Investigations of N-acetyltransferases in human hepatocytes and rat models.

Jason Matthew Walraven

University of Louisville

Follow this and additional works at: http://ir.library.louisville.edu/etd

Recommended Citation
https://doi.org/10.18297/etd/1504

This Master's Thesis is brought to you for free and open access by ThinkIR: The University of Louisville's Institutional Repository. It has been accepted for inclusion in Electronic Theses and Dissertations by an authorized administrator of ThinkIR: The University of Louisville's Institutional Repository. This title appears here courtesy of the author, who has retained all other copyrights. For more information, please contact thinkir@louisville.edu.
INVESTIGATIONS OF N-ACETYLTRANSFERASES IN HUMAN HEPATOCYTES AND RAT MODELS

By

Jason Matthew Walraven
B.A., Mercer University, 2000

A Thesis
Submitted to the Faculty of the Graduate School of the University of Louisville in Partial Fulfillment of the Requirements for the Degree of

Master of Science

Department of Pharmacology and Toxicology
University of Louisville School of Medicine
Louisville, KY

May 2005
INVESTIGATIONS OF N-ACETYLTTRANSFERASES IN HUMAN HEPATOCYTES AND RAT MODELS

By

Jason Matthew Walraven
B.A., Mercer University, 2000

A Thesis Approved on

January 6, 2005

by the following Thesis Committee:

________________________________________
Thesis Director
ACKNOWLEDGMENTS

I would like to thank my mentor, Dr. David Hein, for providing the opportunity to learn and work in his lab, and for his patient guidance during the past two years. I would also like to thank the other committee members, Dr. Christopher States, Dr. Gavin Arteel, Dr. William Pierce, and Dr. Carolyn Klinge, for their comments and assistance in improving and completing this thesis. To my parents, your love and support ultimately paved the way for the opportunities that I have today. To my wife, Beth, none of this would be possible without the patience, understanding, and selfless love you have given throughout this time- and self-consuming pursuit.
This thesis begins by reviewing human N-acetyltransferases (Chapter I), then outlines experiments involving human hepatocytes and rat N-acetyltransferases (Chapters II-V). These experiments facilitated the development of a dissertation project designed to study N-acetyltransferase tissue-specific expression in inbred rat strains (Chapter VI).

Real-time PCR experiments concluded that genetic and/or environmental factors influence N-acetyltransferase expression in cryopreserved human hepatocytes. Therefore, an animal model must be used to study N-acetyltransferase expression. Initial studies of recombinantly expressed rat N-acetyltransferases (Nats) revealed a correlation between rat and human N-acetyltransferases in nucleotide and amino acid sequence, catalytic activity, substrate selectivity and thermostability, suggesting the rat is an acceptable model for such studies. Dissertation aims outlined for studying rat N-acetyltransferase expression in tissues of various inbred rat strains involve analysis of tissue-specific mRNA splicing, mRNA expression, protein expression, and enzyme activity, and a study of cigarette smoke inhalation as a possible environmental influence on Nat expression.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>ACKNOWLEDGEMENTS</th>
<th>iii</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABSTRACT</td>
<td>iv</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>vii</td>
</tr>
</tbody>
</table>

## CHAPTER

I. BACKGROUND AND SIGNIFICANCE ................................................................. 1

II. NAT1 EXPRESSION IN PRIMARY HUMAN HEPATOCYTES ............................ 15

   Introduction ....................................................................................... 15
   Methods ............................................................................................. 20
   Results & Discussion ....................................................................... 25
   Conclusions ..................................................................................... 36
   Future Experiments ......................................................................... 37

III. A RODENT MODEL FOR STUDYING N-ACETYLTRANSFERASE TISSUE-SPECIFIC EXPRESSION IN VIVO ................................................................. 38

IV. IDENTIFICATION, CLONING, AND SEQUENCING OF A NOVEL RAT N-ACETYLTRANSFERASE GENE (NAT3) ................................................................. 41

   Introduction ....................................................................................... 41
   Methods ............................................................................................. 43
   Results & Discussion ....................................................................... 44

V. CHARACTERIZATION OF RAT N-ACETYLTRANSFERASES NAT1*13, NAT2*20, NAT2*21A, NAT2*21B, AND NAT3 BY CLONING AND RECOMBINANT EXPRESSION IN ESCHERICHIA COLI ................................................................. 49

   Introduction ....................................................................................... 49
   Methods ............................................................................................. 52
   Results & Discussion ....................................................................... 60
LIST OF FIGURES

FIGURE

1. Chemical Structures of Some N-acetyltransferase Substrates .............................................. 2
2. Reactions Catalyzed by Arylamine N-acetyltransferases .......................................................... 4
3. Cryopreserved Hepatocyte NAT1 and NAT2 Genotype/Phenotype Correlation .................. 16
4. Real-Time PCR of NAT1*4 and NAT1*10 Allele cDNA ...................................................... 21
5. Quantitative Real-Time RT-PCR Sensitivity for NAT1 Allele Expression Analyses .......... 26
6. NAT1 Allelic Expression in NAT1*4/*10 Human Hepatocytes ............................................ 27
7. Hepatocyte NAT1 Activity and NAT1 mRNA Expression .................................................... 29
8. Creation of a Standard Curve with NAT1*4/*4 & NAT1*10/*10 Genomic DNA Mixtures ............................................................ 31
9. Standard Curve Analysis of Hepatocyte NAT1 Allelic Expression ..................................... 33
10. Rat Nat3 Nucleotide and Deduced Amino Acid Sequences ............................................... 45
11. Amino Acids 125, 127, 129 Influence N-acetyltransferase Substrate Selectivity .............. 46
12. Human, Mouse, and Rat N-acetyltransferase Sequence Homologies .................................. 47
14. Rat Nat N-acetylation of PABA and SMZ ........................................................................ 61
15. Rat Nat N-acetylation of MDA and MOCA ..................................................................... 62
16. Rat Nat N-acetylation of ABP and AF ............................................................................. 63
17. Rat Nat N-acetylation of pABG .................................................................................. 64
18. Kinetics of Rat Nat N-acetylation of 2-Aminofluorene ....................................................... 67
19. Kinetics of Rat Nat N-acetylation of 4-Aminobiphenyl ...................................................... 68
20. Thermostability of Recombinant Rat N-acetyltransferases ............................................. 70
21. Polyclonal Antisera Detection of Rat N-acetyltransferases by Western blot ................. 72
22. Immunoreactive Detection of Recombinantly Expressed Rat Nat2 Enzymes............... 74
23. Real-time RT-PCR Analysis of Recombinantly Expressed Rat Nat2 mRNA............... 75
24. Constructed Sequence of Recombinant Nat mRNA from PKK223-3......................... 78
25. Mfold Predicted mRNA Structures for Recombinant Nat2 Slow Alleles ..................... 79
26. Taqman Primer and MGB Probe Design for RT-PCR Rat Nat Locus Discrimination ...... 95
27. Primer Design for Real-Time PCR Controls and PCR of RACE Products................... 97
CHAPTER I
BACKGROUND AND SIGNIFICANCE

Arylamine N-acetyltransferases NAT1 and NAT2 are xenobiotic metabolizing enzymes that first received attention over 40 years ago when researchers discovered that polymorphic NAT2 modifies individual human variability in the metabolism of the anti-tubercular drug isoniazid (Evans et al., 1960; Peters et al., 1965), making N-acetyltransferases among the first polymorphic genes identified. It was later discovered that this role extended also to other hydrazine drugs including the MAO inhibitor phenelzine and the anti-hypertensive drug hydralazine (Lemke et al., 1995; Weber and Hein, 1985). NATs also detoxify the antibacterial sulfonamides (Parker, 1969) and arylamine drugs such as the antiarrhythmic drug procainamide, the antibiotic dapsone, and the aromatase inhibitor aminoglutethimide (Weber and Hein, 1985) (Figure 1).

The human genome encodes two active N-acetyltransferase isoforms, NAT1 and NAT2, each consisting of a single intronless coding exon 870 base pairs long. These genes lie in close proximity on chromosome 8 (Matas et al. 1997), with high nucleotide sequence identity (86.8%), and which encode proteins that share a high amino acid sequence identity (80.0%). Human NAT1 and NAT2 enzymes are very similar in their sequence and function, with distinct, yet overlapping, substrate selectivities, distinct structural stabilities, and distinct kinetic properties toward their arylamine substrates (Blum et al., 1990; Hein et al, 1993).

In addition to drug detoxification, NATs are implicated as modifiers of cancer predisposition due to their roles in both detoxification and activation of aromatic and heterocyclic amine carcinogens in the environment (Fretland et al., 2002). NATs metabolize carcinogens like
Figure 1. Chemical Structures of Some N-acetyltransferase Substrates.

N-acetyltransferase (NAT) isozymes metabolize aromatic amines, heterocyclic amines, and hydrazines, including many pharmaceutical drugs and environmental carcinogens. (A) Some pharmaceutical drugs metabolized by NATs include the antitubercular drug isoniazid, the antihypertensive drug hydralazine, the MAO inhibitor phenelzine, the aromatase inhibitor aminoglutethimide, the antibiotic dapsone, the antiarrhythmic drug procainamide, and the antibacterial drug sulfamethazine. (B) Some environmental carcinogens metabolized by NATs include 4-aminobiphenyl (ABP), a component of cigarette smoke, and 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP), found in meats cooked well-done.
4-aminobiphenyl (ABP), a component of cigarette smoke (Patrianakos et al., 1979), the carcinogen 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP), and other protein pyrolysis products, formed in meats cooked well-done (Godshalk et al., 2001; Layton et al., 1995). Prior to acting as mutagens and animal carcinogens, these compounds must first undergo metabolic activation by xenobiotic metabolizing enzymes (Wogan et al., 2004).

N-acetyltransferases catalyze N-acetylation (usually detoxification), O-acetylation (activation), and N,O-acetylation (activation) reactions (Hein DW, 1988). These reactions are carried out via a ping-pong reaction mechanism in which the NAT enzymes use acetyl-CoA as the acetyl-group donor (Riddle and Jencks, 1971). All N-acetyltransferases, regardless of species, contain the Cys-His-Asp catalytic triad, which resembles that of cysteine proteases (Sinclair et al., 2000). NATs catalyze N-acetylation (Figure 2) by transferring an acetyl group from acetyl-CoA to the exocyclic nitrogen of an aromatic or heterocyclic amine. This is usually a detoxification step, leading to excretion of the metabolite. For O-acetylation (Figure 2) to take place, the exocyclic amine must first undergo N-hydroxylation by a CYP1A1 or CYP1A2 enzyme. This reaction competes with N-acetylation for the exocyclic nitrogen, and is considered an activation step. N-acetyltransferases will further activate the carcinogen by transferring an acetyl group to the oxygen. The resulting N-acetoxy metabolite is very unstable and spontaneously decomposes to acetic acid and a highly reactive aryl nitrenium ion. This electrophilic ion will then form adducts with nucleophilic sites throughout the cell, including DNA and proteins. N,O-acetylation (Figure 2) is similar to O-acetylation except that the competing N-acetylation and N-hydroxylation reactions both succeed, result in an N-acetylated and N-hydroxylated exocyclic nitrogen. NATs can then catalyze intramolecular O-acetylation, resulting in the same unstable acetoxy species formed by O-acetylation (Hein DW, 1988).

Because NAT1 and NAT2 are highly polymorphic, they contribute to individual cancer susceptibility by modifying the activation and deactivation of arylamine carcinogens. To date, over 25 known human NAT1 alleles and over 35 known human NAT2 alleles have been identified,
Figure 2. Reactions Catalyzed by Arylamine N-acetyltransferases

N-acetylation occurs when an acetyl group is transferred from acetyl-CoA to the exocyclic nitrogen of an aromatic or heterocyclic amine, and is usually a detoxification step. P450 enzymes compete with NATs for metabolism of the exocyclic nitrogen where they catalyzing a hydroxylation reaction, thereby activating the carcinogen. NATs can further activate the compound by subsequently acetylating the oxygen which results in an extremely unstable N-acetoxy derivative. The N-acetoxy form of the molecule spontaneously breaks down to form an electrophilic arylnitrenium ion which can form adducts with DNA and other nucleophilic sites in the cell. Alternatively, the exocyclic nitrogen can be both N-acetylated and N-hydroxylated, in which case NATs will catalyze an intramolecular transfer of the acetyl group to the oxygen, resulting in formation of the reactive arylnitrenium ion.
each of which possess a combination of single nucleotide polymorphisms (SNPs) (Vatsis et al., 1995). Some of these SNPs modify the acetylator phenotype of an individual by altering enzyme stability, enzyme function, and/or mRNA expression, resulting in rapid, intermediate, and slow acetylator phenotypes (Butcher et al., 2002; Fretland et al., 2001a/b; Leff et al., 1999a; Zang et al., 2004). The fact that these enzymes are highly polymorphic makes them a prime research subject for molecular epidemiology studies, from which we can gain insight into how different allelic forms of these enzymes can modify an individual’s cancer risk, drug response, and the susceptibility to adverse drug reactions (Hein et al., 2000b; Spielberg, 1996). While the xenobiotic metabolizing function of NATs applies to pharmaceutical drugs and carcinogens, as covered above, this review will focus on the role of NATs in carcinogen metabolism and cancer susceptibility.

Molecular epidemiology studies that have investigated the relationship between N-acetyltransferase genotype/phenotype and cancer risk suggest that NATs modify predisposition to various cancers. Such studies have investigated the role of polymorphic NAT variants in urinary bladder cancer (Cartwright et al., 1982; Lower et al., 1979), colorectal cancer (Bell et al., 1995a; Chen et al., 1998), breast cancer (Bulovskaya et al., 1978; Millikan et al., 1998), lung cancer (Cascorbi et al., 1996; Bouchardy et al., 1998), head and neck cancer (Gonzalez et al., 1998; Olshan et al., 1997), prostate cancer (Fukutome et al., 1999; Wadelius et al., 1999), pancreatic cancer (Bartsch et al., 1998), and others. Some of these studies suggest a positive correlation between certain NAT genotypes and predisposition to cancer. In general, however, the findings of these epidemiological studies are inconsistent. Apparent inconsistencies may be attributable to the study of different chemical carcinogens, inadequate understanding of the genotype/phenotype relationship of some alleles, small sample size, and/or other confounding factors not accounted for in the study design (Hein et al., 2000a). The most consistently reported molecular epidemiological associations indicate that individuals with a NAT2 slow acetylator phenotype are at higher risk for urinary bladder cancer (Cartwright et al., 1982; Lower et al., 1979), due to
decreased detoxification (N-acetylation) of aromatic amines, and individuals with a NAT2 rapid acetylator phenotype have an elevated risk for developing colon cancer (Gil and Lechner, 1998; Ilett et al., 1987), due to increased activation (O-acetylation) of heterocyclic amines. While we know little about the role of NAT1 and NAT2 in cancer susceptibility, we know less about the role of NAT1 (Hein, 2002). This lack of understanding regarding NAT1 could be due to an incomplete understanding of the relationship between NAT1 genotype and phenotype, and the factors that influence this relationship (Hughes et al., 1998; Butcher et al., 1998). Although such ambiguity is observed for some NAT2 alleles, it is to a lesser extent than for NAT1 (Gross et al., 1999; Meisel et al., 2001; Sim et al., 2000).

The epidemiological observation that NAT influence on cancer risk varies depending on tissue type, as mentioned above, could be explained in various ways. One possible explanation worth considering involves the tissue-specific regulation of NAT isozymes. This could easily account for the differences in susceptibility of different tissues to NAT2-mediated carcinogenesis. Tissue-specific expression could be key to the physiological function of N-acetyltransferases, and therefore also an important factor influencing the ability of physicians to predict tissue-specific cancer risk following aromatic amine exposure. NAT1 and NAT2 tissue-specific expression could play an important role in arylamine-induced cancer risk by determining the ratio of inactive to reactive arylamine metabolites within each cell (Grant et al., 2000), thus modifying the risk of mutagenesis.

The relative expression of NAT1 and NAT2 in different tissues is important because NAT1 and NAT2, while similar in many ways, differ in several important aspects. First, NAT1 and NAT2 have different substrate preferences (Hein et al., 1994; Minchin et al., 1992; Wild et al., 1995). Although there is some overlap, most substrates are preferentially metabolized by one isozyme over the other. For instance, human NAT1 selectively acetylates p-aminobenzoic acid (PABA), whereas human NAT2 selectively acetylates sulfamethazine (SMZ) (Grant et al., 1991). Substrate selectivity is influenced by three key residues in the NAT genes, at positions 125, 127,
and 129, all three of which differ between NAT1 and NAT2 (Goodfellow et al., 2000). Since NAT1 is catalytically more efficient than NAT2 (Grant et al., 1997; Vatsis et al., 1995; Vatsis and Weber., 1997), the pool of activated or deactivated carcinogens in a tissue following any given arylamine exposure is dependent on whether NAT1 or NAT2 or both are expressed and active in that tissue, and at what level they are expressed. For example, a high-NAT1-expressing tissue with barely detectable NAT2 expression may be more at risk from exposure to a NAT1 selective carcinogen than a NAT2 selective carcinogen. In addition to substrate selectivity, NAT1 and NAT2 differ in structural stability, where NAT2 is much more stable than NAT1 (Dupret et al., 1994). This difference, detected experimentally as thermostability, could translate into differences in cellular enzyme turnover. Lastly, differential expression of NAT1 and NAT2 in tissues is important because the catalytic efficiency of the enzymes differs, where NAT1 generally catalyzes reactions much more efficiently than NAT2 (Grant et al., 2000). Therefore, considering the differences between NAT1 and NAT2 in substrate selectivity, structural stability, and catalytic activity, the tissue-specific expression of these isozymes must be better understood if we are to advance our understanding of NAT-mediated cancer risk in vivo.

Tissue-specific gene expression is common in the human genome (Bulun et al., 2003; Shmelkov et al., 2004; Simpson, 2004; Whetstine et al., 2002), and therefore it is reasonable to suspect that N-acetyltransferase expression might be regulated likewise. While apparent tissue-selective epidemiological associations could be explained away by alternative hypotheses, other data lend support to the idea that NAT1 and NAT2 are tissue-specifically and cell-type-specifically expressed, though mRNA and protein expression analyses in a wide range of tissues have yielded inconsistent results (Hickman et al., 1998).

Unfortunately, NAT protein expression analyses and NAT mRNA expression analyses have not produced consistent conclusions regarding the sites of NAT1 and NAT2 expression. Analyses of NAT protein activity in tissues conclude that NAT1 is ubiquitously expressed (Pompeo et al., 2002; Rodrigues-Lima et al., 2003), while NAT2 expression is limited to liver
(Evans and White, 1964) and intestinal epithelium (Hickman et al., 1998). On the other hand, NAT1 and NAT2 transcripts were detected in most tissues using in situ hybridization (Debiec-Rychter et al., 1999; Windmill et al., 1997), suggesting that both NAT1 and NAT2 mRNA are ubiquitously expressed. Several factors contribute to the apparent discrepancies between analyses of protein and mRNA expression.

First, mRNA detection methods and protein detection methods differ with regard to sensitivity. In situ hybridization is a highly sensitive technique capable of detecting even low levels of transcript (Wilson et al., 1997), while measurements of enzyme activity are generally less sensitive. Thus, NAT1 and NAT2 mRNA are more readily detected than the NAT1 and NAT2 enzyme activity.

Second, flaws in experimental design may produce false and confusing results. Correct probe design is crucial for accurate mRNA detection. This point becomes apparent when considering the example of conflicting intestine NAT expression reports. It was concluded based on NAT enzyme acetylation activity measurements that NAT1 is expressed higher than NAT2 in the intestine (Hickman et al, 1998), while mRNA expression analysis suggested that NAT2 is expressed even higher than NAT1 (Windmill et al., 1997). A careful examination of the methods reveals that the NAT1 probes were designed to target to a DNA sequence 5' of the NAT1 start codon, which is a sequence that may not exist in most NAT1 transcripts. Although this 5' sequence comes from a previously reported NAT1 transcript (Ohsako and Deguchi, 1990), its presence in NAT1 mRNA transcripts is questionable. Recent analyses of NAT1 promoters (Butcher et al., 2004b; Husain et al., 2004) do not report this transcript as having even a minor presence among NAT1 transcripts. Perhaps NAT1 expression only appears to be lower than NAT2 expression in Windmill’s mRNA study because the NAT1 probe recognizes only small fraction of NAT1 transcripts at best.

A final factor deserving consideration regarding discrepancies between protein and mRNA expression measurements involves the often difficult and/or misleading interpretation of
NAT1 and NAT2 enzyme activity data. For instance, in muscle NAT2 mRNA was detected but NAT2 enzyme activity was below the limit of detection; on the other hand, NAT1 mRNA enzyme were readily detected (Rodrigues-Lima et al., 2003). Detection of NAT1 enzyme but not NAT2 enzyme could be attributed to higher NAT1 catalytic activity for its selective substrates as compared to that of NAT2 for its selective substrates. This would permit detection of lower levels of NAT1 by enzymatic activity measurements than similarly low levels of NAT2 (Grant et al., 2000). Detection of low enzyme levels using activity assays is problematic, requiring relatively high enzymatic activity. In the example above from muscle tissues, the NAT2 activity level may have fallen below the detection limit, due to its relatively low activity.

