences in electrophoretic mobility observed for 3A12 and 3A26. Similar findings have been reported for cytochromes P450 2B1 and 2B2, which differ by only 14 amino acids from a total of 491 and have been found to generate two distinct immunoreactive bands (Ryan et al., 1982; Waxman et al., 1983). The molecular masses of these two enzymes are also quite similar to one another, differing by only 13 Da from a total of over 55,900 Da each. These observations suggest that some secondary protein structure remains intact even after samples were boiled in SDS and may account for differences in electrophoretic mobility between these enzyme homologs.

In addition to the differences in electrophoretic mobility and heterologous expression levels of P450s 3A26 and 3A12, several distinctions in catalytic activity have been identified between these two enzymes. The major differences in steroid hydroxylase activities identified here clearly demonstrate that cytochrome P450 3A26 is uniformly less active than 3A12. Previous work on human cytochromes P450 3A4 and 3A5 has shown some parallels when compared with canine P450s 3A12 and 3A26. P450s 3A4 and 3A5 exhibit 84% amino acid sequence identity and metabolize many of the same substrates. Both P450s 3A4 and 3A5 have been found to catalyze 6β-hydroxylation of testosterone,
progesterone, and androstenedione, although minor hydroxylation products such as 16α-hydroxyprogesterone were found to comprise approximately 20% of the total metabolites of 3A4 but not 3A5 (Aoyama et al., 1989). Recent site-directed mutagenesis studies have demonstrated that the replacement of Ile-369 in P450 3A4 with the corresponding Val in 3A5 caused progesterone 16α-hydroxylase activity to be suppressed (He et al., 1997). Interestingly, P450 3A26 also has a Val at residue 369 and has no appreciable 16α-hydroxylase activity. Taken together, these experiments suggest that differences in residues of SRS5 found between P450s 3A12 and 3A26 may play major roles in determining steroid hydroxylase activity differences observed for these enzymes.

This study has resulted in the isolation, expression and functional characterization of a cDNA encoding the canine cytochrome P450 3A26 enzyme. This enzyme exhibits marked differences in their respective rates of hydroxylation of steroid substrates. Immunoblot data also confirm the presence of multiple 3A proteins in canine microsomes, with 3A26 corresponding to a previously unknown enzyme of lower apparent molecular weight identified in previous studies. Future experiments will utilize site-directed mutagenesis techniques in an attempt to facilitate increased heterologous expression of 3A26 and to identify the residues responsible for conferring specific metabolism profiles to 3A26 and 3A12. These studies should be invaluable in the determination of P450 3A substrate specificity.
CHAPTER 3

USE OF CHIMERIC ENZYMES AND SITE-DIRECTED MUTAGENESIS FOR IDENTIFICATION OF THREE KEY RESIDUES RESPONSIBLE FOR DIFFERENCES IN STEROID HYDROXYLATION BETWEEN CANINE CYTOCHROMES P450 3A12 AND 3A26

Canine cytochromes P450 3A12 and 3A26 differ by 22 out of 503 amino acid residues. Chimeric constructs and site-directed mutants were employed to investigate which of the residue differences are responsible for the much higher rates of steroid hydroxylation by 3A12. Six initial 3A12/3A26 hybrids were generated using convenient restriction endonuclease sites, and site-directed mutagenesis was used to restore full 3A12 activity to some of the hybrids. One pair of 3A12/3A26 chimeras indicated that the first four residue differences between 3A12 and 3A26 are at least partially responsible for the differences in progesterone hydroxylation. Site-directed mutagenesis of these four residues revealed that Ile-187 in 3A26 decreases progesterone 6β-hydroxylase activity, whereas the Thr residue at this site in 3A12 confers much higher activity. Another chimera made by replacing the internal PstI fragment of 3A12 with that of 3A26 exchanged six amino acid residues, and subsequent site-directed mutagenesis led to the identification of 3A26 residues Ser-368 and Val-369 as major contributors to low activity. Conversion to Pro and Ile, respectively, within the PstI fragment restores the rate of
formation of 6β-hydroxyprogesterone to that of 3A12. Moreover, the simultaneous conversion of 3A26 residues 187, 368 and 369 to those of 3A12 conferred almost half of the progesterone 6β-hydroxylase activity and virtually all of the testosterone and androstenedione 6β-hydroxylase activity of 3A12. This report details the first studies to use catalytically distinct cytochromes P450 3A from the same species in the elucidation of structural variations responsible for differences in function in this important class of enzymes.

Members of the cytochrome P450 superfamily of hemoproteins are responsible for the metabolism of endogenous and exogenous compounds. The 3A enzymes are major contributors to hepatic biotransformation pathways, with human 3A4 accounting for as much as 60% of the P450 found in human liver (Guengerich, 1990). A wide range of clinically relevant drugs are metabolized by 3A enzymes including acetaminophen, codeine, erythromycin, lidocaine, and steroids, as recently reviewed (Guengerich, 1995). In addition, adverse pharmacokinetic drug interactions have been observed clinically with the concomitant use of multiple drugs that are metabolized by 3A enzymes (Periti et al., 1992). Despite the wealth of information on the importance, regulation, and substrate specificity of the cytochrome P450 3A subfamily, until recently relatively little was known about the structure-function relationships of these enzymes (Harlow and Halpert, 1997; He, et al., 1997; Domanski et al., 1998). In contrast to the 2A (Lindberg and Negishi, 1989; Hanioka, et al., 1990; Honkakoski and Negishi, 1997), 2B (Aoyama, et al., 1989; Kedzie et al., 1991), and 2C (Kronbach, et al., 1988; Hsu, et al., 1993) subfamilies, a lack
of functionally distinct natural variants and the high conservation of specificities across species has hindered structure-function analyses of the cytochromes P450 3A.