The lack of consistent data regarding the tissue-specific expression of NAT1 and NAT2 precludes coming to meaningful conclusions about how NAT1 and NAT2 are expressed in the different tissues of the body. A more practical approach must be devised. Understanding how NAT mRNA expression, protein expression, and enzyme activity are regulated has value for advancing our understanding of how NAT1 and NAT2 tissue-specific expression relates to NAT1 and NAT2 phenotype in different tissues. Analyzing only one of these factors alone, however, has little practical value. Due to the possibility that post-transcriptional regulation events modify NAT expression, NAT1 and NAT2 mRNA levels cannot be used as an accurate predictor of NAT phenotype in a tissue. Likewise, due to the possibility that post-translational regulation events modify NAT expression, NAT1 and NAT2 protein levels cannot be used as an accurate predictor of NAT phenotype in a tissue (since it may be that not all NAT protein is active). Also, it is possible that these forms of regulation vary widely from tissue to tissue and cell-type to cell-type, so that it is faulty to assume that the relative levels mRNA expression, protein expression, and enzyme activity will be in the same proportion in different tissues. Aside from the method difficulties discussed above, the fundamental weakness of the studies to-date with regard to their ability to contribute useful information regarding NAT tissue-specific expression is the mistake of analyzing only one measure of NAT expression at a time. No study reported to date provides
concrete data regarding how \textit{NAT1} and \textit{NAT2} tissue-specific expression influences NAT phenotype in tissues because no study to date has analyzed NAT mRNA, protein, and enzyme activity concurrently in different tissues. This type of study is needed for a more complete picture of the translational, transcriptional and post-transcriptional influences on \textit{NAT1} and \textit{NAT2} expression, and how they might vary among different tissues.

Recent analyses of \textit{NAT1} mRNA structure, and continued studies of NAT induction suggest that \textit{NAT1} expression is transcriptionally regulated. (Butcher et al., 2003; Boukouvala et al., 2003; Mitchell and Warshawsky, 2003; Zaher and Svensson, 1994). Identification and characterization of the major \textit{NAT1} promoters and non-coding exons in human breast demonstrated the presence of at least 4 alternative \textit{NAT1} promoters (Butcher et al., 2004b; Husain et al., 2004), which could be key to the mechanism by which NAT tissue-specific expression is regulated (Boukouvala et al., 2003; Rodrigues-Lima and Dupret, 2004). These studies have also demonstrated that alternative splicing of the 9 \textit{NAT1} non-coding exons results in at least 7 different mRNA species, with a single dominant promoter in breast tissue. Many mammalian genes are tissue-specifically expressed through promoter-dependent alternative splicing of non-coding exons, resulting in multiple mRNA species with different transcriptional and translational efficiencies (Bulun et al., 2003; Shmelkov et al., 2004; Ayoubi and Van De Ven, 1996; Whetstine et al. 2002). Since our knowledge of \textit{NAT1} mRNA structure and expression is limited to a single tissue in humans (breast), much work remains to be done for other tissues. Also, much remains to be delineated with regard to these possible expression mechanisms, requiring further study of \textit{NAT1} and \textit{NAT2} promoter usage and mRNA splicing patterns in multiple tissues. In addition to an advanced understanding of NAT expression, promoter analyses may provide a foundation for discovery of an endogenous role for N-acetyltransferases by providing links to biomolecular signaling pathways involved in NAT expression.

Inconsistent molecular epidemiology data regarding the association of \textit{NAT1} and \textit{NAT2} genotype and cancer risk, combined with an unclear relationship between \textit{NAT1} genotype and
phenotype suggest that our understanding of the factors (other than NAT1 and NAT2 SNPs) that influence acetylator phenotype is incomplete. Environmental and other genetic factors may influence acetylator phenotype and arylamine-induced cancer risk (Butcher et al., 2000b; Husain et al., 2004). Advancing our understanding of NAT-mediated cancer risk through successful molecular epidemiology investigations requires consideration of these other possible influences on NAT1 and NAT2 tissue-specific expression.

The literature reviewed above suggests that these factors probably influence NAT1 expression nearly exclusively, considering the observed divergence of NAT1 genotype and phenotype, and the consistent NAT2 genotype and phenotype correlations. Some have postulated that the apparent lack of correlation between NAT1 genotype and phenotype is due to an incomplete knowledge of NAT1 polymorphisms, which would be resolved once a more comprehensive list of NAT1 polymorphisms is compiled (Payton and Sim, 1998). Others suggest that endogenous compounds or NAT substrates and/or products influence NAT1 expression (Butcher et al., 2000a/b; Ward et al., 1992). Yet others propose the possibility that NAT activity and expression are influenced by non-genetic, or environmental factors (Butcher et al., 1998). While we don’t completely understand NAT regulation outside of SNPs and genotype, it is clear that in vivo N-acetyltransferase phenotype is influenced by genetic and environmental factors independently of known NAT genotype (Rodrigues-Lima and Dupret, 2004).

Genetic influences on gene expression include cis-acting and trans-acting genetic factors (Morley et al., 2004). Unknown linked, or cis-acting, genetic factors could include unidentified genetic polymorphisms in NAT1 and NAT2 promoters or other control regions that influence NAT expression. Such SNPs could alter transcription initiation and/or efficiency, or affect mRNA processing, folding, and/or stability. For example, a mutation in the promoter of the low-density lipoprotein receptor gene identified in a familial hypercholesterolemia patient caused an 80% reduction of the promoter activity (Mozas et al., 2002). While cis-acting genetic factors account for up to 35% of the variability in gene expression among individuals, trans-acting loci have an
even greater influence on variability in gene expression throughout the genome (Pastinen and Hudson, 2004). Unlinked, or trans-acting, genetic factors are variable among individuals due to the uniqueness of their genetic backgrounds. These factors include polymorphic genes outside the \textit{NAT1} and \textit{NAT2} loci that influence their expression, such as transcription factors or other expression control proteins. The function of trans-acting genes could vary among individuals as a result of SNPs or other function-affecting factors that influence their expression and/or activity. The expression of HMG-CoA reductase provides an example of this type of gene regulation. HMG-CoA reductase expression in mouse liver is about 5-fold higher in C57BL/6 mice than BALB/c mice. Cis-acting genetic factors affecting the structural gene were ruled out as a cause for this tissue-specific variation of HMG-CoA reductase mRNA expression, and a trans-acting locus that controls the gene’s expression was found to be the determining factor (Hwa et al., 1992). Both cis-and trans-acting genetic factors should be seriously considered for their possible role in regulating the expression of N-acetyltransferases, and \textit{NAT1} in particular.

In addition to genetic influences on NAT expression, environmental stimuli may influence NAT expression. Environmental influences may include occupation-related and/or lifestyle-related factors such as cigarette smoke or exposure to industrial chemicals. Experiments in cell culture have shown that NAT expression is influenced by external stimuli such as curcumin, a spice (Chen et al., 2003), the anticancer drug paclitaxel (Yang et al., 2003), luteolin, a compound found in many common edibles such as celery and peppermint (Su et al., 2003), even Vitamin C (Hung and Lu, 2001) and acetaminophen (Rothen et al., 1998). These influences act to induce or inhibit NAT mRNA expression and/or enzyme activity in the cell culture systems tested. Other cell culture experiments suggest that NAT substrates (p-aminobenzoic acid, p-aminosalicylic acid, ethyl-p-aminobenzoate, and p-aminophenol) down-regulate NAT1 activity by down-regulating NAT1 protein, while mRNA remained unchanged (Butcher et al., 2000b). In similar studies, hydroxylamine-PABA irreversibly inactivated NAT1 though covalent modification of the enzyme active site (Butcher et al., 2000a). Inactivation of NAT1 enzymes by
their substrates and other xenobiotics may also take place at the level of transcription via xenobiotic-inducible regions on NAT1 and NAT2 promoters (Mitchell and Warshawsky, 2003). Other studies suggest substrate-dependent post-translational inactivation via substrate-induced conversion of the NAT1 active site from a stable acetylated state to an unstable deacetylated state, thereby leaving the active site cysteine unprotected and resulting in the ubiquitination and subsequent degradation of the NAT1 enzyme (Butcher et al., 2004a). Oxidative stress and cellular redox stress may also influence NAT1 activity. Reactive oxygen species (ROS), such as hydrogen peroxide, can inactivate NAT1 by reacting with its active site cysteine. This results in a sulfenic acid form that is reversible by reducing agents such as reduced glutathione (Atmane et al., 2003). Reactive nitrogen species (RNS), such as S-nitrosothiols, also inhibit the NAT1 enzyme by targeting and modifying the reactive cysteine at its active site, resulting in a mixed disulfide, a reaction that is also reversible in the presence of reducing agents (Dairou et al., 2003). Pathophysiological processes such as inflammation produce the highly reactive RNS peroxynitrite that reacts with the active site cysteine to form sulfonic and sulfinic acids which, unlike hydrogen peroxide and S-nitrosothiols, irreversibly inactivates NAT1 (Dairou et al., 2004). UVB irradiation is an oxidative process that induces inactivation of NAT1 and NAT2 in human lens epithelial cells, also a reversible reaction (Dairou et al., 2005). Overall, these studies have shown that NAT1 activity and/or expression is/are influenced by non-genetic factors such as pharmaceutical drugs, environmental chemicals, cellular oxidants, and even inflammation-related chemicals. More investigations are needed into assess the importance of these environmental variables on N-acetyltransferase expression in an animal model. Further characterization of these influences may aid in resolving the NAT1 genotype/phenotype inconsistencies and clarify NAT1 molecular epidemiology data (Rodrigues-Lima and Dupret, 2004).

Some potentially important questions regarding the influence of genotype and environment on NAT expression may help shape future investigations NAT1 and NAT2 tissue-specific expression. Can environmental exposures or unknown cis- or trans-acting genetic factors
overwhelm the influence of an individual’s NAT1 and/or NAT2 genotype? Are genetic factors (cis- and/or trans-acting factors, and tissue-specific expression) the primary determinants of NAT1 and NAT2 acetylator phenotype, or do environmental exposures also influence acetylator phenotype? Do environmental exposures and trans-genetic factors influence the expression of NAT1 and NAT2 equally, or is one locus more sensitive to certain regulatory influences than the other locus? The answers to these questions are important for understanding how NAT-mediated individual predisposition to certain cancers is modified in individuals by better understanding the factors that influence interindividual expression of NAT1 and NAT2.

How NAT1 and NAT2 are tissue-specific expressed, and the genetic and environmental influences on that expression, are thus far unknown. The lack of basic information regarding NAT promoters and other expression control regions has precluded the search for cis-acting mutations outside the coding region until lately. Recent identification of human NAT1 promoters (Husain et al., 2004) and the promise of the same for NAT2 (Husain et al., 2003) should speed the discovery of functionally significant polymorphisms in expression control regions (cis-acting genetic factors). A great deal of work remains to be done if we are to understanding N-acetyltransferase tissue-specific expression and the environment and trans-acting genetic influences that modify that expression.
CHAPTER II

NAT1 EXPRESSION IN PRIMARY HUMAN HEPATOCYTES

Introduction

Cryopreserved human hepatocytes are an ideal system for analyzing genotype/phenotype relationships. The advantages of primary cell culture, the ability to work with a single cell type that has not been altered through an immortalization process (Li et al., 1999; Sun et al., 1993), and the convenience of cryopreserved samples that are conveniently accessed at will, are combined into one with cryopreserved hepatocytes. Since the liver is a key site for xenobiotic metabolism, and both NAT1 and NAT2 are expressed in liver (Windmill et al., 2000), hepatocytes are suitable for studying N-acetyltransferase expression.

Our lab obtained 76 cryopreserved human hepatocyte samples, representing as many individuals (In vitro Technologies, Inc., Baltimore, MD, USA). These hepatocytes were genotyped for NAT1 and NAT2 by real-time RT-PCR using previously published methods (Doll and Hein, 2001; Doll and Hein, 2002). The same samples were then tested for NAT1 and NAT2 N-acetylation activity using p-aminobenzoic acid (PABA) as the selective substrate for human NAT1 (Doll et al., 1997), and sulfamethazine (SMZ) as the selective substrate for human NAT2 (Leff et al., 1999b). The results indicated that NAT1 acetylator phenotype did not correlate with expected NAT1 phenotype, as deduced from NAT1 genotype (Figure 3A). In fact, the acetylation rates varied over 600-fold among NAT1*4/*4 individuals (n = 38) and over 350-fold among NAT1*4/*10 individuals (n = 24). The data for NAT1*10/10 individuals were tighter, but the sample size was much smaller (n = 4). The NAT2 data, on the other hand, demonstrated a clear correlation between NAT2 acetylator phenotype and expected NAT2 phenotype, as deduced from NAT2
Figure 3. Cryopreserved Hepatocyte NAT1 and NAT2 Genotype/Phenotype Correlation

Cryopreserved hepatocytes were genotyped for NAT1 and NAT2, then tested for NAT1 and NAT2 N-acetylation activity using NAT1-selective substrate p-aminobenzoic acid (PABA), and NAT2-selective substrate sulfamethazine (SMZ). Activities were grouped according to genotype and plotted as shown above. (A) NAT1*4 is the wild-type allele, and NAT1*10 is a variant allele that demonstrates higher activity than NAT1*4 in vitro (Badawi et al., 1995; Bell et al., 1995b). NAT1*4/*4 homozygotes and NAT1*4/*10 heterozygotes demonstrate a wide range of N-acetylation activities. (B) NAT2*4 is the wild-type rapid allele, and NAT2*5 and NAT2*6 alleles are slow alleles. Combining rapid NAT2*4 with a NAT2*5 or NAT2*6 allele results in intermediate phenotype. Genotyping and enzyme activity assays were performed by Mark Doll.
This is not the first time this phenomenon has been observed for \textit{NAT1}. Other studies have documented a lack of correlation between \textit{NAT1} genotype and phenotype in placenta (Smelt et al., 1998), breast (Williams et al., 2001), and whole blood (Payton and Sim, 1998). Possible reasons for this discrepancy between \textit{NAT1} genotype and expected acetylator phenotype could include the following: (1) the existence of unidentified cis-acting genetic factors, such as polymorphisms in \textit{NAT1} expression control regions; (2) environmental influences on \textit{NAT1} expression; (3) variability in trans-acting genetic factors among individuals who have distinct genetic backgrounds. These factors were discussed in Chapter I.

It is clear from Figure 3 that factors other than \textit{NAT1} genotype influence \textit{NAT1} acetylator phenotype in hepatocytes, as in the other tissue types mentioned above. The background data for the individuals from whom the hepatocytes originated is not sufficiently detailed to draw correlations between environmental exposures and \textit{NAT1} expression. Due to the complexity of biological interactions with the environment, it is doubtful whether such correlations are possible even with the most detailed background information. Since one cannot experimentally analyze the hepatocytes to determine environmental influences on \textit{NAT1} phenotype, the focus must be shifted to genetic influences.

As already mentioned, some have suggested that this problem might be solved following more thorough characterization of \textit{NAT1} polymorphisms (Payton and Sim, 1998). If unknown 5'-UTR SNPs influence \textit{NAT1} expression, characterization of these SNPs may bridge the gap between \textit{NAT1} genotype and phenotype. Since the entire \textit{NAT1} gene is at least 12 kb long (Husain et al., 2004), sequencing this entire region in a large number of individuals to find polymorphisms in this region is practically impossible. Therefore, a different approach is necessary.

While many SNPs may exist in the \textit{NAT1} 5'-UTR, polymorphisms of interest are those that have functional consequences for \textit{NAT1} expression. Functional consequences of coding
region SNPs can be detected as alterations of enzyme activity. Functional SNPs in the 5'-UTR can only be detected at the level of mRNA transcription and possibly protein translation. The fundamental strategy for the experiments described in this chapter is based on the former.

Experiments were designed based on competing hypotheses. The first hypothesis is that the dramatic variation in human hepatocyte NAT1 PABA activity within each genotype is the result primarily of cis-acting SNPs in the 5'-UTR of the NAT1 locus. If the data support this hypothesis, functionally important mutations exist in the 5'-UTR, providing compelling evidence to justify future investigations that analyze the NAT1 locus for unknown SNPs. The alternative hypothesis is that dramatic variation in human hepatocyte NAT1 PABA activity is not caused primarily by genetic differences among individuals at the NAT1 locus. If the data support the alternative hypothesis, environmental or trans-acting genetic factors (genetic variability outside the NAT1 locus) influence NAT1 expression, warranting further investigation into the influence of these factors.

SNPs in the 5'-UTR could directly alter transcription activity by affecting interactions between transcription factors and the NAT1 promoter, by altering exon splicing patterns, altering mRNA structure, etc. Polymorphisms in the 5'-UTR could be as far away as 12 kb or more from the NAT1 coding region. Due to genetic recombination by crossing over, these polymorphisms could recombine independently of the NAT1 coding region. In this case, specific 5'-UTR SNPs would not associate with specific NAT1 alleles, but would be distributed randomly among all known NAT1 alleles. For example, two individuals may have the same NAT1 allele, say NAT1*4/*4, but have different polymorphisms in the 5'-UTR of the NAT1 gene, resulting in drastically different acetylator phenotypes. The complexity of the interactions between 5'-UTR polymorphisms and NAT1 alleles would depend on the number of 5'-UTR polymorphisms in the population and how drastically they influenced activity (we are only interested in functionally important polymorphisms). The experiments described in this chapter were designed based on these ideas.
The strategy implemented to test the competing hypotheses involves analysis of the hepatocytes for functionally important polymorphisms by testing for differences in transcription. Since NATI genotypes will harbor unknown 5'-UTR polymorphisms if the first hypothesis is true, it is necessary to measure the expression level of NATI alleles independently to determine whether the alleles are influenced by some unknown difference that causes them to express at different levels. Significant allelic expression differences will be indicative of unknown function-altering polymorphisms that result in different mRNA transcription rates. Since the first hypothesis requires very large mRNA expression differences in order to account for the very large variability in enzyme activity, the allelic expression differences will not be subtle and therefore identification should be straightforward.

The above strategy requires measurement of NATI alleles independently. NATI genotypes among the hepatocyte were mostly combinations NATI*4 and NATI*10 alleles, with a large portion of these NATI*4/*10 heterozygotes. Since differences cannot be measured in homozygous individuals, the NATI*4/*10 hepatocytes are ideal for this type of analysis. Since the 5'-UTR polymorphisms are far enough away from the coding region to be recombined independently of one another, it is reasonable to think that 5'-UTR polymorphisms will be spread among all NATI alleles. With NATI*4 as the reference allele, NATI*10 has polymorphisms T^{1088}A and C^{1095}A in the 3'-UTR within the first polyadenylation signal. These SNPs make NATI*10 alleles distinguishable from NATI*4 alleles by the sequence recognition techniques described in the methods section. Comparing NATI*10 and NATI*4 mRNA expression in NATI*4/*10 heterozygous hepatocytes will reveal whether polymorphisms in the 5'-UTR result in different versions of the NATI*4 and NATI*10 alleles that have drastically different expression to account for the lack of correlation between NATI genotype and phenotype in these hepatocytes.
Methods

Cryopreserved Hepatocytes

Seventy-six cryopreserved hepatocyte samples were obtained from In Vitro Technologies, Inc. (Baltimore, MD) and stored in liquid nitrogen upon arrival. Due to limited sample availability following other analyses on these hepatocytes, 17 of the 24 original NAT1*4/*10 heterozygote hepatocyte samples were available for analysis at the time of this experiment. The NAT1 enzymatic activities of these 17 hepatocytes varied over 350-fold and therefore included samples with a representative range of enzyme activities.

Determination of Method Sensitivity

Prior to analyzing hepatocyte NAT1*4 and NAT1*10 expression by real-time RT-PCR, it was necessary to test the method’s ability to accurately detect differences in NAT1*4 and NAT1*10 mRNA expression. Analysis of a sample with equal amounts of NAT1*4 and NAT1*10 mRNA should give allele ratio of 1.0. However, this value may deviate from 1.0 due to the characteristic sensitivity of the real-time PCR method, instrumentation, and differences between the NAT1*4 and NAT1*10 mRNA probes in their annealing and cleavage (by polymerase) efficiencies. Therefore, standards were necessary to determine the range of values surrounding 1.0 that should be considered as indicative of a 1.0 allele ratio. Anything outside of this range is considered significantly different from 1.0, and is indicative of a real difference in expression between the NAT1*4 and NAT1*10 alleles. This type of analysis requires a standard guaranteed to have equal amounts of NAT1*4 and NAT1*10 DNA sequence. Genomic DNA is the ideal standard since, by definition, genomic DNA from heterozygous individuals contains equal copies of each allele. In theory, NAT1*4/*10 heterozygous hepatocyte genomic DNA samples should have a 2^{-ACT} value of 1.0 for the ratio of NAT1*4 to NAT1*10.

Genomic DNA from 8 different NAT1*4/NAT1*10 heterozygous individuals was used as template for real-time PCR analysis (Figure 4). The reaction mixture contained NAT1-specific TaqMan primers (forward, 5’-gaaacataaccacaacatttcg3’; reverse, 5’-aatcacaatttcagaat
Figure 4. Real-Time PCR of NAT1*4 and NAT1*10 cDNA

The region of cDNA containing the single nucleotide polymorphisms (SNPs) is amplified using gene-specific primers. Fluorogenic TaqMan probes complementary to the sequence being detected anneal to the specific PCR products as they are created, allowing real-time detection of specific PCR products. TaqMan probes have a fluorogenic reporter dye on the 5' end and a quencher on the 3' end. The quencher reduces the fluorescent signal of the reporter dye by fluorescence resonance energy transfer (FRET). After the probe anneals to the specific target sequence, the 5' nuclease activity of AmpliTaq Gold polymerase cleaves the probe and separates the quencher from the reporter dye, resulting in an increase in fluorescence reading. The probe is cleaved as the polymerase extends the target sequence so that PCR extension of the target sequence is not interrupted.
-aacca-3') (Applied Biosystems, Foster City, CA), FAM-labelled NATI*4-specific (5'-actttaaaagacattttattatta-3') and VIC-labelled NATI*10-specific (5'-catctttaaatacatttttattatta-3') fluorogenic TaqMan probes (Applied Biosystems), hepatocyte genomic DNA and TaqMan Universal PCR Master Mix (Applied Biosystems), which contains AmpliTaq Gold polymerase. Real-time PCR was performed using an ABI Prism 7700 sequence detection system from Applied Biosystems. The reaction conditions were identical to the conditions used for genotyping the hepatocyte samples. These conditions, in addition to the primer and probe design, were described previously (Doll and Hein, 2002).

Allele-Specific Expression in Hepatocyte Samples

Cryopreserved hepatocytes were thawed on ice, then pelleted according to the manufacturer's instructions, and homogenized using QIAshredder columns from Qiagen (Valencia, CA). RNA was isolated using the RNeasy Kit from Qiagen, and then separated by electrophoresis on a 1% agarose gel to test the RNA quality by observing the distinct 28S and 18S ribosomal RNA bands. RNA samples were then treated with DNase using the Turbo DNase kit from Ambion (Austin, TX) and stored at -80°C. RNA was quantified by separating the RNA by electrophoresis on a 1% agarose gel along with RNA standards in known amounts (200 ng, 400 ng, 800 ng). RNA concentration was determined by densitometric analysis based on the standard curve. RNA samples were reverse transcribed using SuperScript III First-Strand Synthesis System for RT-PCR from Invitrogen (Carlsbad, CA). Control reverse transcription reactions were also performed in the absence of reverse transcriptase as controls for DNA contamination. All hepatocyte samples were analyzed for β-actin expression as a normalizing control. Real-time PCR was performed on the hepatocyte cDNA samples, as described above for the genomic DNA samples.

Standard Curve for Allele-Specific Measurements

A standard curve was designed for further analysis of the allelic expression data. Whereas the real-time PCR primers used for amplifying NATI*4 and NATI*10 are identical, the
allele-specific probes are different for \textit{NAT1*4} and \textit{NAT1*10}, and therefore can introduce variability. The standard curve will take into account that variability and allow comparison of the allele ratios minus the influence of the probe differences.

Hepatocyte genomic DNAs from \textit{NAT1*4/*4} homozygotes and \textit{NAT1*10/*10} homozygotes were mixed in \textit{NAT1*4/*4: NAT1*10/*10} ratios of 100:0, 90:10, 70:30, 50:50, 30:70, 10:90, and 0:100 to mimic situations in which the \textit{NAT1*4} and \textit{NAT1*10} alleles are expressed in different ratios in the hepatocyte samples. These standards were designed to test the ability of the method to detect differences in the expression of \textit{NAT1*4} and \textit{NAT1*10} alleles, as well as to provide a standard curve with which to analyze allele-specific expression. These mixtures were analyzed by real-time PCR and data were analyzed in the same way as the genomic DNA controls.

\textbf{Data Analyses}

\textit{NAT1} data were normalized to \(\beta\)-actin by subtracting the \(\beta\)-actin \(C_T\) value from the \textit{NAT1} \(C_T\) value, giving the \(\Delta C_T\) value. The expression of \textit{NAT1*4} and \textit{NAT1*10} alleles in these samples were analyzed as the ratio of \textit{NAT1*4} mRNA to \textit{NAT1*10} mRNA (\(\textit{NAT1*4}/\textit{NAT1*10}\)). In the case of equal expression, the ratio equals 1.0; in the case of 2-fold higher \textit{NAT1*4} than \textit{NAT1*10}, the ratio equals 2.0, etc. Therefore, the ratio of the \(\textit{NAT1*4} \Delta C_T\) to the \(\textit{NAT1*10} \Delta C_T\) gives the \(\Delta \Delta C_T\) value. Since \(C_T\) values are inversely proportional to the amount of mRNA expressed, it is necessary to normalize to a calibrator, resulting in \(2^{\Delta \Delta C_T}\) (calculations derived from Applied Biosystems’ “User Bulletin \#2: ABI Prism 7700 Sequence Detection System,” 2001). This value is mathematically equivalent to the ratio of individual \textit{NAT1*4} to \textit{NAT1*10} \(2^{\Delta C_T}\) values. For real-time PCR of genomic DNA samples, the value \(2^{-\Delta C_T}\) was calculated, since genomic DNA was normalized to DNA concentration and not to an internal standard. This value is equivalent to the ratio of \textit{NAT1*4} to \textit{NAT1*10} \(2^{C_T}\) values.
Statistical Analyses

All assays were performed in triplicate, with each replicate performed on a different day, and the standard error of the means (SEM) calculated for the set of triplicate experiments. Analysis of variance (ANOVA) was used to determine differences among the allele expression ratios for the 17 hepatocyte samples.
Results & Discussion

Determination of Method Sensitivity

Analysis of heterozygous $NAT1^*4/10$ hepatocyte genomic DNA by real-time PCR resulted in ratios ranging from approximately 0.5 to 1.5 (Figure 5). Therefore, when measuring $NAT1^*4$ and $NAT1^*10$ expression in heterozygous hepatocytes, any allele ratio between 0.5 and 1.5 will not be considered as different than 1.0. Any ratio less than 0.5 or greater than 1.5 can be considered different from 1.0, indicating that the alleles are expressed differently.

As is evident from the relatively wide range of ratios considered equal to 1.0 (0.5 – 1.5) this method is not sensitive enough to detect small differences in allele expression. The method is, however, sufficiently sensitive to detect large differences. Explaining the 350-fold range of enzyme activities in the $NAT1^*4/10$ hepatocytes in terms of cis-acting genetic polymorphisms requires that the hepatocytes with the lowest activity possess a combination of very low expressing $NAT1^*4$ and $NAT1^*10$ alleles. Likewise, the hepatocytes with the highest activity possess a combination of higher expressing $NAT1^*4$ and $NAT1^*10$ alleles. If this theory is true, the low expressing and high expressing alleles represented in these samples must have drastically different expression rates. Therefore, although a greater sensitivity might be achieved by optimizing the method, the differences in $NAT1^*4$ and $NAT1^*10$ allele expression that we are trying to detect are very large, eliminating the need for a more sensitive method.

Allele-Specific Expression in Hepatocyte Samples

The ratios of $NAT1^*4$ to $NAT1^*10$ in 17 hepatocyte samples varied from approximately 0.5 to 3.3 (Figure 6). Five of these samples show significantly reduced $NAT1^*10$ expression relative to $NAT1^*4$. The other 12 hepatocyte samples have allele expression ratios that fall within the range of 0.5 – 1.5, and thus are not considered different than 1.0. Analysis of variance (ANOVA) was performed on the data in Figure 6 showing that the data in this group are significantly different ($P = 0.014$).

Instead of a random distribution of high expressing and low expressing alleles, the data
Figure 5. Quantitative Real-Time RT-PCR Sensitivity for NAT1 Allele Expression Analysis

Heterozygous NAT1*4/NAT1*10 human hepatocyte genomic DNA samples were analyzed by real-time PCR. The experimentally determined ratios plotted above, have variability extending about 50% above and below (dashed lines) the theoretically expected 1.0 ratio (solid line). Thus, due to the sensitivity of the method, experimentally determined NAT1*4 and NAT1*10 allele expression ratios below 0.5 or above 1.5 will be considered different from 1.0, while anything within this range falls within the limits of detection for this method.
Figure 6. Relative NAT1 Allele Expression in NAT1*4/*10 Human Hepatocytes

Hepatocyte cDNA samples reverse transcribed from hepatocyte RNA were analyzed for NAT1*4 and NAT1*10 allele-specific expression by real-time PCR. The NAT1*4/NAT1*10 allele expression ratios are displayed above. The solid line corresponds to a theoretical NAT1*4/NAT1*10 expression ratio of 1.0. The region between the two dotted lines, which lie above and below the solid line, represent the range of expression ratios determined to not be different from 1.0 by this method (Figure 5). Several samples demonstrate significant variation from 1.0, indicating unequal expression of the NAT1*4 and NAT1*10 alleles.
suggest a general trend of higher NAT1*4 expression than NAT1*10 expression. It is unclear at this point, however, whether this trend is real or an artifact of characteristic differences between the probes used to detect NAT1*4 and NAT1*10 sequences. It could be that, compared to the NAT1*10 probe, the NAT1*4 probe anneals to its target more readily and/or is cleaved more readily by the polymerase. On the other hand, the NAT1*4 probe could lack complete specificity for NAT1*4, and anneal to NAT1*10 some of the time, thereby artificially boosting the NAT1*4 signal. These factors were tested using a standard curve, as described in the next section.

The greatest difference between NAT1*4 and NAT1*10 expression observed in the hepatocytes is approximately 3-fold. Therefore, there is no evidence to justify a conclusion that enzyme activity differences in heterozygotes (350-fold) are solely caused by forms of NAT1*4 and/or NAT1*10 alleles that express differently. However, the evidence of slight allelic expression differences (~3-fold), possibly caused by 5'-UTR polymorphisms, does not rule out the possibility that different forms of known NAT1 alleles exist or at least contribute to the lack of correlation between NAT1 genotype and phenotype in hepatocytes.

The question then remains: what can account for the inability of NAT1 genotype to predict NAT1 phenotype? Instead of searching for polymorphisms in the 5’-UTR to explain the discrepancy, the strategy described here directly tested the critical question of whether differential mRNA expression can account for NAT1 phenotype. Since this study reveals that differential mRNA expression cannot, in fact, account for NAT1 phenotype, the expectation that identifying and characterizing 5’-UTR polymorphisms will clear up the problem can be set aside. The list of explanations for NAT1 phenotype has now been narrowed down to the influences of environmental factors and/or trans-acting genetic factors on NAT1 mRNA and/or protein expression.

Total NAT1 mRNA expression can be calculated by adding the quantitative expression values for both NAT1*4 and NAT1*10 alleles. No correlation can be observed between total NAT1 mRNA and NAT1 enzymatic activity levels (Figure 7). This further supports the idea that
Figure 7. Hepatocyte NAT1 Activity and NAT1 mRNA Expression

The X-axis of each graph is labeled with hepatocyte sample identification codes. (A) Hepatocyte NAT1 enzymatic activities were measured using the NAT1-selective substrate p-aminobenzoic acid (PABA). (B) Total NAT1 mRNA expression was determined by calculating the sum of quantitative NAT1*4 and NAT1*10 mRNA expression measurements. Comparison of NAT1 enzyme activity and NAT1 mRNA in order of increasing enzyme activity reveals the lack of correlation between the two. In fact, the sample with the lowest enzyme activity has one of the highest levels of NAT1 mRNA expression, while the sample with the highest enzyme activity has a relatively low NAT1 mRNA expression.
factors external to the NAT1 locus, either genetic or environmental, are influencing NAT1 acetylator phenotype post-transcriptionally. Possible mechanisms for such influences were mentioned in the Chapter I, and therefore will not be discussed here.

One concern is that the relatively low stability of the NAT1 enzyme (Dupret et al., 1994), might take some responsibility for the difficulty in correlating NAT1 genotype to acetylator phenotype. However, there is no reason to believe that In Vitro Technologies somehow mishandled the samples. It is more likely that In Vitro Technologies has a great deal of experience with these samples, and that they were prepared and handled consistently. The same reasoning applies to their handling in our lab. The very strong NAT2 genotype/phenotype correlations in these same samples provide evidence for this. It is also important to note that the lack of correlation between NAT1 genotype and phenotype are not novel, but have been observed and reported by others in this field as well (Payton and Sim, 1998; Smelt et al., 1998; Williams et al., 2001), as discussed in Chapter I.

Standard Curve for Allele-Specific Measurements

Creating a standard curve and applying the hepatocyte samples to that curve revealed information that might otherwise have gone unrealized. The standard curve clarified whether the general trend of higher NAT1*4 than NAT1*10 expression in the hepatocytes is real or an artifact of probe characteristics. Data from real-time PCR of NAT1*4/*4 and NAT1*10/*10 genomic DNA mixtures were analyzed the same way as the allele-specific expression analyses in the previous section. NAT1*4/NAT1*10 ratios were calculated (this time as 2^ΔC_T values, since no internal standard is applied) and plotted (Figure 8A). A standard curve was then created by fitting a third order polynomial to these points (R^2 = 0.98), giving an equation with which the hepatocyte allelic expression data could be fitted to the curve (Figure 8B). This curve takes into account the differences between the two probes used to recognize NAT1*4 and NAT1*10 sequences. The hepatocyte allele expression data were fitted to the curve by substituting the ΔΔC_T values (Figure 6) for the “y” variable in the third order polynomial equation and solving
Figure 8. Creation of a Standard Curve with NATI*4/*4 & NATI*10/*10 Genomic DNA Mixtures

Hepatocyte NATI*4/*4 and NATI*10/*10 genomic DNAs were mixed in various ratios to mimic possible expression ratios of NATI*4 and NATI*10 and analyzed by real-time PCR. (A) The ΔC_T values were plotted instead of the 2^{-ΔC_T} values, since the ΔC_T curve gave a polynomial equation that is more convenient to use, and graphically easier to read and understand. (B) The ΔC_T values were then fitted to a third-order polynomial curve (equation shown on graph) with very close fit (R^2 = 0.98). This curve will be the standard curve for analysis of hepatocyte allele expression data relative to genomic DNA standards, and the third order polynomial equation will be used to fit these data to the curve.
for the "x" variable using the equation solver function of a Texas Instruments TI-86 calculator, which equals the value corresponding to the correct NAT1*4:NAT1*10 mixture. These new data were then plotted on the standard curve (Figure 9) along with the genomic DNA samples from Figure 5.

The analysis revealed that the trend of greater NAT1*4 expression than NAT1*10 expression is real and not an artifact of the probe differences. When plotted on this standard curve using the third order polynomial equation, the genomic DNA samples, which should theoretically come to an allele ratio of 1.0, had a mean NAT1*4/NAT1*10 ratio of approximately 1.1, or 53% NAT1*4 and 48% NAT1*10. This suggests that the NAT1*4 and NAT1*10 probes act differently in this real-time PCR method. The hepatocyte allele expression data gave a mean NAT1*4/NAT1*10 ratio of approximately 1.8, or 64/38; this means that 64% of the NAT1 mRNA was NAT1*4 and only 38% was NAT1*10. Mean allele ratios of the hepatocyte samples and the heterozygous genomic DNA samples are significantly different according to a t-test (P = 0.0050).

The difference observed between the NAT1*4 and NAT1*10 alleles is not unusual in that variations in allelic expression are common in the human genome (Shuen Lo et al., 2003; Yan et al., 2002). On the other hand, an observation of lower NAT1*10 mRNA expression than reference NAT1*4 mRNA expression is unusual in light of reports that NAT1*10 is a rapid allele with reference to NAT1*4 (Badawi et al., 1995; Bell et al., 1995b). Since NAT1*10 is different from NAT1*4 only outside of the coding region, these NAT1 alleles code for the exact same enzyme. Thus, the difference must be transcriptional or translational, and not related to enzyme function. These data are apparently inconsistent with reports that bestow rapid allele status on NAT1*10 relative to NAT1*4, but consistent with enzyme activity data from the cryopreserved hepatocytes (Figure 3).

It is, however, unclear from our analysis whether the general trend of low NAT1*10 mRNA expression compared to NAT1*4 is a genuine case of allele-specific expression. It is possible that one of the 3'-UTR SNPs that defines NAT1*10 influences the detection of a certain
Figure 9. Standard Curve Analysis of Hepatocyte NAT1 Allelic Expression

Hepatocyte cDNA ΔΔC_T values, NAT1*4/*10 genomic DNA ΔC_T values, and NAT1*4/*4 and NAT1*10/*10 homozygote genomic DNA ΔC_T values (controls) were all fitted to the standard curve using the third-order polynomial equation that describes the curve (displayed above). When the value for “y” is inserted into the equation, solving for “x” gives a value corresponding to the NAT1*4: NAT1*10 mixture ratio. The mean allele ratio for NAT1*4/*10 genomic DNA standards (blue triangle) gives an NAT1*4:NAT1*10 allele ratio of 53:47. The mean allele ratio for hepatocyte cDNA samples (red triangle) gives a NAT1*4:NAT1*10 allele ratio of 64/36. Error bars on either side of these means indicate that the standard error of the means (SEM) for these two means do not overlap. A t-test reveals the means are significantly different (P = 0.0050).
percentage of \textit{NAT1}*10 transcripts by altering the poly-adenylation signal usage. If the first poly-adenylation signal following the human \textit{NAT1} stop codon were utilized during translation, \textit{NAT1} real-time PCR primers would be unable to amplify the necessary segment of that transcript, since the primers anneal to sequences on either side of the signal. The T$^{1088}$A SNP in \textit{NAT1}*10 DNA sequence changes the thymine to an adenine in the first AATAAA poly-adenylation signal following the stop codon. Such a change can have obvious deleterious effects on the use of this signal (Sheets et al., 1990). However, this SNP creates yet another stop codon, where the AAT on the 5’ end of the original signal become the three 3’ adenine nucleotides on a new AATAAA signal.

Instead of increased usage of this new poly-adenylation signal, which would decrease detection of \textit{NAT1}*10 transcripts and give reason to question the allele-specific \textit{NAT1}*10/\textit{NAT1}*4 expression data, it is expected that the usage of this new signal would decrease instead of increase, or at least remain the same. Although the SNP creates a new signal only 3 nucleotides 5’ of the original signal, the optimal distance between the poly-A signal and the site where cleavage and poly-adenylation occur has been altered. Data suggest that there is a spatial requirement for the distance between the poly-A signal and the actual site of cleavage/poly-adenylation. Unless this requirement is met, efficient mRNA production may be compromised (Nevins, 1983). On the other hand, analyses of mRNA structure using the “Fold” program (Zuker and Stiegler, 1981) and “Squiggles” program (Osterburg and Sommer, 1981) suggest that the \textit{NAT1}*10 polymorphisms do not alter the mRNA secondary structure (de Leon et al., 2000), and it is therefore believed that the altered poly-A site is well-tolerated.

Regardless of whether the T$^{1088}$A SNP in the \textit{NAT1}*10 allele creates a less functional poly-adenylation signal than the original \textit{NAT1}*4 allele or creates no change at all, there is no reason to believe that the T$^{1088}$A SNP creates a more functional poly-A signal, resulting in a fraction of \textit{NAT1}*10 transcripts that could not be detected by the real-time PCR method described above. The discrepancy still exists, however, between the reduced \textit{NAT1}*10 mRNA expression.
described in this chapter and the designation of \textit{NAT1*10} as a rapid acetylator \textit{NAT1} allele by others. Further analyses may determine that the paradox is explained by translational differences due to 5'-UTR SNPs associated with the \textit{NAT1*10} allele specifically. Such analyses are beyond the scope of this study and will not be pursued.
Conclusions

Quantitative real-time RT-PCR was used to detect allele-specific expression differences with moderate sensitivity. Sequence detection using gene-specific primers and allele-specific probes provides the necessary specificity. With some optimization, this method could be improved to allow for even greater sensitivity than was required for these experiments.

Analysis of \textit{NAT1*4} and \textit{NAT1*10} mRNA expression in 17 \textit{NAT1*4/10} cryopreserved human hepatocytes by quantitative real-time RT-PCR provides evidence for cis-acting genetic factors that can cause up to 3-fold difference in mRNA expression. While these cis-acting genetic factors may contribute to the observed discrepancy between \textit{NAT1} genotype and phenotype in primary hepatocytes, differences in allele expression alone are not responsible for the 350-fold difference in \textit{NAT1} enzymatic activity in the hepatocytes examined. Fitting the real-time PCR data to a standard curve reveals possible allele-specific expression of these \textit{NAT1} alleles; \textit{NAT1*10} mRNA expression is significantly lower than \textit{NAT1*4} mRNA expression in the \textit{NAT1*4/10} heterozygotes. This difference in expression appears to be real, and not an artifact of the method, though more should be done to understand the \textit{NAT1*10} allele.

These data point to the conclusion that unknown cis-acting genetic factors, such as \textit{NAT1} polymorphisms in the 5'-UTR, do not account for the general discrepancy between \textit{NAT1} genotype and phenotype in hepatocytes. They also, therefore, provide indirect evidence for the influence of environmental factors and/or trans-acting genetic factors on \textit{NAT1} phenotype.
Future Experiments

To test for a correlation between NAT1 enzyme activity and NAT1 protein level, a Western blot for should be performed on cytosols from the 17 hepatocyte samples analyzed in this study. This experiment will complete the hepatocyte NAT1 story and give an indication of the role of translational and/or post-translational regulation on NAT1 expression in hepatocytes. Due to the lack of remaining sample from the 17 individuals represented in this study, more hepatocytes from the same individuals will be ordered from In Vitro Technologies.
Further investigation into NAT1 and NAT2 tissue-specific expression will require a model that accommodates analysis of tissue mRNA and protein expression, and analysis of both genetic and environmental influences on their expression. Every experimental model has characteristic advantages and disadvantages that should be evaluated based on the purpose of the study and the quality of information that the model can provide for completing the aims of the study.