Canine models have been used extensively in drug metabolism studies, but much remains to be learned about the individual P450 forms. Previous studies have demonstrated that canine P450 3A12 can catalyse the hydroxylation of steroids including progesterone, testosterone, and androstenedione at rates comparable to human P450 3A4 (Born, et al., 1996; Fraser, et al., 1997). In contrast, the 6β-hydroxylase activity of 3A26 with the same substrates was much lower, despite the fact that these two enzymes exhibit 96% amino acid sequence identity (Fraser et al., 1997). The rates of hydroxysteroid product formation were dependent upon the substrate employed, with 3A26 displaying only 2% of the activity of 3A12 for 6β-hydroxyprogesterone formation, but exhibiting as much as 22% of the 3A12 activity in the generation of 2β-hydroxytestosterone (Fraser, et al., 1997). These results indicated that canine cytochromes P450 3A12 and 3A26 might provide an excellent model system for the investigation of the structural basis of 3A substrate specificity.

The general strategy for the current study involved the use of hybrid enzymes in conjunction with site-directed mutants to identify the specific residue differences between 3A12 and 3A26 that account for their differences in steroid hydroxylation. The chimeric enzymes were based on the hypothesis that the loss of function through discreet and limited changes in different regions of 3A12 would be useful in indicating sections that play roles in the differential function of the parent enzymes. Back-mutation of specific
codons encoding the amino acid residue changes incorporated into 3A12 could then be used to restore activity to the hybrid. The information from these chimeric mutants could then be employed in the rational design of alterations to 3A26 in order to generate steroid hydroxylase rates similar to those of 3A12.

Progesterone was chosen as the substrate for the initial experiments employing chimeras and chimeric mutants of 3A12 and 3A26, based on the extreme differences in their ability to catalyse hydroxylation of this steroid. Testosterone and androstenedione were then used in addition to progesterone in the analyses of mutant 3A26 constructs. Our findings indicate that residues 187, 368 and 369 are instrumental in conferring differences in hydroxylation rates observed between canine cytochromes P450 3A12 and 3A26 for progesterone, testosterone, and androstenedione.
Experimental Procedures

Materials. Restriction endonucleases and media for bacterial growth were purchased from GIBCO-BRL (Grand Island, NY). The pSE380 expression vector used in all of these studies was purchased from Pharmacia (Alameda, CA). Primers for PCR amplification and mutagenesis were obtained from the University of Arizona Macromolecular Structures Facility (Tucson, AZ). CHAPS, progesterone, testosterone, androstenedione, NADPH, and DOPC were purchased from Sigma Chemical Co. (St. Louis, MO). [4-¹⁴C]Testosterone was obtained from Amersham Life Sciences (Arlington Heights, IL). [4-¹⁴C]Progesterone and [4-¹⁴C]androstenedione were obtained from Dupont-New England Nuclear (Boston, MA). HEPES was purchased from Calbiochem Corp. (LaJolla, CA). Thin-layer chromatography plates [silica gel, 250 μm, Si 250 PA (19C)] were purchased from J. T. Baker (Phillipsburg, NJ). All other reagents and supplies not listed were obtained from standard sources.

Cloning and expression of 3A12, 3A26, hybrids, and site directed mutants. The P450 3A12 and 3A26 cDNAs were isolated from a λgt11 cDNA library generated from canine liver as described previously (Ciacio, et al., 1991; Fraser, et al., 1997). The N-termini of 3A12 and 3A26 are identical in sequence until the first variation is encountered at base pair 333. Modifications to the N-terminus of 3A12 have been described (Born et al., 1996; Fraser et al., 1997). Restriction endonucleases and subcloning were used in the
modification of 3A26 for expression in *E. coli*. The unmodified N-terminus of 3A26 was removed and replaced with the corresponding fragment from the modified N-terminus of 3A12. These alterations removed 10 amino acids in the signal anchor sequence of 3A26 and changed the second amino acid residue from aspartic acid to alanine, changes that have been shown to facilitate expression in *E. coli* (Barnes *et al.*, 1991). 3A12 and 3A26 constructs and all chimeras and site-directed mutants were maintained in the pSE380 expression vector.

Heterologous expression and preparation of solubilized *E. coli* membranes was done essentially as described previously (John *et al.*, 1994; Born *et al.*, 1996; Fraser *et al.*, 1997). All constructs were maintained in DH5α cells and grown at 37°C with 240 rpm shaking in 250 ml liquid TB media (12 g Bacto tryptone, 24 g Bacto yeast extract, 4 ml glycerol/l) to mid log phase. IPTG (final concentration 1.0 mM) and 80 mg/l ALA were added, and cells were harvested after incubation at 30°C with 190 rpm shaking. Optimal expression of 3A26 was observed at 38-42 h after IPTG/ALA addition, and typical recovery of 3A26 protein ranged from 6-10 nmol per liter of culture. Maximal expression of 3A12 was observed at 72 h after IPTG/ALA addition and yields ranging from 40 to 60 nmol/l of culture were routine. All chimeras and site-directed mutants were incubated for 38-42 h at 30°C after addition of IPTG/ALA to ensure adequate protein recovery based on the results obtained for 3A26.
Generation of chimeras and site-directed mutants. Chimeric combinations of 3A12 and 3A26 were generated using internal restriction sites by standard sub-cloning techniques. Plasmids containing 3A12 and 3A26 were cut with the appropriate enzymes and DNA fragments were separated on 1.0% agarose gels. The desired DNA fragments were purified using the GeneCleanII DNA purification kit. Chimeras of 3A12 and 3A26 were then generated by combining these fragments and religating them into complete constructs (Fig. 2). The DraIII site at bp 798 was used in conjunction with the HindIII site in the multiple cloning site (MCS) of pSE380 to separate the first four residue differences from the remaining eighteen. Similarly, the PpuMI site at bp 1371 was used with the HindIII site in the MCS to separate the first fourteen residue differences from the last eight changes. In addition to these modifications, an internal 450 bp PstI fragment was exchanged between the constructs to separate the six variations found within this region from the sixteen flanking differences (Fig 2).