Since the importance of studying N-acetyltransferases lies primarily in its human application, directly studying human tissues and human NAT genes is preferred. However, the disadvantages of analyzing human tissues for this type of study far outweigh the benefit. Human tissues can be obtained from a variety of sources, including the National Cancer Institute’s Cooperative Human Tissue Network and the Armed Forces Institute of Pathology National Pathology Repository (Eiseman and Haga, 1999). However, the availability of each sample is limited and the quantity and quality is variable and unpredictable. In addition, there are no guarantees that the tissue section obtained is representative of the whole tissue, which is important when comparing tissue-specific expression of multiple individuals. The quality of the tissues is questionable since the method and time of tissue collection are often unknown and highly variable. Due to possible deleterious effects of mishandling on mRNA and protein preservation, tissue quality and the consistency of tissue handling and processing are very important when analyzing mRNA and protein expression. Perhaps more importantly, the natural variability associated with interindividual human environmental exposures, and the distinct genetic backgrounds among humans make human tissues an inappropriate system for studying the
tissue-specific expression of genes whose expression may be influenced by trans-genetic and environmental variables.

Animal models are the ideal system for studying tissue-specific expression in the absence of unpredictable environmental exposures and genetic variability, and they also provide the most direct and effective means of studying the influence of genetic variability and environmental exposures on gene expression. More specifically, the rat model is often used as a system for better understanding human gene expression; many gene expression studies in rat are used to extrapolate meaningful conclusions about what might also be true for expression of analogous human genes (Ariel et al., 2004; Sigfrid et al., 2004; Van Tuyl et al., 2003).

As is the case with human tissues, use of rat tissues as a model for NAT1 and NAT2 expression has advantages and disadvantages. Advantages include the near unlimited availability of any tissue of interest, and the freedom to analyze a specific and consistently chosen section of tissue (or even the whole tissue) to ensure that expression measurements are consistent and representative of the many cell types that make up a tissue. The quality of the tissues that can be obtained from an animal model is ideal, since the procedures used for tissue harvesting can be kept consistent from sample to sample, and the tissues can be preserved immediately following harvest. Other advantages include the controlled environment of animal storage facilities and the genetic consistency that can come from using inbred rat strains. These advantages must be weighed against a disadvantage, common to any animal model, which is the questionable relevance of rat Nats to human NATs.

The relevance of in vivo rat studies to human NAT1 and NAT2 could be addressed by comparing nucleotide and protein sequences. Both rat and human N-acetyltransferases have a single intronless 870 base pair coding region (Matas et al. 1997). Rat and human N-acetyltransferase genes and enzymes have high sequence identities, as much as 80% amino acid sequence identity and 84% nucleotide sequence identity. More importantly, the catalytic triad, Cys68-His107-Asp122 (Sinclair et al., 2000), is identical in rats and humans, thus implying the
same catalytic function. The three amino acids that play an important role in determining substrate selectivity in human NAT1 and NAT2 can be compared to rat protein sequences. In fact, human NAT1 and rat Nat2 have the same three amino acids at these sites, Phe125-Arg127-Tyr129, suggesting that they have similar substrate selectivities. Human NAT2 and rat Nat1, on the other hand, while they share only one identical amino acid, have similarities in their sequences with Ser125-Ser127-Ser129 for human NAT2 and Tyr125-Ser127-Tyr129 for rat Nat1. This would suggest that rat Nat1 and human NAT2 have similar substrate selectivities. Overall, gene and protein sequence comparisons suggest that rat and human N-acetyltransferases share the same biological functions in vivo.

Rats could also provide a good in vivo model for rapid and slow N-acetyltransferases alleles. While most rats are rapid acetylators (Nat2*20 allele), two rat strains have been identified as slow acetylators. Slow Nat2 alleles have been identified in rat strains WKY (Nat2*21A) and NSD (Nat2*21B). The Nat2*21A slow allele has the four transitions G361A, G399A, G796A, and G522A, while the NAT2*21B slow allele has the four transitions G361A, G399A, G796A, and C672T (Hein et al., 1991a,b; Juberg et al., 1991). The mechanism by which these SNPs confer slow acetylator status is unknown and should be studied further.

Although sequence similarities are compelling, a correlation between rat and human N-acetyltransferases must be experimentally determined. This might be accomplished by comparing the catalytic properties, substrate selectivity, and structural stability of the rat and human N-acetyltransferase enzymes, thereby validating the rat model as a legitimate model for studying N-acetyltransferases in vivo. Before this could be done, however, the suspected existence of a third functional rat N-acetyltransferase had to be identified (Chapter IV).
CHAPTER IV
IDENTIFICATION, CLONING, AND SEQUENCING OF A NOVEL RAT N-ACETYLTRANSFERASE (NAT3)

Introduction

N-acetyltransferases are expressed in a wide range of animal species. Certain characteristics are conserved across species, including the Cys-His-Asp cysteine protease-like catalytic triad and the 870 base pair intronless coding region. Other aspects, however, are highly variable, such as the exact coding region nucleotide sequence and even the number of NAT isoforms encoded for within the organism’s genome. The human genome contains two functional N-acetyltransferase genes, NAT1 and NAT2, and also an inactive pseudo-gene called NATP (Blum et al., 1990). NATP resembles a human N-acetyltransferase in its nucleotide sequence, and might have been an active NAT gene at one time. Other animals, however, have more or fewer N-acetyltransferase isoforms; mice have three (Kelly and Sim, 1994), cats have only one (Trepanier et al., 1998), and dogs have none (Trepanier et al., 1997).

The discovery of murine Nat3 hinted at the existence of a similar third Nat gene in rats. This suspicion was confirmed with the appearance of a GenBank entry for “rattus norvegicus similar to mouse Nat3” (XM_224762). This potential rat Nat3 sequence was predicted by automated computational analysis from an annotated genomic sequence (NW_047470) using a gene prediction method called GNOMON (National Center for Biotechnology Information). The sequence comes from rat strain BN/SsNHsd/MCW, which is a Brown Norway substrain from Harlan Sprague Dawley, Inc., and the Medical College of Wisconsin.

The validity of the rat as a model for N-acetyltransferase activity and expression could be affected by the existence of a third rat N-acetyltransferase gene. If rat Nat3 contributes in large
part to acetylator phenotype in rat tissues, the lack of an equivalent human gene could render the rat less valid as a model. On the other hand, the third N-acetyltransferase gene could have similar properties to the third mouse N-acetyltransferase gene. Mouse Nat3 enzyme has very low activity relative to mouse Nat1 and Nat2 (Estrada-Rodgers et al., 1998; Fretland et al., 1997). Therefore, the importance of rat Nat3, which is very similar to mouse Nat3 in nucleotide and amino acid sequence, is questionable. This chapter describes the cloning and sequencing of rat N-acetyltransferase 3.
Methods

Cloning of the Rat Nat3 Gene

The rat Nat3 coding region was amplified from Fisher 344, Sprague Dawley, and Wistar Kyoto genomic DNA by duplicate independent PCR reactions using Taq polymerase (Perkin Elmer Corp., Norwalk, CT, USA). Gene-specific primers were designed using GenBank sequence “rattus norvegicus similar to mouse Nat3” (XM_224762). The forward PCR primer has sequence 5’-gtagcaacctgatggacattgaagcgtactt-3’ and contains an XmaI restriction site (underlined) to facilitate cloning. The reverse PCR primer has sequence 5’-atgcgactgcagtaaatagtaaaagccaatt-3’ and contains a PstI restriction site (underlined) to facilitate cloning. PCR-amplified Nat3 was then ligated into pcDNA3.1 TA cloning vector and transformed into TOP10 competent E.coli using a TA Cloning Kit (Invitrogen). Successfully transformed E.coli colonies were selected for by ampicillin resistance, and then a single colony was grown in 200 ml of liquid broth (100 μg/ml ampicillin) overnight at 37°C. Plasmid DNA was isolated from the E.coli using the Qiagen Plasmid Midi Kit (Qiagen, Inc., Valencia, CA, USA).

Sequencing of the Rat Nat3 Gene

The Nat3 plasmid insert was completely sequenced using commercially available plasmid-specific primers 5’-taatacgactcactatatgg-3’ (T7 - forward), and 5’-tagaaggcacatgcagg-3’ (BGH - reverse), and newly designed gene-specific forward primers (5’-tgccactttacagaaatctccaa-3’, 5’-tgccatctctactgacagaa-3’, 5’-tcttctttactgggctctga-3’), and gene-specific reverse primers (5’-cagtaaagaagatgactctt-3’, 5’-tgcaattcaggtactctccacat-3’, 5’-gtccatttctgggctctga-3’), to ensure complete coverage of both top and bottom strands of the sequence. Sequencing reactions were performed using BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA), and the sequencing analysis performed by an ABI 310 Genetic Analyzer (Applied Biosystems). Sequencing data were analyzed using SeqManII v.5.03 from DNASTAR Inc. (Madison, WI, USA).
Results & Discussion

Rat Nat3 sequencing of both top and bottom strands was successfully accomplished for rat strains Fisher 344 (F344), Wistar Kyoto (WKY), and Sprague Dawley (SPRD) (Figure 10). No SNPs were found among all three strains sequenced. The sequence did, however, differ from the GenBank predicted sequence (XM_224762) by a single nucleotide. The predicted sequence from the BN strain contains a guanine at position 358, while the F344, WKY, and SPRD have an adenine at this position. The difference manifests itself in an amino acid coding change from valine (G at 358) to isoleucine (A at 358). Nucleotide sequence comparison of all known human, mouse, and rat N-acetyltransferase loci reveals that nucleotide position 358 is an adenine in all of them. Since the predicted Brown Norway Nat3 sequence resulted from a large-scale sequencing project instead of direct sequencing of the gene, it is unclear whether the difference between the predicted BN sequence and the F344, WKY, and SPRD sequences is indicative of a single nucleotide polymorphism or a sequencing error from the large-scale sequencing project.

As is true with all known N-acetyltransferases, the Cys-His-Asp catalytic triad is present in rat Nat3, suggesting that it has true N-acetyltransferase catalytic activity. The three nucleotides determined to play a role in substrate selectivity (Goodfellow et al., 2000) in Nat3 are unique among the human NATs and rat Nats, but identical to those found in the published mouse Nat3 sequence (Figure 11). This suggests that Nat3 will have a unique substrate selectivity among rat Nats, but similar, if not identical, to mouse Nat3. Rat Nat3 nucleotide sequence is 76% identical to rat Nat1 and 78% identical to rat Nat2; rat Nat3 amino acid sequence is 71% identical to rat Nat1 and 72% identical to rat Nat2 (Figure 12). The highest amino acid and nucleotide sequence identity to rat Nat3 is with mouse Nat3, at 88.3% and 91.1%, respectively.

Analysis of chromosomal gene location using NCBI Map Viewer (Wheeler et al., 2005) and BLAT from UCSC Genome Bioinformatics (Kent WJ, 2002) reveals that all three rat Nats are located within about 75 kb on rat chromosome 16. The order of the rat genes 5’ to 3’ is Nat1-Nat2-Nat3, with about 10 kb between Nat1 and Nat2, and about 60 kb between Nat2 and Nat3.
Figure 10. Rat Nat3 Nucleotide and Deduced Amino Acid Sequences

The nucleotide sequence for *rattus norvegicus* N-acetyltransferase 3 (Nat3) was determined by automated DNA sequencing for strains Fisher 344, Wistar Kyoto, and Sprague Dawley. Deduced amino acid sequence is shown also. The locations of the catalytic triad (single underline), the sites influential in substrate specificity (double underline), and the one nucleotide/protein sequence discrepancy with reference XM_224762 at nucleotide 358 (bold) are marked as indicated. This sequence was published in GenBank for strains Sprague Dawley (AY253757), Wistar Kyoto (AY253758), and Fischer 344 (AY253759).
Figure 11. Amino Acids 125, 127, 129 Influence N-acetyltransferase Substrate Selectivity

Human NAT1, rat Nat2 and mouse Nat2 share the same amino acids at sites 125, 127, and 129 (phenylalanine-arginine-tyrosine) suggesting that these enzymes have very similar substrate selectivities. Human NAT2, rat Nat1, and mouse Nat1 do not have identical amino acids at these sites; however, the amino acids are somewhat similar, suggesting that substrate selectivities may be similar. Rat and mouse Nat3 enzymes have identical amino acids at these sites (phenylalanine, phenylalanine, cysteine), suggesting that the substrate selectivities will be identical.
**Figure 12.** Human, Mouse, and Rat N-acetyltransferase Sequence Homologies

Both nucleotide (bottom left half) and amino acid (top right half) sequence comparisons for human, mouse, and rat N-acetyltransferases were determined using EMBOSS pairwise alignment algorithms (Rice et al., 2000). The highest sequence identities, for both nucleotide and amino acid sequence comparisons, are between rat and mouse Nats. The lowest nucleotide sequence identities, for both nucleotide and amino acid sequence comparisons, are between Nat3 (rat and mouse) and the human NATs.
This order is the same for the mouse Nats on mouse chromosome 8, and also for human NAT1 and NAT2 on human chromosome 8. Rat Nat3 sequences for strains SPRD, F344, and WKY were published in Genbank with accession numbers AY253757, AY253758, and AY253757, respectively.
CHAPTER V
CHARACTERIZATION OF RAT N-ACETYLTRANSFERASES NAT1*13, NAT2*20, NAT2*21A, NAT2*21B, AND NAT3 BY CLONING AND RECOMBINANT EXPRESSION IN ESCHERICHIA COLI

Introduction

The usefulness of the rat as a model for N-acetyltransferase studies depends on a good correlation between rat and human N-acetyltransferases. Although the comparison of rat and human N-acetyltransferase nucleotide and protein sequences suggests that such a correlation exists, it must be confirmed experimentally. Rat Nat1*13, Nat2*20, Nat2*21A, and Nat2*21B have been characterized already to some extent (Hein et al., 1997; Doll and Hein, 1995).

Previous studies determined that rat Nat1 and Nat2 have unique substrate selectivities that suggest rat Nat1 is similar to human NAT2, and rat Nat2 similar to human NAT1, thereby suggesting a correlation between the rat Nat and human NAT enzymes. This inverse correlation is further supported by intrinsic stability data, which revealed that the trend for rats is Nat1 > Nat2, and the stability of human NATs has the trend NAT2 > NAT1 (Gray et al., 1996). However, since rat Nat3 was recently discovered, the characterization of the rat Nats is incomplete.

The slow acetylator rat Nat2*21A and Nat2*21B alleles have four SNPs each. Two of the SNPs in each slow allele are silent (G$^{399}$A and G$^{522}$A in Nat2*21A; G$^{399}$A and C$^{672}$T in Nat2*21B), while the other two cause V$^{121}$I (G$^{361}$A) and V$^{266}$I (G$^{796}$A) amino acid changes in both slow alleles. Thus, while Nat2*21A and Nat2*21B differ in their nucleotide sequence at two positions (522 and 672), they code for identical proteins. Kinetic data suggest that Nat2*20 is rapid while Nat2*21A and Nat2*21B are slow, but also that there is a significant difference
between the expression of $Nat2^{*21A}$ and $Nat2^{*21B}$ (Doll and Hein, 1995). The validity of the last finding is in question, however, since a difference between $Nat2^{*21A}$ and $Nat2^{*21B}$ would require that the silent nucleotide differences between the two alleles somehow influence mRNA expression, mRNA stability, and/or translational efficiency due to alterations in mRNA structure. Further experiments are needed to delineate the mechanism of the rat $Nat2$ slow acetylator polymorphic alleles, and the difference between the two $Nat2$ slow alleles needs to be confirmed and characterized.

To test the correlation between human NATs and rat Nats, understand the extent of their similarities, and thereby validate the rat as an in vivo model for this research and for future in vivo rat $Nat$ research, the rat N-acetyltransferases were further characterized, as described in this chapter. For the first time all three rat Nat loci, including all three $Nat2$ enzymes, have been characterized in the same expression system, and at the same time. Rat N-acetyltransferase isozymes were characterized for catalytic activity, substrate selectivity, thermostability, and immunoreactive detection by various polyclonal antisera. In addition, experiments were performed to delineate the $Nat2$ slow acetylator mechanism and the relationship between the two slow $Nat2$ enzymes. Substrates tested include p-aminobenzoic acid (PABA), 4,4-methylenebis(2-chloroaniline) (MOCA), N-(p-aminobenzoyl) glutamate (pABG), 4,4-methylenedianiline (MDA), 4-aminobiphenyl (ABP), 2-aminofluorene (AF), and sulfamethazine (SMZ). PABA is a known human NAT1- and murine Nat2-selective substrate, while SMZ is a known human NAT2- and murine Nat1-selective substrate (Estrada-Rodgers et al., 1998; Grant et al., 1991). The first and only suspected endogenous N-acetyltransferase substrate, pABG, is a folic acid catabolite and a human NAT1- and murine Nat2-specific substrate (Estrada-Rodgers et al., 1998; Minchin, 1995). 4-Aminobiphenyl is a bladder carcinogen found in cigarette smoke (Schulte et al., 1988; Vineis, 1994); this carcinogen is acetylated by both human NAT1 and NAT2 and mouse Nat1 and Nat2, but with higher affinity for human NAT2 (Hein et al., 1993) and for mouse Nat2 (Fretland et al., 1997b). 2-Aminofluorene is a carcinogen that has higher affinity for mouse Nat2 than Nat1.
MDA and MOCA are suspected human carcinogens (National Toxicology Program, 2002a/b) whose metabolism may be influenced by N-acetylation.

Characterization of the rat Nats will lay a basic foundation for understanding rat N-acetyltransferases, and facilitate evaluation of the probable importance of Nat3, since nothing is known about its activity and it has no human analog. Since mouse Nat3 has very low activity relative to mouse Nat1 and Nat2 (Estrada-Rodgers et al., 1998; Fretland et al., 1997), the importance of rat Nat3, which is very similar to mouse Nat3 in nucleotide and amino acid sequence, is questionable. The information gained from these experiments is preliminary data to investigations using the rat as an in vivo model for N-acetyltransferase studies.
Methods

Cloning Rat *Nat2* and *Nat3*

Rat *Nat2* alleles were cloned from PCR products into a bacterial expression vector, whereas rat *Nat3* was sub-cloned from the Nat3-pcDNA3.1 vector described in chapter IV into a bacterial expression vector. Rat *Nat2*20, *Nat2*21A, and *Nat2*21B were amplified from the genomic DNA of rat strains F344, WKY, and NSD, respectively, using high fidelity Phusion polymerase (Finnzymes, Finland) and *Nat2*-specific PCR primers. The *Nat2* forward primer has the sequence 5' -tctgaattcatggacattgaagcatactttgaaagaattgttat-3', with an *EcoRI* restriction site (underlined) designed into the 5'-end to facilitate cloning (Doll and Hein, 1995). The *Nat2* reverse primer was newly designed with the sequence 5' -atgcgactgcagctaaatggtaaaaaatcgacaccatgttt-3', with a *PstI* restriction site (underlined) designed into the 5'-end to facilitate cloning. PCR products were purified using the QIAquick PCR Purification Kit (Qiagen). Purified *Nat2* PCR products and the PKK223-3 bacterial expression vector (Pharmacia-LKB Biotechnology, Piscataway, NJ) (GenBank M77749) were digested with *EcoRI* and *PstI* restriction enzymes (New England Biolabs, Inc., Beverly, MA). The *Nat3*-pcDNA3.1 clone described in Chapter IV, and the PKK223-3 bacterial expression vector were digested with *XmaI* and *PstI* restriction enzymes (New England Biolabs). Restriction digest products were separated by electrophoresis on a 0.6% agarose gel, then analyzed under long wavelength UV light (365 nm). For the digestion of PKK223-3, the band corresponding to the open vector was excised from the gel. For the digestion of the *Nat2* PCR products and the *Nat3*-pcDNA3.1 vector, the appropriately sized band (~1000 kb) was excised from the gel. The restriction digested *Nat2* and *Nat3* products were then purified from the agarose gel using the Qiaex II Gel Extraction Kit (Qiagen). Ligation was then carried out with the cut plasmid, approximately 3 molar equivalents of the insert, and T4 DNA-ligase at 37°C for 20 minutes. All clones were created in duplicate from duplicate initial PCR reactions, and water controls were used to ensure that there were no false positive Nat bands due to DNA contamination.
Transformation of Competent *Escherichia coli*

NovaBlue competent cells (Novagen, Inc., Madison, WI) were transformed with ligation reaction products, then plated on LB-agar plates (containing 100 μg/ml ampicillin for selection of *E.coli* containing the circular PKK223-3 vector, which contains the ampicillin resistance gene encoding β-lactamase) and allowed to incubate overnight at 37°C. NovaBlue competent cells were used as an initial cloning host due to their high transformation efficiency. Colonies were tested for PKK223-3 plasmids containing the Nat insert by colony PCR using PKK223-3-specific forward (5’-ttatcagaccgttctgct-3’) and reverse (5’-gctgttgacaattaatcatcgg-3’) primers designed by Dr. David Barker. Colony PCR was performed by heat denaturing transformed *E.coli* (corresponding samples were preserved by streaking on LB-agar plates and incubating overnight at 37°C), followed by PCR using Taq polymerase (Perkin Elmer Corp). PCR products were then subject to electrophoresis on a 1% agarose gel. The presence of Nat-sized bands (~1000 kb) in the gel indicated the presence of the Nat insert. Water controls were used to ensure that there were no false positive Nat bands due to DNA contamination of the PCR reagents used for the PCR. One colony from each of the preserved successful bacterial colony streaks were used to inoculate 200 ml of LB (100 μg/ml ampicillin), then incubated overnight at 37°C with shaking (225 rpm). Cells were centrifuged at 5000 x g, and plasmid DNA isolated using the Qiagen Plasmid Midi Kit (Qiagen), and plasmid DNA quantified using a Beckman DU-650 spectrophotometer.