Site directed mutagenesis was accomplished using two different methods. The mutations at residues 368 and 369 were done using overlap PCR. Overlapping primers used to modify either or both of these residues were designed in the forward and reverse orientations and used with corresponding external primers to generate two fragments that overlap in the region in which the mutation is generated. All mutagenic forward and reverse overlapping primers, with incorporated modifications underlined, are presented in Figure 1. The external primers were designed to overlap the external DraIII and HindIII restriction sites at bp 798 and in the MCS, respectively. Reaction conditions were: 1
cycle of 94°C for 5 minutes followed by 29 cycles of 94°C for 1 minute, 55°C for 1 minute and 72°C for 2 minutes. The resulting two PCR products were then used as template in conjunction with the external non-mutagenic primers in a second PCR reaction to generate a single full length fragment. This fragment was then isolated from an agarose gel and digested with the restriction endonuclease PstI. The 450 bp fragment was then cloned into the appropriate vector fragments digested with PstI, and positive clones were then checked for orientation and sequenced.
FIGURE 1.

Primers used for site-directed mutagenesis.

Each of the primers used for mutagenesis of one or more sites in 3A12 / 3A26 constructs and hybrids is indicated below. The mutation generated is listed in the left column and the primer orientation is indicated in the second column. Matching forward and reverse primer sequences are listed with base pair alterations underlined.

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Orientation</th>
<th>Primer Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>S368P</td>
<td>fwd.</td>
<td>5'-ACTCTCCGATTATACCCAGTCGCTGGTAGACTTGAG-3'</td>
</tr>
<tr>
<td>S368P</td>
<td>rev.</td>
<td>5'-CTCAAGTCTACCCAGCGACTTGATTATAATCGGAGAGT-3'</td>
</tr>
<tr>
<td>V369I</td>
<td>fwd.</td>
<td>5'-ACTCTCCGATTATACCCAGTCGCTGGTAGACTTGAG-3'</td>
</tr>
<tr>
<td>V369I</td>
<td>rev.</td>
<td>5'-CTCAAGTCTACCCAGCGACTTGATTATAATCGGAGAGT-3'</td>
</tr>
<tr>
<td>S368P V369I</td>
<td>fwd.</td>
<td>5'-ACTCTCCGATTATACCCAGTCGCTGGTAGACTTGAG-3'</td>
</tr>
<tr>
<td>S368P V369I</td>
<td>rev.</td>
<td>5'-CTCAAGTCTACCCAGCGACTTGATTATAATCGGAGAGT-3'</td>
</tr>
<tr>
<td>I129M</td>
<td>fwd.</td>
<td>5'-AAGAGAAATGCCAATTTTGCTTGCTTCT-3'</td>
</tr>
<tr>
<td>I129M</td>
<td>rev.</td>
<td>5'-CAAGTCTCGATTCTCTTCACCCTC-3'</td>
</tr>
<tr>
<td>I187T</td>
<td>fwd.</td>
<td>5'-ACCAGCAATCGTTTTGAGTGAACATTG-3'</td>
</tr>
<tr>
<td>I187T</td>
<td>rev.</td>
<td>5'-AAACGATGTCCTGGTAATCAATCCATCCGTGCTAGG-3'</td>
</tr>
<tr>
<td>N209K</td>
<td>fwd.</td>
<td>5'-ACCAAGAAATCTTTTTGATTATCATGAC-3'</td>
</tr>
<tr>
<td>N209K</td>
<td>rev.</td>
<td>5'-TTTAAAAGAGCTTCTTTGATTTTTATCCAACCTC-3'</td>
</tr>
<tr>
<td>L220F</td>
<td>fwd.</td>
<td>5'-CCATTTTTCTCCATATTACT-3'</td>
</tr>
<tr>
<td>L220F</td>
<td>rev.</td>
<td>5'-GAAATGAAATG-3'</td>
</tr>
</tbody>
</table>

The I129M, I187T, N209K, and L220F mutants were generated using overlapping forward and reverse primers (Fig. 1) containing the desired residue changes in conjunction with the high fidelity Pwo polymerase, which displays corrective exonuclease activity. The PCR reaction consisted of 1 cycle of 94°C for 5 minutes followed by 10 cycles of 94°C for 1 minute, 60°C for 1 minute, 68°C for 4 minutes and a final 5 minute extension at 68°C. This protocol resulted in the generation of full length constructs with changes at the desired residues. The PCR products were then digested with DpnII which cuts only DNA...
strands that are methylated, thus removing the template plasmid from the reactions. The PCR reactions were directly transformed into DH5α competent cells. DNA was isolated and analyzed for the desired alterations. DNA dideoxy-sequencing was performed on all constructs to ensure that no errors were incorporated into the constructs as a result of the PCR reactions. Positive mutants were then expressed and solubilized membrane preparations were done as described above.

Functional characterization of chimeras and site-directed mutants. CHAPS-solubilized *E. coli* membrane preparations were used directly in steroid hydroxylase assays as described previously (Born et al., 1996; Fraser et al., 1997). Ten picomoles P450 were reconstituted with 40 pmol *E. coli*-expressed rat NADPH-P450 reductase, 10 pmol rat cytochrome b$_5$, and 0.1 mg/ml DOPC and 0.06% CHAPS in a minimal volume. Assays were performed for 10 minutes at 37°C in 15 mM MgCl$_2$, 50 mM HEPES buffer (pH 7.6), 0.06% CHAPS and 1mM NADPH. Reactions were stopped with the addition of 50 μl THF to each reaction tube. $^{14}$C-Steroid stock solutions were made in 100% methanol. Care was taken so that methanol concentrations in the reaction mixture were equivalent and did not exceed 1% of the total reaction volume. Individual assays were performed using concentrations of testosterone, progesterone and androstenedione ranging from 25 to 250 μM. Identification of 6β-hydroxysteroid metabolites was by relative mobility on TLC and by comparison to authentic standards.
Results

Generation of chimeras of 3A12 and 3A26. Drall, PstI, and PpuMI restriction endonuclease sites used in the generation of hybrid 3A12/3A26 enzymes are outlined in Figure 2. These sites were instrumental in separating small groups of amino acid residue differences between 3A12 and 3A26 and characterizing the contributions to alterations in steroid hydroxylase activity. Progesterone 6β-hydroxylation was chosen as the marker activity for these studies, since it discriminates best between 3A12 and 3A26. Specifically, restriction endonuclease fragments from 3A26 were inserted into 3A12, and the resulting hybrid proteins were examined for loss of progesterone hydroxylase activity. Any major reductions in activity would indicate amino acid residue differences within the fragments exchanged that contribute to differential activity displayed by 3A12 and 3A26. Site-directed mutagenesis was then used to identify which of the incorporated residue differences might be responsible for alterations in catalytic activity.