To verify that the correct sequence was cloned into the plasmid, both strands of the Nat insert were sequenced using gene-specific sequencing primers. For the Nat3 clone, the primers described in Chapter IV were used, in addition to the PKK223-3-specific reverse primer described above. For Nat2 clones, four forward primers (5’-tctgaattcatggacattgaacactttgaaga-attggttat-3’, 5’-tagaagtcatctttgatcaa-3’, 5’-aagagaatggaacctggtac-3’, cagcategtctggattacaagt-3’) and four reverse primers (5’-atgcgactgcagctaaatggtaaaaaatcgatcaccatgttt-3’, 5’-tacctgagttttaggagtta-3’, 5’-agaagacagtttcttcaaatcagctc-3’, 5’-acttagtacggtgaacggt-3’) were used, in addition to the PKK223-
3-specific reverse primer described above. Most of the Nat2 sequencing primers were designed by Mark Doll. Nat2 primers do not overlap with slow Nat2 allele polymorphic sites.

Sequence-verified Nat-PKK223-3 plasmids were then transformed into competent JM105 E.coli. JM105 E.coli glycerol stocks were plated on LB-agar (no drug). A single JM105 colony was then used to inoculate 25 ml of LB (no drug), and grown to log phase by incubation at 37°C with shaking (225 rpm). Following centrifugation at 5,000 x g for 10 minutes, pellets were re-suspended in 1/10 volume cold TSB medium (10% PEG, 5% DMSO, 10 mM MgCl₂, 10 mM MgSO₄, 1 X LB medium), and incubated on ice for 10 minutes. Cells were stored in 100 μl aliquots at -80°C until used for transformations. Competent JM105 E.coli were transformed with Nat-PKK223-3 plasmids and incubated overnight at 37°C on LB-agar (100 μg/ml) plates. The presence of the Nat insert was again verified by colony PCR. A negative control for enzyme assays was also created by transforming JM105 competent E.coli with an empty PKK223-3 plasmid. Nat1*13-PKK223-3 clones were obtained from Mark Doll and transformed into competent JM105 E.coli as described above.

Recombinant Expression in E.coli

From frozen stock, samples were plated on LB-agar plates (100 μg/ml ampicillin), and incubated overnight at 37°C. Colony purification was performed by re-plating a single colony from the overnight incubation on an LB-agar plate (100 μg/ml ampicillin), and incubated overnight at 37°C. A single colony was chosen from the purification and used to inoculate 25 ml liquid broth (100 μg/ml ampicillin), which was then incubated overnight at 37°C with shaking (225 rpm). Subsequently, 200 ml of LB (100 μg/ml ampicillin) was inoculated to an OD₆₀₀ of 0.2. This inoculate was then incubated with shaking (225 rpm) for approximately 3 hours at 37°C to an OD₆₀₀ of 0.5 to 0.7. Immediately, the PKK223-3 tac promoter was induced by adding isopropyl-beta-D-thiogalactopyranoside (IPTG) to 100 mM, then incubated for an additional 3 hours. Following incubation, cells were immediately pelleted by centrifugation at 6,000 x g for 10 minutes, then either processed immediately or stored overnight at -20°C. Bacterial cytosols
were prepared by sonication in 1/20 volume (i.e. 10 ml for 200 ml culture) homogenization buffer (20 mM Na3PO4, 1 mM EDTA, pH7.4, 1 mM DTT, protease inhibitor cocktail), followed by centrifugation at 15,000 x g. Cytosols were aliquoted into 1.5 ml tubes and stored at -80°C. Total protein concentration was determined by the Bradford method (Bio-Rad Laboratories, Inc., Hercules, CA) (Bradford, 1976). Cytosols were aliquoted and stored at -80°C.

Enzymatic Activity Assays

N-acetylation activity was measured using bacterial cytosolic preparations containing recombinantly expressed Nat1 13, Nat2 20, Nat2 21A, Nat2 21B, Nat3, or the PKK223-3 negative control (no insert). Cytosols were incubated with 1 mM acetyl-CoA and 100 μM or 300 μM substrate for 10 minutes at 37°C. Enzymatic reactions were stopped upon addition of acetic acid to 0.1 M to precipitate the protein. Precipitated protein was removed by centrifugation at 13,000 rpm for 10 minutes, and the remaining supernatant analyzed by high performance liquid chromatography (HPLC) using System Gold Software (Beckman-Coulter, Fullerton, CA, USA) for quantification of N-acetylated product. Tested compounds include the following known Nat substrates: p-aminobenzoic acid (PABA), 4,4-methylenebis(2-chloroaniline) (MOCA), N-(p-aminobenzoyl) glutamate (pABG), 4,4-methylenedianiline (MDA), 4-aminobiphenyl (ABP), 2-aminofluorene (AF), and sulfamethazine (SMZ). HPLC methods for each substrate were developed by Mark Doll.

Enzyme activities were normalized to the time of the reaction (10 minutes) and the total protein concentration as determined by the Bradford method (Bio-Rad) resulting in units nmoles/min/mg. Initial enzyme activity measurements were performed using the above reaction conditions for serial dilutions (1:1, 1:5, 1:25, 1:125, 1:625) of each recombinant lysate. For each lysate-substrate reaction combination, the dilution that resulted in less than 15% conversion of substrate to product was chosen to ensure that the enzyme’s activity is not affected by limiting substrate availability. Thus, activities were measured at initial rate conditions and the data are
reported as initial velocities.

N-acetylation activity was measured for each substrate in triplicate, in addition to a no-acetyl-CoA control to ensure that the reactions are acetyl-CoA dependent. The negative control activity was subtracted from the sample activities. Initial velocities are reported in nmoles acetylated substrate per minute reaction time per mg total protein (nmoles/min/mg) with standard error of the means (SEM) calculations for each.

**Kinetic Parameters**

To calculate $K_m$ (μM) and $V_{max}$ (nmoles/min/mg) kinetic properties, initial velocities were measured, as described above, at a minimum of six different substrate concentrations. The data were fitted to a non-linear regression curve using GraphPad Prism V2.01 software (GraphPad Software, Inc., San Diego, CA). $V_{max}$ is the maximal rate of product formation and $K_m$ is the substrate concentration at $\frac{1}{2} V_{max}$ (Michaelis-Menton Constant). A much more informative parameter is the specificity constant, which is the rate of substrate capture, calculated as $V_{max}/K_m$ (Fersht, 1998). In addition to $K_m$ and $V_{max}$, the specificity constant was calculated.

**Thermostability Assays**

Recombinant Nat1, Nat2, and Nat3 lysates were diluted to 1 μg/ml in homogenization buffer, then incubated at 50°C for 5, 10, 20, 40, 60, and 80 minutes. Incubated lysates were transferred to ice immediately following the allotted time and assayed for N-acetylation activity as described above. Activities in nmoles/min/mg were normalized to the activity prior to any inactivation by calculating the percent original activity remaining, with the highest activity at 100%. Data were fitted to a first order decay curve using GraphPad Prism software, with which the half-life ($T_{1/2}$) and heat deactivation constant (k) were calculated.

**Characterization of N-acetyltransferase Polyclonal Antisera for Rat Nat Detection**

The specificity of various N-acetyltransferase polyclonal antisera for rat Nat1 versus Nat2 was characterized by SDS-PAGE and Western blotting using rat Nat recombinant lysates. These antisera were also used to study the mechanism of the rat Nat2 slow alleles. To develop rat Nat3
polyclonal antiserum, a Nat3-specific peptide was designed based on the same strategy used for the rat Nat1 and Nat2 polyclonal antisera production (Figure 13), then developed commercially through Invitrogen (Carlsbad, CA, USA).

Recombinant lysates were diluted with homogenization buffer to 2 µg/ml, and then diluted to 1 µg/ml by adding an equal volume of 2 X Laemmli Sample Buffer (Bio-Rad). Samples were then denatured by addition of β-mercaptoethanol (to 5%) and immersed in boiling water for 5 minutes. To load 20 µg of total protein, 20 µl of 1 µg/ml sample was loaded onto a preformed 12% polyacrylamide gel (Cambrex, East Rutherford, NJ, USA). Samples were electrophoresed at 125 V for 2.5 hours in pre-mixed 1 X Tris/Glycine/SDS electrophoresis buffer (Bio-Rad Laboratories, Inc.). Protein bands were then electrically transferred from the polyacrylamide gel to Hybond-ECL nitrocellulose membrane (Amersham Biosciences, Piscataway, NJ, USA) at 100 V for 1 hour using pre-mixed 1 X Tris/Glycine transfer buffer (Bio-Rad). The membrane was blocked overnight at 4°C using Blocker BLOTTO in TBS (Pierce Biotechnology, Inc. Rockford, IL). The membrane was then washed 3 times for 10 minutes each time with wash buffer (25 mM Tris, 0.15 M NaCl, pH 7.2) (Pierce). The membrane was blotted with blocking buffer, 0.05% Tween 20 and primary antisera (diluted 1:2000) for 2 hours at room temperature. Following another wash cycle (3 x 10 minutes), the membrane was blotted with blocking buffer, 0.05% Tween 20, and goat anti-rabbit horseradish peroxidase-conjugated secondary antibody (Amersham) diluted 1:50,000 for 1 hour at room temperature. Following another wash cycle (3 x 10 minutes), the membrane was incubated in working chemiluminescent solution for 5 minutes using an ECL detection kit (Amersham Biosciences) exposed to Kodak Bio-Max film.

Six different N-acetyltransferase antisera, in addition to the newly developed Nat3 antibody were tested using the above method. The negative control cytosols (lysates of E.coli transformed with empty PKK223-3) were used as negative controls for the Western blot, and
Peptide Design for Rat Nat Antibodies

A

Nat1  N-VLKTTFGIsLEKFKVPKHELVEFTI-C
Nat2  N-VLKTIFGSLEKLFVPKHELVEFTI-C

B

Nat1  N-VLKTTFGIsLEKFKVPKHELVEFTI-C
Nat3  N-VLKRVFGERLETKVPKCGNWLEFTI-C

C

Nat2  N-VLKTIFGSLERKLVPKHDREFTI-C
Nat3  N-VLKRVFGERLETKVPKCGNWLEFTI-C

Figure 13. Peptide Design Strategy for Nat3-specific Polyclonal Antibody Development

Rat Nat1 and Rat Nat2 polyclonal antisera were produced previously using a peptide identical to the C-terminal rat Nat1 and Nat2 amino acid sequence. Since these antibodies successfully recognize their targets, this strategy was mimicked in designing peptide for producing rat Nat3-specific polyclonal antiserum. The peptide used for production of a Nat3 antibody originated from the C-terminal end of the Nat3 amino acid sequence. (A) The Nat1 and Nat2 peptides (underlined) differ by only six of the 17 amino acids shared between the two. (B) The rat Nat1 and Nat3 peptides differ by seven of the 16 amino acids shared between the two, and also differ by additional three N-terminal amino acids in the Nat3 peptide and one additional amino acid on the N-terminal end of the Nat1 peptide. (C) The rat Nat2 and Nat3 peptides differ by eight of the 16 amino acids shared between the two, and also differ by an additional three N-terminal amino acids in the Nat3 peptide. Since the differences between Nat3 and Nat1, and between Nat3 and Nat2 are greater than the differences between Nat1 and Nat2, the resulting Nat3 polyclonal antiserum should be as selective as the Nat1 and Nat2 antisera since it provides a seemingly sufficient number of Nat3-specific epitopes.
yeast cytosols containing recombinantly expressed NAT1 and NAT2 were used as the positive controls. Quantitation of Nat bands was achieved by normalization to non-specific bands. Protein bands were analyzed by densitometry.

**Bacterial RNA Isolation**

Total bacterial RNA was isolated from JM105 *E.coli* using the RNeasy Kit from Qiagen. To test for quality, RNA was separated by electrophoresis on a 1% agarose gel and analyzed for distinct 23S and 16S bacterial ribosomal RNA bands. DNA was removed from the RNA samples using the Turbo DNase kit from Ambion (Austin, TX), then re-analyzed by electrophoresis to confirm disappearance of DNA contamination, as is evident by the high molecular weight DNA bands visible at the top of the agarose gel. During this electrophoresis step, RNA was also quantified by including RNA standards in known amounts (200 ng, 400 ng, 800 ng). The bands were quantitated by densitometry. The concentration of the samples was then calculated using a standard curve. RNA samples were stored at -80°C.

**Real-Time RT-PCR**

Bacterial RNA was reverse transcribed to cDNA using SuperScript III First-Strand Synthesis System for RT-PCR from Invitrogen (Carlsbad, CA). Control reverse transcription reactions were performed in the absence of reverse transcriptase as controls for DNA contamination. The reaction mixture contained newly designed rat Nat-specific TaqMan primers (forward, 5'-agatgtggagctctggaatt-3'; reverse, 5'-gcaggcacctgag-3') from Applied Biosystems (Foster City, CA), a VIC-labelled rat Nat2-specific fluorogenic MGB probe (5'-cttccctgatgtaattc-3') from Applied Biosystems, bacterial cDNA and TaqMan Universal PCR Master Mix from Applied Biosystems, which contains AmpliTaq Gold polymerase. Real-time PCR was performed using an ABI Prism 7700 sequence detection system from Applied Biosystems. The reaction conditions used for real-time PCR were previously described (Doll and Hein, 2002). Total RNA concentration was used to normalize the real-time PCR data.
Results & Discussion

Cloning, Transformation and Recombinant Expression

The coding exon of rat N-acetyltransferases *Nat1*13, *Nat2*20, *Nat2*21A, *Nat2*21B, and *Nat3* were successfully cloned into bacterial expression vector PKK223-3 (Pharmacia), as verified by sequencing with primers that span the entire top and bottom strands of the Nat sequence as well as the 5’- and 3’- sequence-vector interfaces. Successful transformation of JM105 *E.coli* with each clone was verified by the ampicillin resistance that PKK223-3 conferred to the successfully transformed bacteria, and subsequently by colony PCR with gene- and plasmid-specific primers and gel electrophoresis. Recombinant expression was successful as verified by measurement of N-acetyltransferase activity in lysates and by immunoreactive detection using Nat-specific polyclonal antibodies. Bacterial expression vector PKK223-3 without insert was used as a negative control beneficial for cloning, transformation, and recombinant expression verification.

Enzymatic Activity Assays

N-acetylation activity was measured using recombinantly expressed *Nat1*13, *Nat2*20, *Nat2*21A, *Nat2*21B, and *Nat3*. Initial velocities were measured as described above for substrates p-aminobenzoic acid (PABA), 4,4-methylenebis(2-chloroaniline) (MOCA), N-(p-aminobenzoyl) glutamate (pABG), 4,4-methylenedianiline (MDA), 4-aminobiphenyl (ABP), 2-aminofluorene (AF), sulfamethazine (SMZ) (Figures 14-17).

Initial velocity measurements confirmed that rat *Nat3* is a functional N-acetyltransferase enzyme with distinct substrate selectivity, but with relatively low activity. *Nat3* catalyzed the N-acetylation of ABP, AF, MDA, and MOCA at low levels relative to *Nat1* and *Nat2*, with its highest activity toward MDA and lowest activity toward MOCA. *Nat3*-catalyzed N-acetylation products were not detected with substrates pABG, PABA, and SMZ. No *Nat3* selective substrate could be identified from the substrates tested.

*Nat1* catalyzed the N-acetylation of every substrate tested, with the exception of pABG.
Enzymatic activities were measured in triplicate. Error bars represent the standard error of the means. (A) PABA is selectively acetylated by rat Nat2 20 (very low activity for rat Nat1 not visible on graph) over Nat1, and Nat3 acetylation of PABA was not detected. (B) SMZ slightly prefers acetylation via Nat1, although Nat2 activity is substantial. Nat3 acetylation of SMZ was not detected. A significant difference between the activities of two Nat2 slow enzymes was observed for both PABA ($p < 0.0001$) and SMZ ($p < 0.0001$).
Enzymatic activities were measured in triplicate. Error bars represent the standard error of the means. (A) MDA is selectively acetylated by Nat2 20. Nat3 acetylation was detected, resembling the activity of a Nat2 slow acetylator enzymes. (B) MOCA is selectively acetylated by Nat1. Nat3 activity was detected for MOCA at levels similar to Nat2 slow acetylators. A significant difference between the activities of the two Nat2 slow enzymes was observed for both MDA (p < 0.0001) and MOCA (p < 0.0001).

Figure 15. Rat Nat N-acetylation of MDA and MOCA
Figure 16. Rat Nat N-acetylation of ABP and AF

Enzymatic activities were measured in triplicate. Error bars represent the standard error of the means. (A) ABP is a selective substrate for Nat2 20, but with substantial activity also via Nat1. Nat3 acetylation of ABP was detected at a rate similar to the Nat2 slow enzymes. (B) AF is selectively acetylated by Nat2. Nat3 acetylation of AF was detected at a rate similar to the Nat2 slow alleles. A significant difference between activities of the two Nat2 slow enzymes was observed for both ABP (p < 0.0001) and AF (p = 0.0007).
Figure 17. Rat Nat N-acetylation of pABG

Enzymatic activities were measured in triplicate. Error bars represent the standard error of the means. The potentially endogenous Nat substrate pABG was selectively acetylated by Nat2 enzymes with no activity detected for Nat1 or Nat3. The ratio of Nat2 rapid to Nat2 slow activities is much smaller here than for the other substrates tested, for unknown reasons. A significant difference between the Nat2 slow acetylator enzymes was observed (p = 0.0001).
Natl demonstrated the highest catalytic activity toward MDA, though MOCA was most selectively N-acetylated by Nat1. According to the comparison of amino acids residues influential on substrate selectivity (Figure 11), it might be expected that rat Nat1 would mimic the substrate preference of human NAT2. The human NAT2 selective substrate SMZ (Grant et al., 1991) was acetylated at a higher rate by rat Nat1 than Nat2, though not to the degree required for classification as a rat Nat1 selective substrate. MOCA appears to be a superior rat Nat1 selective substrate, both for its selectivity and relatively high catalytic activity, and should be used for \textit{in vivo} studies of rat Nat1 enzymatic activity instead of SMZ. The apparent difference in SMZ substrate preference between human NAT2 and rat Nat1 may reflect the influence of other differences between the enzymes that also play a role in substrate selectivity.

For all substrates tested, rat Nat2 20 was a rapid acetylator relative to Nat2 21A and Nat2 21B, which were slow acetylators. This relationship is consistent with previous findings regarding these rat Nat2 enzymes (Doll et al., 1995; Doll and Hein, 1995). All three rat Nat2 enzymes acetylated every substrate tested, though all three acetylated PABA at the highest rate; in fact, PABA was highly selective for rat Nat2 over Nat1 and Nat3. This relationship is consistent with the comparison in Figure 11, since PABA is selective for human NAT1 over NAT2, confirming the suspected correlation between the substrate selectivities of rat Nat2 and human NAT1. Of those substrates tested, PABA is the clearly the substrate of choice for studies of rat Nat2 activity \textit{in vivo}.

An apparent paradox is observed in the difference between the activities of slow acetylators Nat2 21A and Nat2 21B. This difference has been observed with multiple different clones by different people and at different times (Hein et al., 1997). The observed differences are significant (GraphPad Prism unpaired t-test) for all substrates tested; ABP (P < 0.0001), MDA (P < 0.0001), MOCA (P < 0.0001), PABA (P < 0.0001), pABG (P = 0.0001), SMZ (P < 0.0001), AF (P = 0.0007). Since these enzymes are identical in their primary amino acid sequence, the different activities suggest differences at the level of transcription and/or translation. Since the
differences between the two alleles at the nucleotide level are silent (G\textsuperscript{522}A in \textit{Nat2*21A}; C\textsuperscript{672}T in \textit{Nat2*21B}), one or both of these silent nucleotide polymorphisms may contribute to the activity difference between the alleles. This activity difference could come by way of alternative codon usage and/or alterations in mRNA expression and/or structure. This issue will be addressed further in experiments to follow.

**Kinetic Parameters**

The Michaelis-Menten constant (K\textsubscript{m}), the maximum velocity (V\textsubscript{max}), and the specificity constant (V/K) were calculated by fitting the data to a non-linear regression curve using GraphPad Prism software for the acetylation of AF by Nat1 13, Nat2 20, Nat2 21A, Nat2 21B, and Nat3 (Figure 18), and of ABP for Nat1 13, Nat2 20, and Nat3 (Figure 19). The data demonstrate substrate-dependent Nat catalytic activity.

N-acetylation of AF is accomplished most efficiently at all substrate concentrations by Nat2 20, which has both the largest V\textsubscript{max} and the largest specificity constant. The other rat Nats have comparable V\textsubscript{max} values to one another, with Nat3 having the lowest of them all. The two slow Nat2 enzymes have the lowest specificity constant due to their relatively large K\textsubscript{m} values, although they have higher V\textsubscript{max} values than Nat3; Nat3 apparently acetylates AF better than the Nat2 slow alleles at lower concentrations, but the Nat2 slow enzymes are more efficient acetylators than Nat3 at relatively higher concentrations.

Nat2 slow acetylator polymorphisms appear to both reduced V\textsubscript{max} and increased K\textsubscript{m} of the enzymes, resulting in a smaller selectivity constant. The reduced V\textsubscript{max} may indicate reduced protein, though that should be confirmed with a Western blot analysis. Here, the apparent difference in activity between the Nat2 slow enzymes (Figures 14-17) manifests itself as significant differences in V\textsubscript{max}, but not K\textsubscript{m}. Reduced V\textsubscript{max} suggests reduced protein for Nat2 21A relative to Nat2 21B. Reduced Nat2 21A protein should be confirmed by Western blot.