In Figure 2, the 6β-hydroxylase activity of the hybrid enzymes is presented as a percentage of 3A12 wild-type activity. The chimeric enzymes A and B, generated with Drall, separate the first 4 amino acid differences from the remaining 18 and exhibited decreased activity when compared with wild-type 3A12. As seen with hybrid B, a 66% decrease in activity resulted from the presence of only four 3A26 residues in the N-terminal region.
Chimeras of 3A12 and 3A26.

Hybrid constructs of canine cytochromes P450 3A12 and 3A26. 3A12 is shown with white bars and 3A26 is shown in black bars. The hatched lines between wild-type 3A12 and 3A26 represent the relative positions of the 22 amino acid residue differences shared by the two enzymes. The left column identifies each hybrid according to the nomenclature used in the text. Hybrids are represented by segments corresponding to each of the parent proteins. Restriction endonuclease sites employed in chimera generation are indicated at the bottom. The right column represents percent of 3A12 6β-hydroxyprogesterone formation.

The second set of hybrids, C and D, was generated using a PpuMI site that separates the final 8 amino acid residue differences from the 14 upstream changes.

Chimera C retained 75% of the 6β-hydroxylase activity of 3A12 despite the replacement
of 8 C-terminal residues with those of 3A26. However, construct C no longer exhibited any 16α-hydroxyprogesterone activity (data not shown), indicating that some differences in stereo- and regioselectivity do result from these alterations. Additionally, chimera D exhibited only 2% of 3A12 activity, indicating that the eight C-terminal differences are not sufficient to confer progesterone hydroxylase activity on 3A26. Overall, the data from construct C eliminated the final 8 amino acid residue differences from consideration as major contributors to the 6β-hydroxylase activity of 3A12.

An internal 450 base pair PstI fragment containing 6 amino acid residue differences was then exchanged between the two wild-type constructs, generating E and F in Figure 2. The activities of these chimeras were both extremely low, representing only 2% and 5% of 3A12 activity. These results indicate that residues both within and outside of the PstI fragment contribute to the 6β-hydroxylation of progesterone. Significantly, the activity of the 3A12 hybrid E containing these 6 changes dropped by 95%, indicating that essential residue differences could be found in this region.

**Site-directed mutagenesis of hybrid constructs.** The results obtained with hybrid 3A12/3A26 proteins primarily implicated two regions of 3A12, the N-terminal and internal PstI regions as being important for 6β-hydroxylation of progesterone. The next set of experiments employed site-directed mutagenesis of individual and multiple codons encoding residue differences within the regions that were predicted to play roles in the observed catalytic variability of 3A12 and 3A26. Based on a number of previous reports,
substrate recognition sites (SRSs) for the 3A enzymes are similar to those reported for the family 2 enzymes (Gotoh, 1992; Szklarz and Halpert, 1997; Harlow and Halpert, 1997; Domanski et al., 1998). As such, alterations were made with special consideration of differences that fall within the putative family 3 SRS regions.

There are six differences in amino acid sequence found in the PstI fragment within 3A12 and 3A26, including changes at residues 344, 348, 368, 369, 400, and 406. Of these, residues 368 and 369 fall within the proposed SRS5 of the 3A enzymes (Szklarz and Halpert, 1997). Construct E was used as template for the conversion of Ser-368 and Val-369 of 3A26 to Pro and Ile of 3A12, respectively. These changes were made individually and in combination. The results, presented in Figure 3A, indicate that each of the two residues regenerate 25-40% of the 6β-hydroxylase activity independantly and act together in the regeneration of 98% of the activity of wild-type 3A12. The regeneration of activity by the back-mutation of residues 368 and 369 to those found in 3A12 indicates that these residues are both required for high progesterone hydroxylase activity, and that they may play significant roles in conferring activity upon 3A26.
Site-directed mutagenesis of chimeras.

Mutagenesis of 3A12 / 3A26 chimeras was used to restore rates of 6β-hydroxylation of progesterone to those of wild type 3A12. Progesterone hydroxylase rates are shown as a percentage of wild-type 3A12 activity. In Figure 3A, mutants were generated from chimera E by mutating residues 368 and 369 together as well as individually. Figure 3B represents the alteration of chimera A by converting residues 129, 187, 209, or 220 from 3A26 to 3A12 individually.
Site-directed mutagenesis of chimeras.

Mutagenesis of 3A12 / 3A26 chimeras was used to restore rates of 6β-hydroxylation of progesterone to those of wild type 3A12. Progesterone hydroxylase rates are shown as a percentage of wild-type 3A12 activity. In Figure 3A, mutants were generated from chimera E by mutating residues 368 and 369 together as well as individually. Figure 3B represents the alteration of chimera A by converting residues 129, 187, 209, or 220 from 3A26 to 3A12 individually.
Construct B was used in similar studies presented in Figure 3B. Single mutations were generated at residues 129, 187, 209, and 220 in chimera B. The only residue difference of the four targeted amino acids found to be within an SRS (SRS2) was 209. Interestingly, the Ile-187 → Thr change restored the activity of the chimera back to that of 3A12, whereas the other changes at residues 129, 209 and 220 had no effect on the ability of the chimeric mutants to hydroxylate progesterone.

*Site-directed mutagenesis of 3A26.* The findings from hybrid and mutant hybrid studies indicated that residues 187, 368, and 369 contribute significantly to the differences in 6β-hydroxyprogesterone production by 3A12 and 3A26. It was therefore of interest to determine whether the conversion of these three residues in 3A26 to the corresponding 3A12 residues would confer 3A12-like activities. Using the same *DraIII* and *PstI* restriction endonuclease sites, fragments containing the mutant regions were back-cloned into the wild type 3A26 construct. The single I187T mutant construct did not express well and was not employed in steroid hydroxylase assays. The S368P/V369I double mutant and I187T/S368P/V369I triple mutant, however, did express well in our heterologous system. The data from steroid hydroxylase assays are presented in Table 1. The double mutant was shown to recover 20% of the 3A12 6β-hydroxylase activity and the triple mutant 36%. In comparison, less (16 and 18% respectively) of the 16α-hydroxylase activity was regenerated by these substitutions. These data demonstrate that
residues 187, 368, and 369 are major contributors to the differences in 6β-hydroxylation of progesterone observed between 3A12 and 3A26.

TABLE 1

Progestosterone hydroxylase activities of P450 3A12 and 3A26 double and triple mutants

Solubilized E. coli membrane preparations containing 10 pmol of 3A12, 3A26 S368P/V369I, or 3A26 H187T S368P/V369I mutants were reconstituted with 40 pmol cytochrome P450 reductase and 10 pmol cytochrome b5 and analyzed for progesterone, testosterone, and androstenedione hydroxylase activity. Substrate concentrations of 250 mM were used throughout. Rates are given in nmol product/min/nmol P450 and represent the mean of 2-4 incubations with two separate preparations of each enzyme. Numbers in parentheses represent 3A26 activity as a percentage of 3A12 activity.

<table>
<thead>
<tr>
<th>P450</th>
<th>Progestosterone</th>
<th>Testosterone</th>
<th>Androstenedione</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>6β</td>
<td>16α</td>
<td>15β</td>
</tr>
<tr>
<td>3A12</td>
<td>58, 6, 6</td>
<td>84, 91</td>
<td>21, 30</td>
</tr>
<tr>
<td>3A26 S368P/V369I</td>
<td>70, 98 (14)</td>
<td>14, 14 (16)</td>
<td>22, 26 (94)</td>
</tr>
<tr>
<td>3A26 H187T S368P/V369I</td>
<td>2,1, 2, 4 (36)</td>
<td>16, 15 (18)</td>
<td>69, 62 (257)</td>
</tr>
</tbody>
</table>

Hydroxylation of testosterone and androstenedione. Based on the findings with progesterone, it was of interest to examine the catalytic profiles of the 368/369 double and 187/368/369 triple mutants with testosterone and androstenedione as substrates. These findings, presented in Table 1, indicate that the triple mutant catalyzed the formation of 6β-hydroxytestosterone and 6β-hydroxyandrostenedione at the same rate as 3A12, whereas the double mutant displayed lower activity. Interestingly, the rates of 2β- and 15β-hydroxytestosterone and of 16β-hydroxyandrostenedione formation by the triple
Mutants are higher than those of wild type 3A12. The results indicate that residues 187, 368, and 369 are the major contributors to the differences in 6β-hydroxysteroid production catalysed by canine cytochromes P450 3A12 and 3A26.

Multiple hybrid constructs. Based on the findings that N-terminal and SRS5 amino acid residue differences contribute to steroid hydroxylation rate differences between 3A12 and 3A26, it was expected that combinations of these regions and/or residues might result in the generation of a majority of the 3A12 progesterone 6β-hydroxylase activity in multiple hybrid and hybrid/mutant constructs. Therefore, a set of multiple hybrid and hybrid-mutant combinations (G, H, J, and K) was generated using a combination of PpuMI and PstI restriction fragments of wild type and mutagenized constructs (Fig. 4). Multiple regions and/or residues of 3A12 were inserted into the 3A26 construct and the resulting chimeric/mutant proteins were analyzed for the ability to 6β-hydroxylate progesterone.

Construct G combines the four N-terminal residue differences from 3A12 with the SRS5 Pro-368 and Ile-369 residues of 3A12, forming a hybrid mutant that exhibits 70% of the 6β-hydroxyprogesterone formation of wild-type 3A12. Chimera H combines the same N-terminal four residue differences from 3A12 with the internal PstI fragment containing six residue differences from 3A12 including residues 368 and 369. Interestingly, the additional four 3A12 residues in the PstI region compared with construct G reduce the rate of 6β-hydroxyprogesterone formation to 38% of 3A12. This unexpected result
suggests that residue-residue interactions involving this region might play a role in the steroid hydroxylase rate differences between 3A12 and 3A26.

FIGURE 4

Combination chimeras and chimera / site-directed mutants of 3A12 and 3A26.

Additional constructs used in the identification of regions and residues responsible for differences in the 6β-hydroxylation of progesterone. As before, 3A12 regions are noted with white bars and 3A26 regions are indicated by black bars. Changes generated by site-directed mutagenesis are listed above the construct modified. Relative progesterone 6β-hydroxylase rates are indicated in the column on the right.
Further evidence for such interactions is found in the examination of hybrids J and K. Hybrid J is a combination of the N-terminus of 3A26 (containing eight residue differences) and the C-terminus of 3A12 containing fourteen differences. This hybrid displays only 20% of 3A12 6β-hydroxylase activity of progesterone. In comparison, chimera K consists of the N-terminal portions of 3A12 and the C-terminal regions of 3A26. The ability of this protein to 6β-hydroxylate progesterone is much lower than that of the previously described chimera A, in which only four N-terminal 3A12 residues were observed to generate more than a third of 3A12 activity. Overall, the data in Figures 2 and 4 indicate that the region from the Dralll site at codon 268 to the Pstl site at residue 331 and from the Pstl site at codon 331 to the Ppuml site at residue 459 must originate from the same enzyme for optimal activity. The possibility of residue-residue interactions between these two regions is supported by our molecular modeling of human 3A4, which indicates that residues 312, 313, 368, and 369 are within 4 Å of one another. These findings help to explain why only 36% of the progesterone 6β-hydroxylase activity of 3A12 is generated by the 3A26 I187T/S368P/V369I triple mutant and underscore the complexity of interpretation of 3A structure-function studies.