ABP is N-acetylated most efficiently by Nat1 at relatively low concentrations and by Nat2 at relatively high concentrations. Although Nat1 has smaller V\textsubscript{max} than Nat2, it has a higher
Figure 18. Kinetics of Rat Nat N-acetylation of 2-Aminofluorene (AF)

Rat N-acetyltransferases acetylate 2-aminofluorene with varying kinetics. Initial velocities were measured in triplicate (error bars and “±” values following data indicate SEM) for various dilutions of substrate. The non-linear regression fit and the calculated kinetic constants for each enzyme are shown above. The original location of the inset magnified portion is surrounded with a dotted line. Nat2 20 acetylates AF most efficiently, followed by Nat1 and Nat3. Nat2 slow alleles have the lowest specificity constants due to their high $K_m$ values.
Figure 19. Kinetics of Rat Nat N-acetylation of 4-Aminobiphenyl (ABP)

Initial velocities were measured in triplicate (error bars and “±” values following data indicate SEM) for various substrate dilutions. The non-linear regression fit and the calculated kinetic constants for the data are shown above. The original location of the inset magnified portion is surrounded with a dotted line. While Nat2 20 has the largest $V_{\text{max}}$, Nat1 has the largest specificity constant for AF N-acetylation; at relatively low substrate concentrations, Nat1 is a more efficient acetylator of ABP than Nat2, whereas the opposite is true at higher ABP concentrations. Nat3 has relatively low $V_{\text{max}}$ and selectivity constant.
specificity constant. Nat2 has the largest $V_{\text{max}}$, but also has a very large $K_m$, resulting in a smaller specificity constant than Nat1. Nat3 has the smallest specificity constant, but acetylates ABP at Nat1-saturating substrate concentrations.

These kinetic characterizations of recombinant rat enzymes in an artificial bacterial system are useful for general comparisons of the enzymes’ activity and stability, but are limited in application since they do not necessarily reflect the characteristics of the enzymes in vivo.

**Thermostability**

Structural stability of the rat Nat enzymes was tested as thermostability (Figure 20). Nat1 is the most stable of all rat Nat isozymes, showing no decrease in its activity after 80 minutes incubation at 50°C. Rat Nat3 is less stable than Nat1, having lost most of its activity by 80 minutes of incubation, but considerably more stable than any of the Nat2 alleles. Nat2 20 is very unstable, having lost most of its activity within 5 minutes of incubation.

The Nat2 slow alleles have reduced thermostability relative to Nat2 20. In fact, the rates of inactivation are so high that their activity was completely eliminated before the first time point (5 minutes) was taken. Significance between the inactivation rates of the two slow alleles could not be tested due to the rapid drop off of the enzyme activity following incubation. This was not pursued further since the two slow alleles produce identical enzymes.

These data are consistent with inactivation data reported previously for all rat Nats except Nat3, which was not known at the time (Gray et al., 1996). Here, it was determined that stability from highest to lowest is Nat1 $\gg$ Nat3 $>$ Nat2 20 $>$ Nat2 21A/21B. The general relationship between Nat1 and Nat2 thermostability (Nat1 $\gg$ Nat2) shows an inverse correlation with human NAT1 and NAT2 enzymes, where human NAT2 is much more stable than human NAT1. Reduced enzymatic stability is apparently part of the mechanism for slow Nat2 acetylator phenotype.

**Characterization of N-acetyltransferase Antisera for Rat Nat Detection**

A number of polyclonal antibodies have been developed for detection of
Heat Inactivation of Rat N-acetyltransferases

Recombinant rat N-acetyltransferases were heat inactivated by incubation in at 50°C for 0, 5, 10, 20, 40, 60, 80 minutes, then assayed for N-acetylation of 2-aminofluorene. Resulting N-acetylation activities were normalized to the enzyme’s original activity prior to incubation (time = 0). In the plot shown above is a non-linear fit for one phase exponential decay (GraphPad Prism), with 100% on the Y-axis represents the activity prior to incubation. Rat Nat1 is highly stable, with no detectable decrease in activity during the entire incubation. Rat Nat3 is of intermediate stability. The rat Nat2 enzymes, however, are very unstable, with Nat2 20 losing most of its activity within the first five minutes of incubation, and Nat2 21A and Nat2 21B losing their activity almost immediately upon incubation.
N-acetyltransferase enzymes by Western blot. These include human, murine, hamster, as well as rat polyclonal antisera. The Nat3-specific polyclonal antiserum recently obtained from Invitrogen was included in these analyses. Once characterized, successful antibodies will be utilized for detection of Nats in tissues, where the concentration of Nat enzymes may be relatively low. Since rat tissues contain complex mixtures of multiple rat Nat isozymes, it is important that the antisera are isozyme-specific. Recombinant lysates containing rat Nat1 13, Nat2 20, Nat2 21A, Nat2 21B, and Nat3 offer the benefit of high Nat expression. Individually expressed recombinant rat Nats in a high-expression system is ideal for characterization of these antibodies. For the first time, all seven polyclonal antisera were characterized for rat Nat1, Nat2, and Nat3 specificity by Western blot (Figure 21).

The polyclonal antibodies raised against rat Nat1 were sufficiently specific for rat Nat1 versus Nat2, and likewise the polyclonal antibodies raised to rat Nat2 were sufficiently specific for rat Nat2 versus Nat1. The newly designed and produced rat Nat3 antiserum did not detect any of the rat Nats under the conditions tested. Of the other antibodies tested, one did not detect any of the rat Nats (human NAT2), and the rest of them detected both enzymes (hamster Nat2, mouse Nat1, mouse Nat2) to varying degrees. Since these antibodies were tested with bacterial cytosols, their specificity with regard to non-specific bands in rat tissues is not known, and background bands in these bacterial lysates are not of practical importance to this study.

The rat Nat3 antiserum titer as determined by the company that developed the antibody (Invitrogen) indicates that polyclonal Nat3 antibodies are present in the antiserum in amounts comparable to the rat Nat1 and Nat2 antibodies in their antisera, although there are no guarantees that the antiserum will work for Western blotting. More Western blotting conditions should be tested to further characterize the Invitrogen Nat3 antiserum. The dilution of antibody, concentration of tween 20, time and temperature of incubation, and time of film exposure should be optimized to attempt to detect recombinant Nat3 using this antiserum.

Since the epitope for rat Nat2 polyclonal antiserum production is located at the C-
Figure 21. Polyclonal Antisera Detection of Rat N-acetyltransferases by Western blot

Recombinant lysates containing rat Nat1 13, Nat2 20, and Nat3 were analyzed by Western blot using the available polyclonal antisera. The negative control is from lysates of recombinant E.coli expressing empty PKK223-3 vector. The rat Nat1 polyclonal antiserum selectively recognizes Nat1 over Nat2 and Nat3, with a very small band for Nat2 (< 2% of the Nat1 band). The rat Nat2 antiserum specifically recognizes Nat2 over Nat1 and Nat3. Unfortunately, the newly developed rat Nat3 antiserum was unsuccessful at recognizing any of the rat Nats under the conditions tested. Other antisera recognized either none of the rat Nats or both rat Nat1 and Nat2 to varying degrees. Mouse and hamster antibodies were obtained from Edith Sim, University of Oxford, UK.
terminus of the protein (Figure 13), and since none of the slow Nat2 polymorphisms affect amino acids region (there are only two amino acid differences between the rapid allele and either of the slow alleles), it is reasonable to think that all three Nat2 enzymes will provide identical epitopes for antibody detection. Therefore, any differences observed by densitometric analysis between the amounts of the rapid Nat2 enzyme and the two slow Nat2 enzymes will reflect a real protein difference and not a difference in the number of epitopes provided by the rapid enzyme versus the slow enzymes.

The Nat2 Slow Acetylator Mechanism

The mechanism behind the Nat2 slow acetylator alleles is unknown, and thus more experiments are required. Kinetic measurements (Figure 18) and thermostability measurements (Figure 20) suggest that Nat2 21A and Nat2 21B protein levels are reduced compared to Nat2 20 protein. A Western blot of recombinantly expressed Nat2 enzymes using the rat Nat2-specific polyclonal antiserum clearly revealed that Nat2 slow acetylator alleles do in fact have dramatically reduced protein levels compared to Nat2 20 (Figure 22). Combining what is known about Nat2 slow acetylator kinetics, thermostability, and protein level, points to the conclusion that Nat2 21A and Nat2 21B protein are reduced due to decreased thermostability (Figure 20), and the catalytic capability of the enzyme is reduced due to increase $K_m$ (Figure 18), the combination of which results in slow acetylator status.

In addition to the difference between rapid and slow rat Nat2 catalytic activities, significant catalytic activity differences were observed between the cytosols expressing the two slow Nat2 alleles (Figures 14-17). The difference is also suggested by Western blot (Figure 22), in which the Nat2 bands are normalized to non-specific bands; nonspecific bands should be present in equal amounts across all samples in the same expression system. However, another Western blot must be performed and normalized to a bacterial housekeeping gene, or some other normalizing factor, to ensure that the small difference between recombinantly expressed Nat2 21A and Nat2 21B protein is real and significant.
Recombinant lysates containing Nat2 20, Nat2 21A, and Nat2 21B were analyzed by SDS-PAGE and Western blot. Nat2 bands and a set of nonspecific bands were quantitated by densitometric analysis. Nat2 bands were normalized to the nonspecific bands, which were used as a loading control. Nat2 slow alleles produce drastically less protein in this system than the Nat2 rapid alleles. An apparent significant difference exists between the expression of the Nat2 slow enzymes. These data need to be confirmed by a Western blot normalized to an internal control, or other normalizing standard.
Figure 23. Real-time RT-PCR Analysis of Recombinantly Expressed Rat Nat2 mRNA

Total bacterial RNA containing recombinant Nat mRNA was subjected to real-time RT-PCR. The same primers and Nat2-specific fluorogenic probe were used for detection of all three alleles. The differences observed among the three alleles are not reflective of activity or apparent protein differences. The total mRNA levels do not reflect the corresponding enzyme activities, indicating that differences in mRNA expression do not cause the slow acetylator phenotype. In addition, the differences between the two slow acetylator alleles in activity, and possibly also protein, are not reflected in these mRNA expression data, but rather the opposite trend is observed. While the apparent reduction in mRNA expression for the slow alleles is numerically significant, it is most likely an artifact of the real-time PCR method.
The influence of Nat2 slow allele polymorphisms on mRNA expression was tested by real-time RT-PCR (Figure 23). These data show that the affect of Nat2*21A and Nat2*21B polymorphisms on protein expression level cannot be explained by reduction in mRNA expression. They also show that the apparent difference between Nat2 21A and Nat2 21B protein expression is not due to transcriptional differences, since the opposite trend is suggested. The apparently significant difference between mRNA levels for the rapid allele and the two slow alleles could be related to differences in real-time PCR efficiency, since the G399A polymorphism in Nat2*21A and Nat2*21B is within a region to which one of the PCR primers anneals, thereby reducing PCR efficiency for the slow alleles.

Thus, if the protein expression differences can be verified by a Western blot analysis normalized to a bacterial internal standard, the mRNA data indicate that in this system the silent SNPs that characterize the Nat2 slow alleles influence translation of the Nat2 21A and Nat2 21B enzymes. Possible translational differences might be caused by differences in codon usage and availability in this system, or altered mRNA structure which could result in differences in translational efficiency.

Codon usage in E.coli was analyzed based on the two codons that differ between the two slow alleles (Table 1) (Fuhrmann et al., 2004; Nakamura et al., 2000). This analysis led to the conclusion that the opposite trend (Nat2 21A > Nat2 221B expression) would be expected if codon usage differences significantly influenced the translational efficiencies of the Nat2*21A and Nat2*21B mRNAs. In addition, the codon usage is practically reversed in rattus norvegieus for one of the codon pairs, indicating that if there were an influence of codon usage on translational efficiencies in the recombinant system it would be reversed in vivo.

Since codon usage is apparently not involved in modifying translational efficiencies, recombinant mRNA sequences (Figure 24) were analyzed for folding differences using the Mfold program (Figure 25) (Mathews et al., 1999; Zuker, 2003). Although the polymorphisms that distinguish Nat2*21A (G522A) from Nat2*21B (C672T) are silent in the amino acid sequence of the
Table 1. Codon Usage Comparison for Nat2*20, Nat2*2IA, and Nat2*2IB

To analyze the possible influence of differences in codon usage on Nat2*2IA and Nat2*2IB enzyme activity and possibly protein expression, the usage of the relevant codons was compared for this system. To test the correlation of any codon usage differences in this E.coli system to an in vivo rat model, the fraction of these codons for rattus norvegicus was also compared. Since the fraction of the GAA codon in E.coli is twice the fraction of GAG, codon usage comparisons suggest that Nat2*2IA might be expressed more readily than Nat2*2IB, which is the reverse of what is observed experimentally. Although the Serine codon trend is correct, the difference between them is small and not likely significant. The codon usage in rat seems to be the reverse of what is seen in E.coli, suggesting that any effect of codon usage in this recombinant system would be negated in vivo.
**Figure 24. Constructed Sequence of Recombinant Nat2 mRNA from PKK223-3**

Promoter and terminator sequences from the PKK223-3 bacterial expression vector sequence (Genbank M77749) were added to the 5’-and 3’-regions (respectively) of the Nat genes for analysis of predicted mRNA structures (Figure 25). (A) PKK223-3 contains an *E. coli* tac promoter of the sequence shown (Amann et al., 1983; De Boer et al., 1983). The tac promoter start site is underlined (solid), and the *Xmal* restriction site is underlined (dashed). (B) PKK223-3 contains an rrnB T₁T₂ ribosomal terminator (Brosius et al., 1978). These constructs were designed based on termination use of the T₁ terminator (double underline) (Orosz et al., 1991). The *PstI* restriction site used for cloning is underlined (dashed) and the suspected termination codon underlined (solid) (Lyakhov et al., 1997). (C) Suspected mRNA sequence for recombinantly expressed Nat2*21A, with PKK223-3 promoter and terminator sequences in lower case (bold).
Figure 25. Mfold Predicted mRNA Structures for Recombinant Nat2 Slow Alleles

The RNA folding prediction software, Mfold, was used to predict the mRNA structures of recombinantly expressed rat Nat2*21A and Nat2*21B. Differences between the predicted mRNA structures are circled, with the associated polymorphism indicated where appropriate. These two alleles differ only by these two nucleotide changes, which are silent in the amino acid sequence. While this structure prediction does not take into account the tertiary structural interactions within the mRNA species', it gives some clues about what might happen to the secondary structure. The influence of the G^{522}A polymorphism in Nat2*21A may be to reduce the complexity of the folding slightly, whereas the influence of the C^{672}T polymorphism in Nat2*21B seems to relax some of the structural complexity in that region.
coded proteins, they may have some influence on mRNA folding (Shen et al., 1999) and thereby influence mRNA stability (Capon et al., 2004) or translational efficiency. The Mfold server predicts folding changes associated with the two polymorphisms that distinguish the alleles. The G$^{522}$A polymorphism in Nat2*21A may reduce the complexity of the folding slightly relative to the Nat2*21A structure. On the other hand, the C$^{672}$T polymorphism in Nat2*21B seems to release many of the structural folds in that region, relative to Nat2*21B. A change to lower complexity in this region affected by the C$^{672}$T polymorphism is consistent with an increased translational efficiency for this allele, since ribosomal unfolding of this section might require less energy and time during translation. This change may also be consistent with a slightly decreased mRNA stability for the Nat2*21B recombinant mRNA, as suggested with real-time RT-PCR analysis (Figure 23). This would require the Nat2*21B silent mutations to be more influential (relative to Nat2*21A) on translational efficiency than on mRNA stability. Due to the complex nature of an mRNA molecule, these predicted mRNA structures amount to speculation, an educated guess, and verification would require further experiments; such experiments are beyond the scope of this study and will not be performed.
Conclusions

Rat *Nat1*13, *Nat2*20, *Nat2*21A, *Nat2*21B, and *Nat3* genes encode functional Nat enzymes with characteristic substrate selectivities, catalytic activities, and thermostabilities *in vitro*. MOCA is the best Nat1-specific substrate, and PABA is the best Nat2-specific substrate. Although Nat3 is a functional N-acetyltransferase, it has relatively low activity toward every substrate tested and no comparable human N-acetyltransferase gene. Future *in vivo* Nat expression studies using rats need not include rat *Nat3*, since it does not appear to significantly influence overall N-acetylation and is relatively unrelated to human NATs. However, *Nat3* mRNA expression should be measured to verify that low rat Nat3 activity is not compensated for by high *Nat3* expression *in vivo*.

A correlation exists between rat Nat1 and human NAT2, and between rat Nat2 and human NAT1. Evidence for this correlation was found in sequence similarity (Figure 11), substrate selectivity (Figures 14-17), and thermostability (Figure 20). These similarities indicate that the rat is a suitable model for *in vivo* N-acetyltransferase studies.

Rat Nat antisera are sufficiently specific for rat Nat1 and Nat2 (Figure 21). The newly designed and developed rat Nat3 antiserum was unsuccessful at identifying any of the rat Nats at the conditions tested.

The mechanism of the rat Nat2 slow acetylators, Nat2 21A and Nat2 21B, involves elevated *K_m*, thus reduced specificity constant (Figure 18), and reduced protein (Figure 22) due to reduced enzyme stability (Figure 20). Due to the polymorphic nature of rat *Nat2*, rats may serve as useful *in vivo* rapid and slow acetylator models.

Although the *Nat2* slow alleles differ only by silent nucleotide polymorphisms, their enzymatic activities differ significantly (Figures 14-17) possibly due to significant differences in protein expression (Figure 22). The possible differences in protein expression are not due to differences in mRNA transcription or mRNA stability (Figure 23). Possible translational differences cannot be explained by codon usage (Table 1), but they may be explained by
differences in translation efficiency due to differences in mRNA folding (Figure 25).
Future Experiments

Of the substrates tested, PABA, SMZ, MOCA, MDA, and pABG need to be tested for kinetic parameters $K_m$ and $V_{max}$. Additional prospective substrates have been identified, including p-aminoacetanilide, 3-amino-4-methoxyacetanilide, 3-ethylaniline, 2-amino-5-nitrophenol, 4-amino-2-nitrophenol, 2,6-dimethylaniline, o-toluidine, and 3,5-dimethylaniline. These will also be assayed for N-acetylation. Metabolic activation assays will be performed on N-OH-PhIP and N-OH-ABP with the new HPLC method developed by Mark Doll.

Instead of relying on previously published data regarding human NAT activity, a more direct comparison of human NAT1 and NAT2 versus rat Nat1 and Nat2 may be necessary. Human NATs will be recombinantly expressed in the same bacterial system using expression plasmids designed previously in this lab by Mark Doll, and assayed with substrates PABA and MOCA, which are rat Nat2 and Nat1 selective, respectively. This will allow direct comparison of rat Nats to human NATs expressed in the same system.

Optimization of Western blotting conditions will be tested to determine whether the Nat3 antiserum will be useful for Nat3 detection. In addition, the rat Nat antisera will be tested for their detection of rat Nat proteins in vivo. Western blot will also be performed on the rat Nat2 recombinant lysates and normalized to an internal standard. Since protein expression of the two slow alleles is very similar, the difference must be verified by normalization to an internal standard.
CHAPTER VI

TISSUE-SPECIFIC EXPRESSION OF RAT N-ACETYLTRANSFERASES: DISSERTATION AIMS AND EXPERIMENTAL METHODS

Introduction

The goal of my dissertation project is to investigate the influence of genetic background and cigarette smoke exposure on *rattus norvegicus* N-acetyltransferase 1 (*Nat1*) and 2 (*Nat2*) mRNA and protein expression in various tissues. These studies will utilize rats as *in vivo* models to characterize and predict a mechanism for human N-acetyltransferase tissue-specific expression and the influence of both genetics and environmental exposures on human N-acetyltransferase tissue-specific mRNA and protein expression. These objectives will be accomplished upon completion of the following three specific aims: (1) Characterize the catalytic activity, substrate specificity, and thermostability of Sprague Dawley rat N-acetyltransferase isozymes by recombinant expression in *Escherichia coli*; (2) Characterize *Nat1* and *Nat2* mRNA and protein expression in multiple tissues of different rapid acetylator rat strains; (3) Investigate the influence of cigarette smoke inhalation on *Nat1* and *Nat2* mRNA and protein expression in multiple tissues of different rapid acetylator rat strains.
Specific Aim I

Introduction

Specific Aim I will characterize the catalytic activity, substrate specificity, and thermostability of Sprague Dawley rat N-acetyltransferase isozymes by recombinant expression in *Escherichia coli*. While much of this aim was accomplished as described in chapters four and five, further experiments were proposed for completion of the work. The methods described in this section were not presented in chapter four or five, but will be utilized in addition to the methods outlined in chapter four and five for completion of analyses related and unrelated to Specific Aim I. Since a formal introduction to Specific Aim I can be found in chapters four and five, an extensive introduction will not be repeated here.

The purposes of Specific Aim I are as follows: (1) Verify similarities in biological function between rat and human N-acetyltransferases (suggested by high sequence identities) in an effort to validate the rat as a suitable *in vivo* model for this research; (2) Sequence and characterize the previously unidentified rat Nat3 and determine its relevance to this study.

The hypotheses that drive this aim are as follows: (1) Rat Nats are comparable to human NATs with regards to catalytic activity, substrate specificity, and thermostability, and therefore may serve an analogous physiological role; (2) Rat Nat3 is a functional N-acetyltransferase enzyme, similar in function to Nat1 and Nat2, nevertheless with distinct catalytic activity, substrate selectivity, and thermostability.