Human 3A4 mutagenesis and catalytic profiles. The finding that residue 187 contributes significantly to the ability of canine cytochromes 3A to catalyze steroid hydroxylations has some bearing on human cytochrome P450 3A4 activity. A threonine residue is found at this position in all mammalian 3A enzymes including 3A4. Site-directed mutagenesis was
used to convert Thr-187 in 3A4 to Ile in order to examine the role of this residue in the catalysis of steroid hydroxylations catalyzed by 3A4. The data from these studies are presented in Table 2 and show that a reduction in activity similar to that observed for the canine enzymes. The alteration, which occurs outside of any of the proposed SRSs, is intriguing and may be of interest in future investigations of human 3A enzymes.

**TABLE 2**

**Steroid hydroxylase activities of human P450s 3A4 and 3A4 T187I single mutant**

Solubilized *E. coli* membrane preparations containing 10 pmol of either wild type 3A4 or 3A4 T187I mutant were reconstituted with 40 pmol cytochrome P450 reductase and 10 pmol cytochrome b5 and analyzed for progesterone, testosterone, and androstenedione hydroxylase activity. Substrate concentrations of 50 μM were used throughout. Rates are given in nmol product/min/nmol P450 and represent the mean of 2–4 incubations with two separate preparations of each enzyme. Numbers in parentheses represent mutant activity as a percentage of wild type activity.

<table>
<thead>
<tr>
<th>P450</th>
<th>Progesterone (50 μM)</th>
<th>Testosterone (50 μM)</th>
<th>Androstenedione (50 μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3A4</td>
<td>7.2, 8.6</td>
<td>6.9, 7.4</td>
<td>2.8, 2.7</td>
</tr>
<tr>
<td>3A4 T187I</td>
<td>3.4, 3.7 (45)</td>
<td>2.9, 2.7 (39)</td>
<td>1.3, 1.2 (45)</td>
</tr>
</tbody>
</table>
Discussion

The results presented here describe for the first time the use of highly related but functionally distinct cytochromes P450 3A in structure-function analyses of this important class of enzymes. The design of these experiments involved the concerted use of chimeras and site-directed mutants to identify specific residues that contribute to the observed catalytic differences between 3A12 and 3A26. The basic design of introducing a loss of 3A12 function through the insertion of 3A26 restriction fragments into 3A12 coupled with back mutations to restore 3A12-like activity was a useful strategy in these studies of two highly related enzymes.

The identification of residues 187, 368, and 369 as major contributors to the steroid hydroxylase activity exhibited by 3A12 and 3A26 resulted in the generation of a 3A26 triple mutant that displayed certain catalytic activities similar to those of 3A12. A 20-fold increase in progesterone 6β-hydroxylase activity was observed for the triple mutant when compared with the 3A26 wild-type along with 10-fold increases in rates of 6β-hydroxytestosterone and 6β-hydroxyandrostenedione production. It is interesting to note that the 3A26 triple mutant regains more progesterone 6β-hydroxylase than 16α-hydroxylase activity. It was observed in hybrid C of Figure 2 that this product was lost in 3A12 with the exchange of eight C-terminal 3A26 amino acid residues, suggesting that residues in this region contribute to the formation of the 16α-hydroxy product (data not shown). Molecular modeling studies of residue differences in this region may provide
some indications of the exact nature of these interactions, potentially implicating specific residues that contribute to the observed differences in regio- and stereospecificity.

Differences in the testosterone hydroxylase profiles were also identified between 3A12 and the 3A26 triple mutant, with substantial (2-3 fold) increases in 2β-hydroxy and 15β-hydroxy product formation observed for the mutant when compared with 3A12. The data are reminiscent of the effects of α-napthoflavone on 3A4, which preferentially stimulates the 2β- and 15β-hydroxytestosterone products when compared with the 6β-hydroxy products (Harlow and Halpert, 1997). 3A12 and the 3A26 triple mutant, however, did not display any marked differences in relative stimulation of hydroxytestosterone products by α-napthoflavone (data not shown). These findings indicate that the mechanisms of differential product formation by α-napthoflavone for 3A4 and the canine 3A26 triple mutant are different.

The results presented here also indicate that other residue differences may play roles in the stereo- and regiospecific hydroxylation of steroidal compounds by canine 3A12 and 3A26. Chimeras A, C, and K demonstrate one intriguing example of the possible role of residue interactions that contribute to the activity of these enzymes. It was found in A that 35% of progesterone hydroxylase activity is obtained when only four N-terminal residues of 3A12 are inserted into 3A26. Additionally, increasing the number of N-terminal 3A12 residues by ten to a total of fourteen (Chimera C) increased activity two fold. The mutagenesis studies indicated that residues 368 and 369 in these ten additional residues should be responsible for the increases observed in hybrid C.
Interestingly, the inclusion of four N-terminal 3A12 variable residues along with six of the ten middle 3A12 variable residues as in chimera H reduced the activity to that of hybrid A. Moreover, the combination of the first four N-terminal changes in conjunction with the 368 and 369 mutations in hybrid/mutant G resulted in the restoration of 70% of 3A12 activity. These findings suggest that some residue interactions affecting the overall catalytic rates of these enzymes must be taking place, possibly by changing the conformation of the active site or by altering the potential interactions of the substrate with the heme moiety. A molecular model examining these amino acid differences between 3A12 and 3A26 would be useful in predicting potential residue interactions that might be contributing to the observed variations in activity.