Methods

**Colorimetric Determination of Enzyme Activity.** Since HPLC assays are generally developed only for commonly assayed substrates, a different assay will be utilized for the less common substrates p-aminoacetanilide, 3-amino-4-methoxyacetanilide, 3-ethylaniline, 2-amino-5-nitrophenol, 4-amino-2-nitrophenol, 2,6-dimethylaniline, o-toluidine, and 3,5-dimethylaniline. Incubation of recombinant lysates with acetyl-CoA and substrate will be performed as described in chapter five. Following incubation, guanidine HCl and sodium phosphate (pH 6.8) will be
added, followed by dithionitrobenzene (DTNB), sodium phosphate (pH 6.8), and ethylenediaminetetraacetic acid (EDTA) and incubation at room temperature for 5 minutes. During these incubations, CoASH is produced by reaction of free CoA, a byproduct of the N-acetylation reaction and indicator of substrate conversion to product, with thiol-containing DTNB, resulting in a yellow color that will be quantified using a Beckman DU-650 spectrophotometer to measure the absorbance of these samples at 412 nm (De Angelis et al., 1998). Control reactions containing no substrate will be included for each sample and each dilution to control for the absorbance of the total protein present in the reactions. Dilution buffer (homogenization buffer) must contain no dithiothreitol (DTT) or β-mercaptoethanol since they produce a yellow color that will interfere with absorbance readings. Nmoles of acetylated product is inferred by multiplying the absorbance at 412 nm by the CoASH molar absorption coefficient 0.0137 (Riddles et al., 1983). Activities will be calculated as nmoles/min/mg, and kinetic parameters will be determined as described in chapter five.

**Metabolic Activation Assay.** Metabolic activation assays measure the formation of DNA-adducts from activated O-acetylation products (Figure 2). These assays will use recombinant bacterial lysates containing Nat1 13, Nat2 20, Nat2 21A, Nat2 21B, Nat3, or the PKK223-3 negative control (no insert). Various enzyme dilutions will be assayed initially to determine the dilution necessary to ensure that < 15% total substrate concentration is consumed during the reaction. Cytosols will be incubated with 0.5 mM acetyl-CoA, 0.37 mM 2-deoxyguanosine (Sigma) and 300 μM substrate for 10 minutes at 37°C. Reactions will be stopped by adding water saturated ethyl acetate to precipitate the protein. Precipitated protein will be removed by centrifugation at 13,000 rpm for 10 minutes. Fifty μl of the upper phase (organic) will be transferred to an HPLC vial, without disrupting the interface, then the solvent evaporated to dryness in a speed-vac for about 15 mintues. After drying, the adducts will be resuspended in 10% acetonitrile with vortexing and analyzed by high performance liquid chromatography.
(HPLC) using System Gold Software (Beckman-Coulter, Fullerton, CA, USA) for quantification of N-acetylated product. Compounds to be tested include N-OH-PhIP and N-OH-ABP. Methods for this assay were developed by Mark Doll. Data will be analyzed as described for the N-acetylation assays in chapter five.

**Western Blot.** Western blots will be performed on recombinantly expressed rat Nat2 20, Nat2 21A, and Nat2 21B in an attempt to confirm the small difference between the two slow acetylator alleles (Figure 22). This will require use of a loading control. A re-probing step will be added to the previously described Western blot protocol in chapter five,. Instead of normalizing to a bacterial housekeeping gene, a commercially available monoclonal antibody against bacterial β-lactamase (Abcam Inc., Cambridge, MA, USA) will be utilized. This antibody was raised in mouse, whereas the Nat antibodies were raised in rabbit, thus the membrane can be re-probed without a rigorous stripping protocol. Since β-lactamase is expressed by the PKK223-3 expression vector, this antibody will provide a way to directly normalize the Nat bands to the level of PKK223-3 expression in each preparation. The resulting protein bands will be quantitated by densitometry and the Nat bands corrected relative to β-lactamase as the standard.
Specific Aim II

Introduction

This aim will investigate the mechanism behind the tissue-specific expression of rat Nats, which will lay the groundwork for aim three. As described in chapter one, little is known about the \textit{in vivo} mRNA and protein expression of rat \textit{Nat1} and \textit{Nat2} in any tissue. Experiments are proposed to analyze Nat expression in multiple tissues from multiple inbred rat strains to characterize N-acetyltransferase expression and the influence of genetic variability (trans-acting) on Nat expression. Only one publication to date reports rat \textit{Nat1} and \textit{Nat2} mRNA sequences (from pineal gland), and asserts that rat \textit{Nat1} and \textit{Nat2} share a single promoter (Ebisawa et al., 1995). This is not the expected result of these proposed studies. In fact, Ebisawa's findings have not been duplicated by other laboratories, or for other tissues, and are inconsistent with more recent studies of human N-acetyltransferase expression (Husain et al., 2004). If, however, Ebisawa's results are duplicated in other tissues, the regulation of rat Nats will still have relevance for understanding the regulation of NAT expression with regard to promoter-dependent mRNA splicing and tissue-specific expression.

Following exposure by ingestion, inhalation, or through the skin, many factors influence the \textit{NAT1} and \textit{NAT2} mediated metabolism of arylamines including: (1) the combined \textit{NAT1} and \textit{NAT2} genotypes, which determine rapid and slow acetylator status for each isozyme; (2) tissue-specific \textit{NAT} expression, which determines the tissue distribution of \textit{NAT1} and \textit{NAT2} isozymes; (3) the characteristic tissue distribution of each arylamine, which determines each tissue's level of exposure to arylamine carcinogens; (4) Cytochrome P450 and other oxidation enzymes in each tissue, which determines the fraction of activated arylamine carcinogen; (5) competitive metabolism by other polymorphic xenobiotic metabolizing enzymes such as glutathione S-transferases (Meisel P, 2002). Combinations of these factors will no doubt influence DNA-adduction and possibly mutagenesis. DNA-adduct levels are a convenient gauge for the metabolic conversion of arylamine carcinogens to reactive species, and are directly relevant since
DNA-adducts could be predictive of tumor incidence (Otteneder and Lutz, 1999). Therefore, *in vivo* DNA-adduct formation following arylamine exposure in rats will be the criterion used to prioritize the tissues selected for Nat expression analyses. This aim will focus initially on rat Nat tissue-specific expression in lung, liver, pancreas, bladder, kidney, prostate (male only), mammary gland (female only), and colon (Cummings et al., 1994; Fretland et al., 1997; Fretland et al., 2003; Kaderlik et al., 1994). Although enzymes other than N-acetyltransferases are involved in activation of amines, the tissues listed above are among the most critical sites for arylamine-induced cancer risk. Since N-acetyltransferases catalyze the activation and deactivation of amines, these tissues demand the highest priority for a study related to Nat-mediated cancer risk.

In addition to tissue-specific expression, this study will investigate the influence of genetic variability (trans-genetic background) on Nat expression. Since single nucleotide polymorphisms in the coding region, even polymorphisms that are silent in the amino acid sequence, could influence Nat expression and compromise our ability to draw meaningful conclusions from differences in the expression of different alleles (Capon et al., 2004; Shen et al. 1999), only rapid acetylator rat strains will be chosen, each possessing the same Nat1*13, Nat2*20, and Nat3 coding region sequences. A number of rat strains were screened by automated sequencing of the complete Nat1, Nat2, and Nat3 coding regions. Since the inbred strains used in this aim will not vary in their Nat genotypes, any variations in Nat gene expression must be due to the influence of either cis- (outside the coding region) or trans-acting genetic factors on rat Nat expression. Further experiments could allow distinction between cis- and trans-genetic influence.

The specific purposes of this aim are as follows: (1) Quantify the expression of Nat1, Nat2, and Nat3 mRNA and protein in various rat tissues; (2) Characterize the use of differential promoters and alternative splicing patterns in rat Nat1 and Nat2 mRNA expression, and observe any associations with rat Nat tissue-specific expression; (3) Investigate the influence of genetic
background on rat Nat expression by comparing \textit{Nat1} and \textit{Nat2} tissue-specific mRNA and protein expression in different inbred rat strains.

The hypotheses that drive this aim are as follows: (1) Rat Nats are tissue-specifically expressed with characteristic mRNA and protein expression patterns for each tissue; (2) Nat expression is tissue-specifically regulated through differential promoter usage and alternative splicing of non-coding exons; (3) Cis- and/or trans-acting genetic factors may modify the expression of rat N-acetyltransferases at the mRNA and/or protein level.

\textbf{Methods}

\textbf{Genotyping Rat Strains.} Rat genomic DNA from various inbred strains was isolated from blood samples (performed by Dr. David Barker) or ear clippings (performed by Mark Doll) that were purchased from Charles Rivers Laboratories, Inc. (Wilmington, MA) and Harlan Bioproducts for Science (Indianapolis, IN, USA). \textit{Nat1}, \textit{Nat2}, and \textit{Nat3} were amplified from the genomic DNA by duplicate PCR reactions using high-fidelity Phusion polymerase (Finnzymes). The PCR primers used for amplification of \textit{Nat2} and \textit{Nat3} were described in chapters five and four, respectively. The PCR primers used to amplify rat \textit{Nat1} (Forward, 5’-ctggaattcatggacatcgaagcatacttcgaaggattggttac-3’; Reverse, 5’-agagcatgcatggagacccaatttaccctaaatagt-3’) were designed by Mark Doll. Duplicate PCR reactions were combined, then PCR products purified using the QIAquick PCR Purification Kit (Qiagen). Purified PCR products were then run on a 1% agarose gel to verify the presence of PCR product. Following verification, the purified PCR products were sequenced (top and bottom strands) by automated DNA sequencing using the BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems), and reaction products analyzed on an ABI 310 Genetic Analyzer (Applied Biosystems). Sequencing primers were described in chapters V and IV for \textit{Nat2} and \textit{Nat3}, respectively. \textit{Nat1} sequencing was carried out using the two primers mentioned above, plus five additional forward and reverse primers (Forward, 5’-tctgctgtactggcctctga-3’, 5’-aagagaatggacactgtggttac-3’, 5’-gcctgtttgaaggac-3’; Reverse, 5’-agaagactgtttgcacctgctta-3’, 5’-acggttaaagtgacagt-3’) designed
Of all the rat strains sequenced, PVG, MW, WF, DA, Lou/M, CDF, BN, and LEW, in addition to F344 and SPRD, possess the wildtype Nat allele at all three loci (Table 2). Strains ACI and COP contained a new polymorphic Nat3 allele that will be submitted to GenBank upon verification. The predominance of the original Nat3 sequence (AY253757) among the rat strains supports its designation as the wild-type Nat3 allele.

Rat Selection and Care. For the following analyses, male and female rats from six different rat strains will be sacrificed without any prior experimental treatment. To eliminate variability in conditions with which the rats are raised, all rats will be ordered from Harlan (Indianapolis, IN) to ensure that they are raised under the same conditions. Rats will be obtained at an approximate age of 5 weeks, acclimatized to the University animal facilities for at least 5 days prior to sacrifice or exposure (see Specific Aim III). Rats will be housed in plastic cages with wire bar tops. Ground corn cob bedding (1/4” deep) is changed at least two times a week. Diet will consist of Purina Lab Chow #5001. Room temperature will be maintained between 72-74°C and humidity between 40-70%, with light/dark cycles of 12 hours (lights on at 6 a.m. and off at 6 p.m.).

All experiments will be replicated in four animals (per strain). The total number of animals required for completion of this aim is approximately 48 (6 strains X 4 replicates X 2 sexes). Rats will be sacrificed by exsanguination, making it possible to clear the rat tissues of blood prior to harvest. According to the recommendations of the Panel on Euthanasia of the American Veterinary Medical Association, death by exsanguination is an acceptable form of euthanasia if the animal is anesthetized prior to the procedure (AVMA Panel on Euthanasia, 2001). To minimize the stress involved in administering anesthesia to the rats, the head will be covered with a cloth or towel, providing a dark environment while also preventing the animal from biting (Rasmussen and Ritskes-Hoitinga, 1999).

Exsanguination and Tissue Isolation. Rats will first be anesthetized by IM injection of
Inbred Strain | Nat1*13 | Nat2*20 | Nat3 (AY353757)
--- | --- | --- | ---
ACI† | ✓ | ✓ | G<sup>619</sup>T
PVG | ✓ | ✓ | ✓
MW | ✓ | ✓ | ✓
WF | ✓ | ✓ | ✓
PETH | ✓ | ✓ | ?
DA | ✓ | ✓ | ✓
LOU/M | ✓ | ✓ | ✓
CDF | ✓ | ✓ | ✓
BN | ✓ | ✓ | ✓
LEW | ✓ | ✓ | ✓
COP† | ✓ | ✓ | G<sup>619</sup>T
(F344) | ✓ | ✓ | ✓
(SPRD) | ✓ | ✓ | ✓
(NSD) | ✓ | 2*21B | ✓
(WKY) | ✓ | 2*21A | ✓

Table 2. N-acetyltransferase Loci Sequenced for Various Inbred Rat Strains

Rat Nat loci from various inbred rat strains were sequenced to determine their genotype. Previously sequenced rat Nat loci from four other strains are also shown (parentheses). Of the above inbred rat strains, 10 possess wild type Nat1, Nat2, and Nat3 alleles (underlined). Two strains possess identical polymorphic Nat3 alleles (†).
approximately 1 ml/kg + 50 μl ketamine HCL (800 mg)/xylazine HCl (120 mg) solution (Sigma) (Flecknell P, 1996). Rats will then be sacrificed and exsanguinated by cardiac puncture. Krebs-Henseleit bicarbonate buffer (Krebs et al., 1932) saturated with a mixture of 95% O₂ and 5% CO₂ will be perfused into the aorta using a 16 gauge needle for 3 minutes at 30 ml/min. The ascending vena cava will be cut immediately following puncture to allow the blood to drain. Lung, liver, pancreas, bladder, kidney, prostate (male only), mammary gland (female only), and colon tissues will be harvested. Triplicate representative tissue sections will be isolated from each tissue, then flash frozen in liquid nitrogen. Tissues will be stored at -80°C until further processing, with the exception of pancreas tissue, which, due to its high levels of ribonucleases, will be processed for RNA extraction immediately.

Initial experiments will be performed to compare tissues harvested without exsanguination versus tissues that have been exsanguinated to determine whether blood Nat expression contributes significantly to mRNA and protein quantification. Since the extent of blood perfusion varies among tissues, blood could contribute to Nat expression measurements in a disproportionate manner, resulting in an inaccurate measurement of tissue-specific expression.

**RNA Isolation.** Frozen tissue sections will be thawed in RNA STAT-60 (Tel-Test Inc., Friendswood, TX, USA) and disrupted for 1 minute in a bead-beater homogenizer. RNA will then be isolated from the tissue homogenates according to RNA STAT-60 protocol. Contaminating DNA will be removed from RNA using Turbo DNA-free (Qiagen). RNA will be stored at -80°C. RNA will be analyzed for quality by electrophoresis on a 1% agarose gel. RNA standards of known concentration will be run on the same gel as the samples, and will be used to create a standard curve for quantification of RNA concentration.

**Protein Determinations.** Frozen tissue sections will be thawed in ice-cold homogenization buffer, then disrupted for 1 minute in a bead-beater homogenizer. Cell lysates will then be centrifuged at 100,000 x g for 1 hour, and cytosol aliquots stored at -80°C. Total protein concentration will be determined using the Bradford method (Bio-Rad).
Measurement of Relative mRNA Expression. Although it was determined that Nat3 should not be included in expression studies due to its lack of relevance to human NATs and relatively small contribution to N-acetylation activity, Nat3 will be included in initial determinations of relative rat Nat mRNA expression in tissues. This will enable further characterization of the contribution of rat Nat3 to overall N-acetylation in rat tissues, and examine the possibility that high Nat3 mRNA expression compensates for the low activity of rat Nat3 enzymes.

SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen) will be used to synthesize cDNA from rat tissue total RNA. RT-PCR reactions without reverse transcriptase will be performed as controls for DNA contamination. Nat1, Nat2, and Nat3 mRNA levels will be measured by locus-specific quantitative real-time RT-PCR, normalized to 18S ribosomal RNA (Applied Biosystems), using TaqMan Universal PCR Master Mix from Applied Biosystems and an ABI Prism 7700 sequence detection system (Applied Biosystems). A single set of primers were designed to amplify of all three Nat loci simultaneously, and gene-specific fluorescent probes for detection of relative levels of Nat1, Nat2, and Nat3 in the same sample (Figure 26).

PCR standards will be used to correct for differences among the three locus-specific MGB probes in sequence recognition and cleavage properties. Primers that flank the real-time PCR target region (Figure 27) were designed in order to amplify this region for creation of PCR standards. With these primers, PCR amplification will be carried out using high fidelity Phusion polymerase (Finnzymes), and PCR products then purified using the QIAquick PCR Purification Kit (Qiagen). PCR products will be quantified, then dilutions made and analyzed by RT-PCR to create a standard curve for quantification of Nat mRNA.

The relationship $2^{-\Delta C_T}$ will be used to convert C_T values from inverse logarithmic to linear scale, where $\Delta C_T$ is the 18S ribosomal RNA C_T subtracted from the Nat-specific C_T from the same sample. Control samples lacking reverse transcriptase and water controls (no cDNA) to test
Figure 26. Taqman Primer and MGB Probe Design for RT-PCR Rat Nat Locus Discrimination

Nucleotide sequence comparison of rat Nat1, Nat2, and Nat3 reveals regions of nucleotide identity among the three loci surrounding a small region containing differences among the loci (highlighted). Non-specific Nat real-time PCR primers were designed based on the identical regions (capitalized). Locus-specific MGB fluorogenic probes were designed to distinguish the three Nat loci based on the nucleotide differences (underlined). Since the sequence recognized by the probes overlaps with the forward primer (dashed box), the probes were designed to recognize the opposite strand without overlapping the reverse primer.
for DNA contamination will be included.

**Rat Nat mRNA Transcripts.** The 5' RLM-RACE kit (RNA Ligase Mediated Rapid Amplification of cDNA Ends) from Ambion will be used to characterize Nat1 and Nat2 mRNA species in tissues of F344 strain inbred rats. This is a superior method for determining the complete 5' mRNA sequence because only mature, capped mRNA are amplified. Identification of the 3' poly-adenylation site(s) will be determined using Ambion's 3' RACE kit, which amplifies only mature, poly-adenylated mRNA. RACE products will be amplified by dual rounds of PCR using locus-specific nested PCR primers (Figure 27) and RACE adapter-specific primers. PCR products will be cloned using a pcDNA3.1/V5-His TOPO TA Cloning kit (Invitrogen). Clones will be used to transfect TOP10 E.coli (Invitrogen). Plasmids will then be amplified by growth in TOP10 E.coli, followed by plasmid isolation using the Qiagen Plasmid Midi Kit. Plasmid inserts will be sequenced using plasmid-specific T7 (Forward) and BGH (Reverse) primers. If transcript length exceeds the range of these primers, new primers will be designed based on the known transcript sequence. Sequencing data will be analyzed for transcription start site and mRNA splicing patterns. Transcript sequences will be submitted for publication in GenBank.

Due to the cost of RACE analysis and the large number of samples to be analyzed, rat strain F344 RACE results will be utilized to design Northern blot and RT-PCR analyses. Heterogeneity in Nat mRNA transcript profiles among different strains will be determined by comparison of the Nat transcripts by Northern blot. A high degree of variability in Nat transcript sizes among rat strains will justify performing RACE experiments on strains other than F344. If Northern blot analyses do not indicate such variability among strains, quantitative real-time RT-PCR experiments can be designed using the F344 RACE data for quantification of the specific transcripts detected.

**Northern Blot.** Total RNA (15-20 µg) will be separated on an agarose gel made from NorthernMax 10X denaturing gel buffer (Ambion, Inc.) and run using NorthernMax running
Figure 27. Primer Design for Real-Time PCR Controls and PCR of RACE Products

Nested Primers A and B were designed for 5′ RLM-RACE product PCR; E and F were designed for 3′ RACE product PCR. Primers C and D, which encompass the real-time PCR target region (highlighted) (Figure 26) will be used for creation of real-time PCR standards. Primer location is indicated by bold and/or lower case text.
buffer (Ambion, Inc.). RNA will then be transferred to BrightStar-Plus Positively Charged Nylon Membranes (Ambion, Inc.) using NorthernMax One-Hour Transfer Buffer (Ambion, Inc.). Probes will be labeled, hybridized, and detected using the North2South Direct Labeling and Detection Kit (Pierce, Inc.). Nat RNA bands will be analyzed by densitometric analysis. Probes specific to the Nat coding exon of the two Nat loci will be used to identify locus-specific transcripts.

**Quantification of Relative Protein Expression.** Western blotting will be used to quantify the relative expression of Nat1 and Nat2 protein in rat tissue cytosols. SDS-PAGE and Western blotting will be carried out as proposed under Specific Aim I, normalizing to α-tubulin as the internal control. For quantification, a correction factor will be calculated using purified Nat proteins of known concentrations. This correction factor will be applied to the band intensity for each isozyme to correct for differences in antibody concentration. Nat enzymes will be recombinantly purified using the IMPACT-CN Protein Fusion and Purification System (New England Biolabs, Inc.). In this system, enzymes are recombinantly expressed as fusion proteins with a self-cleaving intein and chitin binding affinity tag. The affinity tag permits purification using a chitin column, and the intein self-cleaves under reducing conditions (dithiothreitol) leaving only the native enzyme. Enzyme concentration will be measured using the Bradford method (Bio-Rad). The ECF detection kit (Amersham Biosciences, Inc.), in combination with a Molecular Dynamics Storm 860 phosphorimager (Amersham Biosciences, Inc.) enables quantitative measure protein bands directly from the membrane. Dilutions of purified Nat enzyme will be used to create a standard curve for quantification of protein from Nat band intensities. These standards will be included on each gel.