Findings presented here also indicate that residue 187 contributes significantly to the ability of canine cytochromes P450 3A to catalyse steroid hydroxylations. It has been reported that an Ile/Met difference at residue 178 of rabbit P450 2C3 alters the $K_m$ of this enzyme for progesterone (Hsu, et al.). Ile-178 in rabbit aligns with residue 184 at the end of the E-helix in human and canine 3A enzymes, and was proposed to influence the I-helix. Our experiments did not indicate an alteration in $K_m$ of 3A4 I187T (data not shown). However, reductions in activity similar to those found in the canine 3A12/3A26 systems were observed. Molecular modeling has demonstrated that residue 187 resides in the E helix of 3A4 and may directly influence the position of the I-helix situated just above it (Szklarz and Halpert, 1997). The insertion of a much larger isoleucine in place of the native threonine may generate alterations in the positions of portions of the E and I
helices, thus potentially affecting the active site of 3A4. These changes may contribute to a difference in substrate binding capacity, access to the heme group, or even access to the binding pocket. While the exact mechanisms for the observed differences in activity of 3A4 and 3A4 T187I are unknown, these alterations are intriguing and may be of great interest in future investigations of human 3A enzymes.

This study has resulted in the identification of three key residues that play a role in differences in steroid hydroxylase activities of canine 3A12 and 3A26. Through the judicious use of chimeric constructs and site-directed mutants, the structure-function determinants of these two highly related but catalytically distinct enzymes were examined. Of the 22 amino acid differences between 3A12 and 3A26, three were found to be major contributors to the modulation of their catalytic activity. The addition of 3A12 residues 187, 368, and 369 to 3A26 resulted in a 10-20 fold increase in the ability of 3A26 to hydroxylate steroids. Moreover, some of these residue differences have also demonstrated altered progesterone hydroxylase activity across species, underscoring the importance of canine studies as a model for human drug metabolism. These experiments are the first to employ these techniques to investigate highly related 3A enzymes from the same species exhibiting widely varying substrate specificities and should be invaluable in the design of future studies involving this important class of enzymes.
CHAPTER 4

Summary and Conclusions

The objectives of the studies described in this dissertation were to isolate and characterize novel canine cytochromes P450 3A. The cytochromes P450 are a superfamily of proteins that catalyze the oxidation of endogenous and exogenous compounds. These reactions are of great biological significance in that they are an integral portion of metabolism mechanisms that are involved in the removal of potentially toxic substances from living systems. Cytochromes P450 are ubiquitous in nature and serve as a first line of defense in the conversion of hydrophobic chemical compounds to more hydrophilic products, thus enhancing removal from living tissues via excretory systems.

The most abundant of the cytochromes P450 are the members of the 3A subfamily, which can comprise as much as 60% of the cytochrome P450 content in human liver. The 3A enzymes are of great pharmacological interest because they exhibit broad substrate specificity, having been shown to catalyze the alteration of over 100 different substrates, and also because of clinically significant pharmacokinetic interactions. For these reasons, members of the 3A subfamily have been the focus of numerous and diverse scientific studies examining the regulation and function of these important enzymes.

Unfortunately, a number of different characteristics of the 3A cytochromes P450 have made rigorous examination of their activity and regulation difficult. First, the expression of these enzymes is regulated by a wide range of inducing agents including
such chemically diverse compounds as glucocorticoids such as dexamethasone and the barbiturate phenobarbital. Additionally, these inducing agents tend to be very general effectors, causing the proliferation of the endoplasmic reticulum and increases in overall cellular protein content. Additionally, the lack of a known receptor for these inducing agents has hampered studies of the regulation of these enzymes.

The mechanistic aspects of 3A activity have also been difficult to elucidate. Structure-function studies are difficult for a number of reasons, including broad substrate specificity and a lack of catalytically distinct members. These limitations have delayed the identification of the structural determinants that are responsible for conferring specific activities upon members of this subfamily. Moreover, with only slight catalytic differences to distinguish individual members coupled with the presence of a wide range of chemically diverse substrates, the progress in identifying specific regions and/or amino acid residues that directly affect catalytic activity has been limited.

It was therefore of great interest to initiate studies that might result in the identification of catalytically distinct cytochromes P450 3A. The dog has been used extensively as a model system in drug metabolism studies in industry, yet relatively little is known about their enzyme profiles. Additionally, previous studies in our laboratory resulted in the identification and cloning of the canine 3A12 enzyme. However, differences in catalytic profiles had been observed between expressed 3A12 and canine liver microsomes, suggesting the presence of multiple 3A enzymes. In addition, complex Southern blots and multiple bands on western blots probed with a 3A-specific polyclonal
antibody indicated that at least one additional catalytically distinct canine 3A enzyme could be found in the canine system. The dog was therefore chosen as a model for these studies in the isolation and characterization of novel hepatic canine cytochromes P450.

Initial studies using 3A-selective primers in conjunction with PCR and employing a canine λgt11 cDNA library as template resulted in the identification of a DNA fragment encoding a portion of a new cytochrome P450 3A enzyme. Further probing of the library with primers specific to the novel sequence resulted in the isolation of the entire cDNA encoding cytochrome P450 3A26. This sequence exhibited 33 nucleotide differences encoding 22 amino acid residue variations when compared to the previously isolated 3A12. As both sequences are 503 amino acids in length, they share 98% sequence identity and 96% amino acid residue identity, making these two proteins excellent candidates for structure-function studies.

Subsequent experiments were designed to facilitate the heterologous expression of 3A12 and 3A26 and the characterization of their activity using \textit{in vitro} methods. Expression of cytochromes P450 in heterologous \textit{E. coli} systems can be difficult and requires some modification of the enzyme to facilitate high product yields. Some members of the 3A subfamily of cytochromes P450 are resistant to heterologous expression in different systems, but based on our previous success with 3A12, modification and expression of 3A26 in \textit{E. coli} was undertaken. Unfortunately, expression levels of functional 3A26 protein were limited and many experiments were required to optimize the relatively low recoveries.
With the successful expression of 3A26, it was of great interest to characterize the enzyme. Previous studies had demonstrated that multiple bands were present in immunoblots of canine microsomes when probed with a 3A-specific polyclonal antibody. It was therefore of great interest whether 3A26 differed in electrophoretic mobility when compared to 3A12. Western blots of heterologously expressed 3A12 and 3A26 in conjunction with PB-induced canine liver microsomes demonstrated that 3A26 is distinct from 3A12, having a greater mobility on SDS-PAGE and corresponding to the lower band previously identified. In comparison, 3A12 displayed reduced electrophoretic mobility and corresponded to the upper band in the probed microsomal preparations. Thus, 3A26 was a distinct P450 enzyme that is expressed in canine liver.

In addition to differences in mobility on SDS-PAGE, experiments comparing the ability of 3A12 and 3A26 to metabolize substrates was examined. Steroids are often used for the examination of 3A enzyme activity due to the relative ease of identification of metabolites on TLC and the high catalytic activity that can be obtained. Moreover, previous experiments with 3A12 done by Stephanie Born in our laboratory had demonstrated that 3A12 exhibits high rates of steroid hydroxylase activity. Studies with 3A26 clearly showed that this enzyme had markedly reduced catalytic activity with a number of steroidal compounds including testosterone, progesterone, and androstenedione. Generally, the rates of steroid hydroxylation exhibited by 3A26 were about 10% of those for 3A12, with progesterone 6β-hydroxylase activity the lowest (2% of 3A12) and 2β-hydroxytestosterone products the most abundant (22% of 3A12). These
findings suggest that 3A12 and 3A26 would be a good system to examine structure-function relationships of 3A enzymes based on the limited number of amino acid residue differences and the distinct catalytic functions displayed.

The differences in steroid hydroxylase activity and the limited number of residue differences led to the design of a project to examine which of the variations are responsible for the observed differences in substrate specificity. These studies were based on the presence of convenient restriction endonuclease sites that allowed for the combination of different sections of each enzyme and the subsequent expression of the hybrids. The fundamental premise was that the addition of discreet portions of 3A26 to 3A12 may cause a reduction in steroid hydroxylase activity, indicating that the regions being analyzed contained important residue differences that impact the ability of the enzyme to catalyze these reactions. Once regions of importance had been identified, site-directed mutagenesis could be used to examine specific amino acids by converting them back to 3A12 residues in an attempt to restore the activity of the chimera to that of wild-type 3A12.

This systematic approach was employed to examine 3A26 and 3A12, leading to the identification of two regions of the enzyme that had a marked impact on the differences in their ability generate hydroxyprogesterone products. Progesterone was used as the substrate for these studies because of the extreme difference in the hydroxylase activity exhibited by 3A12 and 3A26 on this particular compound. Studies involving ten different hybrids led to the identification of two regions of these proteins which contained residue variations that alter the progesterone hydroxylase ability of 3A12 drastically. The
first 4 N-terminal residue differences and a group of six changes within and internal PstI restriction fragment were demonstrated to contain 3A26 residues that reduced the activity of 3A12 by as much as 95%.

Site-directed mutagensis was then used to convert these target residues back to their 3A12 counterparts in an attempt to restore enzyme activity to 3A12 levels. The conversion of residue 187 in the N-terminal region and residues 368 and 369 in the PstI fragment led to the regeneration of high progesterone hydroxylase activity in their respective chimeras. This finding indicated that these 3 residues may be significant in the catalytic activity differences of 3A12 and 3A26. Thus, mutagenesis of the DNA encoding wild type 3A26 was undertaken to convert each of these residues to their 3A12 counterparts both individually and in combination in order to assess their impact on the catalytic rates of steroid hydroxylation. These studies resulted in 10-20 fold increases in rates for the triple mutant when compared to wild-type 3A26. These results indicate that these 3 residues are primarily responsible for the profound differences in the activity exhibited by 3A12 and 3A26.

It has been reported in rabbit cytochrome P450 2C systems that amino acid changes corresponding to residue 183 of human 3A4 have a profound impact on the biochemistry of these enzymes. Because the difference at residue 187 in canine systems had a demonstrated effect on the ability of these enzymes to catalyze steroid hydroxylase reactions, it was hypothesized that this residue in 3A4 might also be of some significance. Site-directed mutagenesis converting Thr-187 in 3A4 to Ile was undertaken to examine
the role of this residue on the ability of 3A4 to catalyze the hydroxylation of progesterone. While no $K_m$ effects were observed as in the rabbit 2C system, a marked reduction in 6β-hydroxyprogesterone production was demonstrated. The mechanisms that cause these differences are unknown. Molecular modeling suggests that residue 187, which resides in the E-helix of 3A4, may impact the I helix which has been shown to contain residues that directly contact the substrate in the active site. Thus, the addition of a larger, hydrophobic residue in the E helix may impact the Helix position or conformation, thus affecting the binding pocket and potential substrate interactions.

The studies described in this dissertation have resulted in the identification of 3A26, a novel member of the cytochrome P450 3A subfamily of proteins. Due to marked differences in substrate specificity and a limited number of residue differences between this enzyme and canine 3A12, these enzymes were used as a model system for the identification of structure-function determinants of catalytic activity. Chimeras and site-directed mutants were used in conjunction with one another to identify 3 residue variations that are responsible for the majority of the differences observed in catalytic activity between 3A12 and 3A26. These findings led to studies indicating similar responses to alterations in human 3A4, demonstrating the potential for canine cytochromes P450 to be used as a model system for human enzyme activity. Moreover, enzyme profiles are relevant in drug development and metabolism studies, where the ability to predict specific metabolism profiles is of great importance. The studies described here contribute to that body of knowledge and may be useful in the determination of these parameters.
REFERENCES