**Enzymatic Activity Assays.** N-acetylation assays will be performed as described in chapter five, but tissue cytosols will be used in the place of recombinant bacterial lysates. Isozyme-selective substrates will be used to distinguish Nat1 enzyme activity from Nat2 activity. These substrates will be the most selective for each isozyme, as determined using the substrate-selectivity data from Specific Aim I.
Metabolic activation assays will be performed as described above under Specific Aim I with substrates N-OH-PhIP and N-OH-ABP, but tissue cytosols will be used in the place of recombinant bacterial lysates. The negative control will be a boiled tissue cytosol sample at the same concentration as the normal tissue cytosols, since the activated arylamines will react with protein and DNA.

**Distinction of Cis- and Trans-Genetic Influence.** Although not a primary goal, it may be possible to distinguish between cis- or trans-acting genetic factors with an F1 hybrid cross between two strains that differ in their Nat expression. We can quantitatively measure the total Nat mRNA for the locus of interest (locus “NatX”) in the F1 hybrid and compare with the expression in both of the parent strains. If the total NatX mRNA in the F1 hybrid is identical to the total NatX mRNA in one of the parent strains, we can conclude that functionally important dominant trans-acting genetic factors are influencing NatX expression in that parent strain. If, however, the hybrid NatX mRNA level is intermediate between the two parent strains, we cannot distinguish between cis-acting and trans-acting genetic factors.

**Statistical Analyses.** Standard error of the means (SEM) will be calculated for triplicate measurements of Nat mRNA expression, protein expression, and enzyme activity in each tissue tested. Likewise, SEM will be calculated to test the variability of mRNA expression, protein expression, and enzyme activity mean values for each tissue among four replicate animals. Tukey parametric one-way analysis of variance will be performed to test the influence of strain differences on expression in each tissue. This same analysis (Tukey) will be utilized to analyze the differences among different tissues within each strain for significance of tissue-specific differences. This test compares every possible pair of measurements within the group for significant differences, and eliminates errors associated with performing many individual t-tests. Statistical significance will be determined at values of $p < 0.05$.

**Potential Additional Analyses and Controls.** If significant differences are found in the expression of Nat loci among different strains, microarray analysis of tissue RNA from different
rat strains could provide clues regarding biomolecular pathways that may be involved in Nat regulation. Since different strains typically model a specific pathology or trait, possible differences in the regulation of Nat expression in these strains relative to other important gene families could provide important information about the endogenous physiological function of Nat and its connections to biomolecular pathways (Staudt and Brown, 2000; Le Roch et al., 2003). The Brown Cancer Center Microarray Facility provides equipment and expertise necessary for successful microarray experiments. GeneChip Rat Genome 230 2.0 Array (Affymetrix, Inc) provides comprehensive coverage of the rat genome, analyzing over 30,000 rat transcripts and variants, and therefore is very useful for analysis of genetic variability and for comparisons of Nat expression with the expression of other biomolecular pathways.

**Potential Setbacks and Alternate Methods.** In the event of failure to purify the rat Nat isozymes using the IMPACT-CN system (New England Biolabs), two alternative methods are available. Recombinant expression of tagged proteins can produce large amounts of protein that are easily purified. Although purification of tagged proteins is relatively simple experimentally, it is unclear whether the tag will alter native enzyme activity. Enzyme assays can be used to determine the effect of a tag on enzymatic activity by comparing tagged enzyme Western blot intensities and enzyme activities to recombinantly expressed Western blot intensities and enzyme activities. If tagging the enzymes alters their activity, Western blot analyses of recombinant expressions correlated to recombinant enzyme activity can be used to develop correction factors. Correlation of quantified Nat protein to enzyme activity will enable quantification of active Nat1 and Nat2 enzyme from enzyme activity assays with tissue cytosols, thereby also allowing comparison of Nat1 and Nat2 active protein levels. Also, since detection of the purified enzyme by Western blot does not require catalytically active enzyme, the tag should pose no threat to its effectiveness as a standard for Western blot analyses. Tags should not interfere with the peptide epitope used for production of the antibody. Alternatively, a synthetic peptide identical to the peptide against which the antisera were produced can be used as an arbitrary standard for
quantitation of the protein amount and the enzyme activity. Inability to quantify Nat protein and inability to compare active Nat1 and Nat2 enzyme are the major disadvantage of this approach.
Specific Aim III

Introduction

This study will characterize the influence of one possible environmental influence, cigarette smoke inhalation, on rat Nat mRNA and protein expression in multiple tissues. Humans are regularly exposed to cigarette smoke either actively or passively. If the components of cigarette smoke influence NAT expression, the high prevalence of this exposure could be one possible cause for variable NAT phenotypes in a population. Cigarette smoke inhalation influences the expression of a number of genes, including the induction of oxidative stress-responsive, Phase II-responsive, and Phase I-related genes (Gebel et al., 2004; Roos et al., 2003). Since cigarette smoke contains many different compounds, this aim will not attempt to delineate which chemicals influence Nat expression. The aim is simply to determine whether cigarette smoke, as a single environmental exposure, influences Nat expression.

Cigarette smoke could be expected to influence Nat tissue-specific expression by several mechanisms. Cigarette smoke could specifically influence lung Nat expression since the smoke is introduced into the body directly through the lungs. Smoke components could tissue-specifically influence the expression of trans-acting genes that in turn influence Nat expression. Cigarette smoke exposure could influence Nat expression in a strain-dependent manner due to genetic background differences. Any influence on Nat expression will most likely be very complex, but it will not be the goal of this study to delineate the mechanism(s) of cigarette smoke influence on Nat expression.

The specific purpose of this aim is to test whether cigarette smoke exposure influences rat Nat1 and/or Nat2 mRNA expression, protein expression, and/or enzyme activity in vivo. The hypothesis is that cigarette smoke inhalation influences Nat1 and/or Nat2 mRNA and protein expression in a tissue specific and/or strain specific manner.

Methods

Rat Studies. Male and female rats will be obtained and handled as described under
Specific Aim II. Following the 5 day acclimatization period, rats will be exposed to cigarette smoke, then immediately sacrificed as described under Specific Aim II. Completion of this aim will require a minimum of 48 animals (6 strains X 4 replicates X 2 sexes).

**Smoking Apparatus.** A TE-10 fully automated smoking machine connected to two rat exposure chambers large enough to hold four cages each (Teague Enterprises, Davis, CA, USA) (Teague et al., 1994) will be utilized to expose rats to smoke from filtered 2R4F research cigarettes (Kentucky Tobacco Research and Development Center, University of Kentucky, Lexington, KY). This cigarette was developed by the NCI Agricultural Research Service-USDA and the Tobacco and Health Research Institute for delivery of approximately 9.2 mg of tar and 0.8 mg of nicotine. The NCI standard cigarette for experimental work, 1R3F, is going out of production. The TE-10 smoking machine holds up to 30 cigarettes which it can automatically load, light, puff, and discard. It can smoke up to 10 cigarettes simultaneously at variable flow rates, thus providing multiple mechanisms for controlling smoke exposure rates. Chamber air flow rates can be monitored and the contents of the air trapped on a filter for easy testing of the composition and quantity of chemicals in the chamber air. For each experiment, one chamber will be used for smoke exposure (4 rat cages) and the other chamber (4 rat cages) for a control environment, with a maximum of four rats per cage.

**Exposure Levels and Times.** Rats will be exposed to a single 8 hour smoke exposure without a recovery period. In the case that N-acetyltransferases are immediate response genes, this time frame should be enough for *de novo* N-acetyltransferase protein synthesis. The animals will then immediately be sacrificed for analysis of the influence of cigarette smoke on Nat expression. This large initial exposure is designed to test for any possible influence of cigarette smoke inhalation on rat Nat gene expression. If no effect is seen on mRNA levels, protein levels, or enzyme activities following the 8 hour exposure, it could be an indicator that cigarette smoke exposure does not influence Nat gene expression, or it could indicate that Nats are not immediate response genes, and extra time is required for *de novo* synthesis of transcription factors. If no
effect is observed at the mRNA level, a longer exposure (12, 24, 48 hours) or a longer lag time between exposure and sacrifice may be necessary to allow time for de novo synthesis of transcription factors.

**Determination of Exposure Level and Exposure Effect on Health.** Immediately prior to exsanguination, a blood sample will be collected from each animal. A commercially available enzyme linked immunosorbant assay (ELISA) will be used to measure serum cotinine levels (Cozart Biosciences, Ltd, Oxfordshire, UK) as an indication of the smoke exposure of each animal. Chamber smoke concentrations will be monitored during each exposure by weighing the total filtered suspended particles from a known volume of air over a 5 minute period. Rat weights will be recorded before and after exposure as a gauge the general health of the animals.

**Measurement of Tissue mRNA and Protein Expression.** The analyses described in the previous section (“Specific Aim II”) will be followed for analyses of tissue mRNA and protein expression in smoke-exposed rats. Since it is up-regulated in response to cigarette smoke exposure in rats, CYP1A1 mRNA levels will be measured in treated versus untreated rats as a control for the effectiveness of our smoke exposure methods. CYP1A1 expression will be measured by RT-PCR with the primers and probes designed as previously reported (Gebel et al., 2004).

**Statistical Analyses.** Standard error of the means (SEM) will be calculated for triplicate mRNA, protein, and enzyme activity measurements in each tissue from four replicate animals for each strain. Likewise, SEM will be calculated to test the variability of mean mRNA expression, protein expression, and enzyme activity values for each tissue among four replicate animals. Differences in mRNA expression, protein expression, and enzyme activity between treated and untreated animals will be tested for significance using a t-test, since only single sets of data will be compared. Statistical significance will be determined at values of p < 0.05.

**Potential Additional Analyses and Controls.** Microarray analysis of tissue RNA from treated and untreated rats could aid in identification of genes with expression altered similarly to
Natl and/or Nat2 in response to cigarette smoke components. This type of information could provide clues toward determining an endogenous physiological function for Nat in addition to the mechanism of Nat regulation (Staudt and Brown, 2000; Le Roch et al., 2003). This information would also validate the methods used here by giving a view of the overall affect of the cigarette smoke exposure methods used, enabling comparison of the results to those found in similar experiments previously reported in the literature (Gebel et al., 2004). The Brown Cancer Center Microarray Facility provides the equipment and expertise necessary for successful microarray experiments. GeneChip Rat Genome 230 2.0 Array (Affymetrix, Inc) provides comprehensive coverage of the rat genome, analyzing over 30,000 rat transcripts and variants.

**Potential Setbacks and Alternate Methods.** If no differences are detected between the Nat1 and Nat2 mRNA and protein expression/activity in treated versus untreated rats following the initial exposure, we will conclude that cigarette smoke does not influence N-acetyltransferase expression/activity in rat. This may provide opportunity to extend the analyses performed in Specific Aim II or explore other environmental exposures.
REFERENCES


Chen JC, Hwang JM, Chen GW, Tsou MF, Hsia TC, Chung JG. Curcumin decreases the DNA adduct formation, arylamines N-acetyltransferase activity and gene expression in human colon tumor cells (colo 205). In vivo 2003; 17: 301-309.


Fretland AJ, Doll MA, Gray K, Feng Y, Hein DW. Cloning, sequencing, and recombinant expression of NAT1, NAT2, and NAT3 derived from the C3H/HeJ (Rapid) and A/HeJ (Slow) acetylator inbred mouse: functional characterization of the activation and deactivation of aromatic amine carcinogens. Toxicol Appl Pharmacol 1997b; 142: 360-366.


Hein DW, Rustan TD, Bucher KD, Martin WJ, Furman EJ. Acetylator phenotype-dependent and -independent expression of arylamine N-acetyltransferase isozymes in rapid and slow acetylator inbred rat liver. Drug Metab Dispos 1991b; 19: 933-937.


Lemke LE, McQueen CA. Acetylation and its role in the mutagenicity of the antihypertensive agent hydralazine. Drug Metab Dispos 1995; 23: 559-565.


National Toxicology Program. 4,4'-Methylenebis(2-chloroaniline). Rep Carcinog 2002a; 10; 149-151.

National Toxicology Program. 4,4'-Methylenedianiline and its dihydrochloride salt. Rep Carcinog 2002b; 10; 152-153.


Rothen JP, Haefeli WE, Meyer UA, Todesco L, Wenk M. Acetaminophen is an inhibitor of hepatic N-acetyltransferase 2 in vitro and in vivo. Pharmacogenetics 1998; 8: 553-559.


Shen LX, Basilion JP, Stanton VP. Single nucleotide polymorphisms can cause different structural folds of mRNA. Proc Natl Acad Sci USA 1999; 96: 7871-7876.


Vineis P. Epidemiology of cancer from exposure to arylamines. Environ Health Perspect 1994; 102 (suppl. 6); 7-10.


Walraven JM, Doll MA, Martini BD, Hein DW. Rattus norvegicus strain Spague Dawley arylamine N-acetyltransferase 3 (Nat3) gene, complete cds. GenBank 2003a; AY253757 (PubMed)

Walraven JM, Doll MA, Martini BD, Hein DW. Rattus norvegicus strain F344 arylamine N-acetyltransferase 3 (Nat3) gene, complete cds. Genbank 2003b; AY253758 (PubMed)

Walraven JM, Doll MA, Martini BD, Hein DW. Rattus norvegicus strain WKY arylamine N-acetyltransferase 3 (Nat3) gene, complete cds. Genbank 2003c; AY253759 (PubMed)


Zang Y, Zhao S, Doll MA, States JC, Hein DW. The T341C (Ile114Thr) polymorphism of N-acetyltransferase 2 yields slow acetylator phenotype by enhanced protein degradation. Pharmacogenetics 2004; 14: 717-723.


<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>adenine</td>
</tr>
<tr>
<td>ABP</td>
<td>4-aminobiphenyl</td>
</tr>
<tr>
<td>Ac-</td>
<td>acetylated</td>
</tr>
<tr>
<td>ACI</td>
<td>agouti rat from Columbia University Institute for Cancer Research</td>
</tr>
<tr>
<td>AF</td>
<td>2-aminofluorene</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>Arg</td>
<td>arginine</td>
</tr>
<tr>
<td>Asp</td>
<td>aspartic acid</td>
</tr>
<tr>
<td>AVMA</td>
<td>American Veterinary Medical Association</td>
</tr>
<tr>
<td>BN</td>
<td>Brown Norway rat</td>
</tr>
<tr>
<td>bp</td>
<td>base pair(s)</td>
</tr>
<tr>
<td>°C</td>
<td>degrees Celsius</td>
</tr>
<tr>
<td>CT</td>
<td>threshold cycle</td>
</tr>
<tr>
<td>CDF</td>
<td>F344/DuCrI rat</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary DNA</td>
</tr>
<tr>
<td>CO₂</td>
<td>carbon dioxide</td>
</tr>
<tr>
<td>CoA</td>
<td>coenzyme A</td>
</tr>
<tr>
<td>CoASH</td>
<td>thiol-containing coenzyme A</td>
</tr>
<tr>
<td>COP</td>
<td>Copenhagen rat</td>
</tr>
<tr>
<td>CYP1A1</td>
<td>cytochrome P450 1A1</td>
</tr>
<tr>
<td>CYP1A2</td>
<td>cytochrome P450 1A2</td>
</tr>
<tr>
<td>Cys</td>
<td>cysteine</td>
</tr>
<tr>
<td>C</td>
<td>cytosine</td>
</tr>
<tr>
<td>DA</td>
<td>d blood group, agouti colored rat</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethylsulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>DNase</td>
<td>deoxyribonuclease</td>
</tr>
<tr>
<td>DTNB</td>
<td>5,5'-dithiobis(2-nitrobenzoic acid)</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>E.coli</td>
<td>Escherichia coli</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbant assay</td>
</tr>
<tr>
<td>et al.</td>
<td>et alii (latin for “and others”)</td>
</tr>
<tr>
<td>etc.</td>
<td>et cetera (latin for “and so on”)</td>
</tr>
<tr>
<td>F344</td>
<td>Fisher 344 rat</td>
</tr>
<tr>
<td>FAM</td>
<td>5,6 carboxyfluorescein</td>
</tr>
<tr>
<td>FRET</td>
<td>fluorescence resonance energy transfer</td>
</tr>
<tr>
<td>g</td>
<td>gram(s)</td>
</tr>
<tr>
<td>G</td>
<td>guanine</td>
</tr>
<tr>
<td>x g</td>
<td>relative centrifugal force</td>
</tr>
<tr>
<td>Glu</td>
<td>glutamic acid</td>
</tr>
<tr>
<td>HCl</td>
<td>hydrogen chloride</td>
</tr>
<tr>
<td>Term</td>
<td>Definition</td>
</tr>
<tr>
<td>--------</td>
<td>------------------------------------------------</td>
</tr>
<tr>
<td>RACE</td>
<td>rapid amplification of cDNA ends</td>
</tr>
<tr>
<td>RLM-RACE</td>
<td>RNA ligase mediated RACE</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>RNS</td>
<td>reactive nitrogen species</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>rpm</td>
<td>revolutions per minute</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>real time PCR</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecylsulfate</td>
</tr>
<tr>
<td>SEM</td>
<td>standard error of the mean</td>
</tr>
<tr>
<td>Ser</td>
<td>serine</td>
</tr>
<tr>
<td>SMZ</td>
<td>sulfamethazine</td>
</tr>
<tr>
<td>SNP</td>
<td>single nucleotide polymorphism</td>
</tr>
<tr>
<td>SPRD</td>
<td>Sprague Dawley rat</td>
</tr>
<tr>
<td>T</td>
<td>thymine</td>
</tr>
<tr>
<td>$T_{1/2}$</td>
<td>half-life</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris-buffered saline</td>
</tr>
<tr>
<td>TET</td>
<td>6-Tetrachlorofluorescein</td>
</tr>
<tr>
<td>TSB</td>
<td>transformation and storage buffer</td>
</tr>
<tr>
<td>Tyr</td>
<td>tyrosine</td>
</tr>
<tr>
<td>UCSC</td>
<td>University of California, Santa Cruz</td>
</tr>
<tr>
<td>USDA</td>
<td>United States Department of Agriculture</td>
</tr>
<tr>
<td>UTR</td>
<td>untranslated region</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet</td>
</tr>
<tr>
<td>V</td>
<td>Volt(s)</td>
</tr>
<tr>
<td>$V_{max}$</td>
<td>maximum velocity</td>
</tr>
<tr>
<td>WF</td>
<td>Wistar Furth rat</td>
</tr>
<tr>
<td>WKY</td>
<td>Wistar Kyoto rat</td>
</tr>
<tr>
<td>µ-</td>
<td>micro-</td>
</tr>
</tbody>
</table>
CURRICULUM VITAE

Jason Matthew Walraven
Department of Pharmacology and Toxicology
University of Louisville School of Medicine
Louisville, KY 40202
j0walr01@louisville.edu
(502) 762-1564

EDUCATION

2003-2008 University of Louisville School of Medicine, Louisville, KY
Department of Pharmacology and Toxicology
M.S. Pharmacology and Toxicology (2005)
Ph.D. Pharmacology and Toxicology (Projected Graduation 2008)

2000-2002 The Southern Baptist Theological Seminary, Louisville, KY
Completed Three Semesters

1996-2000 Mercer University, Macon, GA
B.A. Chemistry (Pre-Medicine)
Magna Cum Laude

HONORS

Dean’s List – Mercer University
President’s List – Mercer University
Phi Kappa Phi – National Honor Society
Beta Beta Beta – Mercer University Biology Honor Society
Gamma Sigma Epsilon – Mercer University Chemistry Honor Society
Graduated Magna Cum Laude - Mercer University

GRANT SUPPORT

National Institute of Environmental Health Sciences (NIEHS) Training Grant (2004)
(T32-ES011564)

ABSTRACTS

Walraven JM, Doll MA, Hein DW. Identification of a novel rat arylamine N-acetyltransferase by cloning, sequencing, and recombinant expression in vitro. 95th Annual Meeting of the American Association for Cancer Research, March 27-31, 2004; Orlando, FL.

Walraven JM, Barker DF, Doll MA, Hein DW. Indirect evidence for extragenic and/or environmental influence on human NAT1 expression: analysis of primary hepatocytes by allele-specific quantitative real-time RT-PCR. 44th Annual Meeting of the Society of Toxicology, March 6-10, 2005; New Orleans, LA.

GENBANK

AY253759 (2003); Walraven JM, Doll MA, Martini BD and Hein DW. Rattus norvegicus strain WKY arylamine N-acetyltransferase 3 (Nat3) gene, complete cds. (PubMed)

AY253758 (2003); Walraven JM, Doll MA, Martini BD and Hein DW. Rattus norvegicus strain F344 arylamine N-acetyltransferase 3 (Nat3) gene, complete cds. (PubMed)

AY253757 (2003); Walraven JM, Doll MA, Martini BD and Hein DW. Rattus norvegicus strain Spage Dawley arylamine N-acetyltransferase 3 (Nat3) gene, complete cds. (PubMed)

PROFESSIONAL SOCIETIES

2003-2005 Ohio Valley Chapter of the Society of Toxicology (SOT)

INVITED PRESENTATIONS

Walraven JM, Doll MA, Hein DW. Identification of a novel rat arylamine N-acetyltransferase by cloning, sequencing, and recombinant expression in vitro. Annual Meeting of the Ohio Valley Society of Toxicology, November 7, 2003; Cincinnati, OH.